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POST-PROLINE CLEAVING ENZYME

SYNTHESIS OF A NEW FLUOROGENIC SUBSTRATE AND DISTRIBUTION OF THE ENDOPEPTIDASE IN RAT TISSUES AND BODY FLUIDS OF MAN

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Summary

Synthesis and application of the first fluorogenic substrate, *N*-carbobenzoxy-glycylprolyl-4-methylcoumarinyl amide (Z-Gly-Pro-MeCouNH) for the determination of the post-proline cleaving enzyme (EC 3.4.21.—) were reported. Maximal activity of the enzyme purified from lamb kidney for the new substrate was observed at pH 7.0. This substrate showed a higher affinity (K_m = 0.02 mM) for the enzyme than the proline containing substrates studied previously and allowed the detection of 10—50 ng post-proline cleaving enzyme activity per ml sample after a 1 min incubation period. Distribution of post-proline cleaving enzyme and other proline specific peptidases in rat tissues was studied using Z-Gly-Pro-MeCouNH and other proline-containing substrates. High post-proline cleaving enzyme activity was observed in testis, liver and skeletal muscle. Inhibition experiments indicated that post-proline cleaving enzyme activity was completely inactivated by 0.1 mM diisopropylphosphofluoridate and Z-Gly-Pro-chloromethylketone, as had been found in the case of the enzyme isolated from lamb kidney. Activity in human body fluids was also tested for levels of post-proline cleaving enzyme activity using Z-Gly-Pro-MeCouNH and semen was found to show the highest cleaving activity.

Constituent amino acids of the substrates used here were all of the L-configuration, unless otherwise specified.

Abbreviations: Cou, coumarinyl; NNap, naphthylamide.

Introduction

Post-proline cleaving enzyme, a proline specific endopeptidase (EC 3.4.21.—), was discovered by Walter et al. [1] in human uterus as an oxytocin-degrading enzyme which hydrolyzes the Pro^7-Leu^8 bond of the hormone. Subsequently, the distribution of this activity was qualitatively assayed using ^{14}C-labeled oxytocin or vasopressin and found to be ubiquitous in vertebrates. The enzyme has been purified to homogeneity from lamb kidney and characterized as the first known endopeptidase with a high specificity for cleaving -Pro-X- peptide bond [3,4] and tentatively characterized as a serine protease [5]. Substrate specificity [6], size and stereospecificity of its active site [7] were also studied.

Recently, Yoshimoto and Tsuru [8] screened microorganisms capable of producing proline specific peptidases and found that only species of pathogenic *Flavobacterium* accumulate intracellularly a proline specific endopeptidase. On the other hand, the distribution of this enzyme in animal organs and tissues has not yet been systematically investigated.

To detect low levels of activity of a protease, the use of substrates with high sensitivity is essential. For this purpose, fluorogenic compounds have recently been prepared as substrates for several peptide bond-hydrolyzing enzymes [9—11]. Thus, we prepared a new fluorogenic substrate, Z-Gly-Pro-4-MeCouNH and systematically studied the distribution of post-proline cleaving enzyme in rat tissues and human body fluids. Proline-containing β-naphthylamides and ester were also used to test for post-proline cleaving enzyme activity. Testis, liver and skeletal muscle of rat as well as human semen were found to show high activity for the hydrolysis of Z-Gly-Pro derivatives of 4-methyl-7-amino-coumarin, β-naphthylamine and *p*-nitrophenol.

Materials and Methods

Materials. Gly-Pro-β-naphthylamide (Gly-Pro-2-NNap) and Z-Pro-2-NNap were purchased from Vega-Fox Chemicals (AZ, U.S.A.). Fast Garnet GBC salt, Pro-2-NNap, bovine pancreas trypsin, α-chymotrypsin, carboxypeptidase A, elastase and swine kidney leucine aminopeptidase were obtained from Sigma Chemical Co. (St Louis, U.S.A.). Human thrombin and urokinase were from Midori Juji Co. (Osaka, Japan). Post-proline dipeptidyl aminopeptidase and post-proline cleaving endopeptidase were purified from lamb kidney by the methods of Yoshimoto and Walter [12] and of Koida and Walter [4], respectively. Diisopropyl phosphofluoridate and Cys-di-2-NNap were from Nakarai Chemicals (Kyoto, Japan). Z-Gly-Pro-Leu-Gly, Ala-2-NNap and Leu-2-NNap were obtained from Protein Research Foundation (Minoh, Japan). Z-Gly-Pro-chloromethylketone (Z-Gly-Pro-CH_2Cl) was prepared as described [5], and Z-Gly-Pro-2-NNap and Z-Gly-Pro-*p*-nitrophenylester (Z-Gly-Pro-ONp) were from the same batch as synthesized previously [6].

Z-Gly-Pro-4-MeCouNH. 7-Amino-4-methylcoumarin was synthesized by the method of Pechman and Schwarz [13]. Z-Gly-Pro-4-MeCouNH was synthesized as follows: Z-Gly-Pro · OH (0.31 g, 1 mmol), hydroxybutyltoluene (0.23 g, 1.5 mmol) and dicyclohexylcarbodiimide (0.21 g, 1 mmol) were dissolved in 6 ml

of dimethylformamide and cooled to 0°C. Then 7-amino-4-methylcoumarin (0.19 g, 1.1 mmol) was added. The reaction mixture was allowed to warm up to room temperature and stirring was continued for two days. Dicyclohexylurea was filtered off and filtrate was evaporated to dryness. The residue was dissolved in ethyl acetate and cooled to 0°C, additional dicyclohexylurea and excess 7-amino-4-methylcoumarin being removed by filtration. The filtrate was diluted with ethyl acetate (30 ml) and the organic layer washed with 1 N HCl (three times), H_2O (twice), 8% $NaHCO_3$ (three times), H_2O (three times) and dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The oily residue was triturated with hexane in an ice-bath, whereupon it solidified. The crude product, isolated by filtration, was purified by silica gel column chromatography. Fractions containing the product were collected and evaporated. The product was solidified from ether, m.p., 115—120°C, $[\alpha]_D^{26} = -102°$ (C = 1.2% in dimethylformamide). For $C_{25}H_{25}N_3O_6 \cdot H_2O$ (M_r 482)

Calculated: C, 62.4; H, 5.65; N, 8.72
Found: C, 62.4; H, 5.79; N, 8.41

Amino acid analysis: Pro, 0.93, Gly, 1.00.

Analytical methods. The concentration of post-proline cleaving enzyme from lamb kidney was calculated on the basis of specific activity of 45 units/mg purified enzyme [5]. In the calculation of k_{cat}, the molecular weight of the enzyme was taken as 58 000 [5]. Kinetic parameters were calculated by a computer Facom 270-20, using the program by Bliss and James [14] based on the Michaelis-Menten equation.

Enzyme activity assays.

(1) Hydrolysis of Z-Gly-Pro-MeCouNH: To 3 ml 0.1 M phosphate buffer (pH 7.0) were added 50 µl 0.5 mM Z-Gly-Pro-MeCouNH in dioxane and 50 µl enzyme solution at 25°C. The fluorescence of 7-amino-4-methylcoumarin released was monitored using an Hitachi 5 spectrofluorometer, excitation at 370 nm, emission at 440 nm [11].

(2) Hydrolysis of peptide- or amino acid-2-NNap: Enzyme activity was assayed by a slightly modified method of Yoshimoto and Walter [12]. A 0.25-ml aliquot of enzyme solution was mixed with 0.25 ml 1 mM substrate in 40% dioxane and 1 ml 0.1 M Tris-HCl buffer (pH 7.0) at 30°C. After incubation for 10 min, 0.5 ml Fast Garnet GBC salt (1 mg/ml in 1 M acetate buffer, pH 4.0, 10% Triton X-100) was added to the reaction mixture. The color of the diazo dye developed was measured at 550 nm with a Hitachi spectrometer type 101.

(3) Hydrolysis of Z-Gly-Pro-ONp: 50 µl 2.1 mM Z-Gly-Pro-ONp in dioxane was added to 50 µl enzyme and 1 ml 10 mM phosphate buffer (pH 7.0) at 25°C, and incubated for various periods of time. The initial velocity of the reaction was assayed by the increase in absorbance at 410 nm using a double-beam spectrophotometer, Shimadzu UV-200.

(4) Hydrolysis of Z-Gly-Pro-Leu-Gly: This was assayed by a ninhydrin method using Leu-Gly as standard [3].

Extraction of enzyme from rat tissues. 2 g tissues (except skin) were homogenized in 4 ml 20 mM Tris-HCl buffer (pH 7.0) at 4°C for 10 min and then

centrifuged at 12 000 × *g* for 1 h. Supernatants were used for post-proline cleaving enzyme measurements. In the case of skin, the tissue was frozen at −80° C in a solid CO_2/acetone bath, sliced and soaked in cold acetone for 30 min. The skin slices were dried in vacuo and then extracted with 20 mM Tris-HCl buffer (pH 7.0) containing 5% KCl. After centrifugation at 8000 × *g* for 15 min, the supernatant was used to determine the level of activity.

DEAE-Sephadex A-50 column chromatography. 20 g tissue was homogenized (three times at 1-min intervals for a total of 10 min) in 40—80 ml 20 mM Tris-HCl buffer (pH 7.0) by a Homo Blender (Sakuma Seisakusho, Tokyo, Japan) and the pellet was centrifuged at 12 000 × *g* for 30 min. The supernatant was fractionated with 30—80% saturation $(NH_4)_2SO_4$. The resulting precipitates were dissolved in small volumes of 20 mM Tris-HCl buffer (pH 7.0) and then desalted with Sephadex G-25. Desalted solutions were applied to a column (2.5 × 25 cm) of DEAE-Sephadex A-50 equilibrated with the above buffer. After being washed with the same buffer, the enzyme was eluted with a linear gradient of 0—0.5 M NaCl (flow rate, 40 ml/h)

Effects of diisopropylphosphofluoridate, Z-Gly-Pro-CH_2Cl and EDTA on enzyme activity. 0.2 ml enzyme solution was incubated with 0.2 ml of these chemicals at 30° C, and after 30 min the residual activities were assayed by the standard method using an appropriate aliquot of the enzyme solution.

Results

Hydrolysis of coumarin derivative by lamb kidney post-proline cleaving enzyme

In order to establish optimal hydrolysis conditions of the new substrate, Z-Gly-Pro-MeCouNH, the enzyme reaction was carried out at various pH values at 25° C. For comparison, the other three substrates of post-proline cleaving enzyme were also subjected to the enzymatic hydrolysis. The results are summarized in Table I. Z-Gly-Pro-MeCouNH was readily split at the optimal pH value of 7, liberating 7-amino-4-methylcoumarin. The K_m value was 20 μM, which is a value significantly lower as compared to those for the other three substrates tested (Table I).

Comparison of sensitivity of substrates to enzymatic hydrolysis

Hydrolysis of the four substrates by post-proline cleaving enzyme from lamb

TABLE I

REACTION CONDITIONS AND KINETIC PARAMETERS IN HYDROLYSIS OF SEVERAL PROLINE DERIVATIVES BY LAMB KIDNEY POST-PROLINE CLEAVING ENZYME

Substrate	Optimum pH	Reaction pH	[S] (mM)	K_m (mM)	k_{cat} (s^{-1})
Z-Gly-Pro-MeCouNH	7.0	7.0	[0.006—0.065]	0.020 ± 0.006 **	557.3 ± 22.7 **
Z-Gly-Pro-2-NNap	7.0	7.0	[0.026—0.83]	0.135 ± 0.007	10.3 ± 0.12
Z-Gly-Pro-ONp *	8.9	7.0	[0.016—0.25]	0.08	25.4
Z-Gly-Pro-Leu-Gly *	7.8	7.8	[0.025—0.2]	0.06	60.5

* See Ref. 6.

** ± S.D.

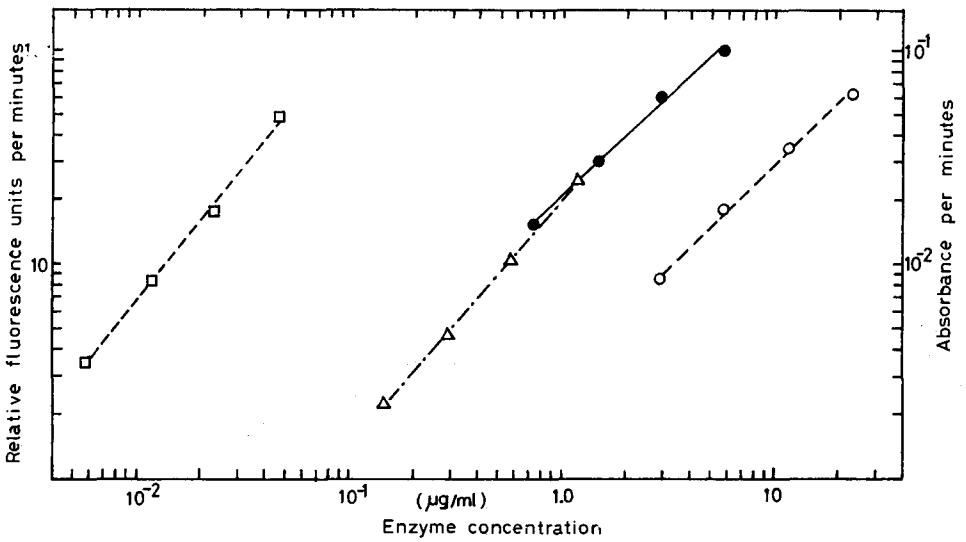

Fig. 1. Initial velocity versus concentration of post-proline cleaving enzyme. The experimental details are described in the text. □- - - - -□, Z-Gly-Pro-MeCouNH (assayed fluorimetrically); △· — · — ·△, Z-Gly-Pro-ONp; ●————●, Z-Gly-Pro-2-NNap; ○- - - - -○, Z-Gly-Pro-Leu-Gly.

kidney was measured. As shown in Fig. 1, linearity between the initial reaction rate and the enzyme concentration was observed for all substrates. Z-Gly-Pro-MeCouNH was the most sensitive substrate, and amounts as small as 5 ng of the enzyme were detectable by using this substrate. On the other hand, 2 μg of the enzyme was detectable using Z-Gly-Pro-Leu-Gly, 0.5 μg of enzyme using Z-Gly-Pro-2-NNap and 100 ng of enzyme using Z-Gly-Pro-ONp, respectively. With Z-Gly-Pro-MeCouNH, Z-Gly-Pro-ONp and Z-Gly-Pro-2-NNap, no detectable hydrolysis was observed upon incubating with relatively high concentrations (0.1—0.5 mg/ml) of trypsin, α-chymotrypsin, elastase, thrombin, urokinase, leucine aminopeptidase, carboxypeptidase A and post-proline dipetidyl aminopeptidase. However, Z-Gly-Pro-Leu-Gly was hydrolyzed by carboxypeptidase A.

Distribution of post-proline cleaving enzyme in rat tissues

The three compounds, Z-Gly-Pro-MeCouNH, Z-Gly-Pro-ONp and Z-Gly-Pro-2-NNap, were found to be sensitive and specific substrates for the post-proline cleaving enzyme from lamb kidney (Fig. 1). Thus, these substrates were used for an investigation of the distribution of this type of enzyme in rat tissues. The activities of post-proline dipeptidyl aminopeptidase (substrate: Gly-Pro-2-NNap), prolinase (substrate: Pro-2-NNap) and of leucine aminopeptidase or arylamidase (substrate: Ala-2-NNap, Leu-2-NNap and Cys-di-2-NNap) were also checked for comparison. The results are summarized in Table II. Extraction recoveries were adequate, since there was observed no detectable activity in each precipitate (cell debri).

The hydrolytic activities toward Z-Gly-Pro-MeCouNH, Z-Gly-Pro-ONp and Z-Gly-Pro-2-NNap were roughly parallel among the rat tissues studied, except

TABLE II

DISTRIBUTION OF AMIDASE AND ESTERASE ACTIVITIES IN VARIOUS TISSUES OF RAT

Enzyme activities were determined as μmol/min per 1.0 g of wet weight, except that the activities of skin and serum were determined as μmol/min per 1.0 g dry weight and 1.0 ml, respectively. The average values of four experiments are shown. Z-Pro-2-NNap, zero activity in each case. -MeCouNH, 4-methylcoumarinylamide; -ONp, *p*-nitrophenyl ester; -2-NNap, β-naphthylamide.

Substrate	Z-Gly-Pro-Me-CouNH	Z-Gly-Pro-ONp	Z-Gly-Pro-2-NNap	Gly-Pro-2-NNap	Pro-2-NNap	Ala-2-NNap	Leu-2-NNap	Cys-di-NNap
Kidney	0.208	0.155	0.037	1.133	0.058	0.111	0.093	0.069
Heart	0.341	0.180	0.087	0.031	0.062	0.104	0.259	0.019
Pancreas	0.106	0.140	0.014	0.032	0	0.080	0.164	0
Spleen	0.211	0.152	0.034	0.089	0	0.063	0.147	0.022
Testis	0.685	0.410	0.142	0.046	0.067	0.134	0.306	0.037
Jejunum	0.101	0.059	0.021	0.079	0	0.133	0.295	0.018
Liver	0.710	0.393	0.047	0.059	0.008	0.049	0.120	0.030
Lung	0.370	0.250	0.102	0.155	0.018	0.135	0.271	0.023
Skeletal muscle	0.529	0.299	0.117	0.026	0.082	0.157	0.314	0.034
Skin	0.008	0.081	0.103	0.108	0	0.076	0.160	0
Brain	0.443	0.234	0.159	0.014	0.096	0.146	0.315	0.036
Gland vesiculosa	0.044	0.171	0.060	0.041	0.010	0.110	0.181	0.025
Serum	0.007	0.107	0	0.013	0	0.021	0.024	0

for liver, skin, gland vesiculosa and serum. Of the tissues tested, testis and liver showed the highest activity followed by skeletal muscle, brain and lung. Some post-proline cleaving enzyme activity was observed, however, in every tissue tested. Very high activity capable of hydrolyzing Gly-Pro-2-NNap was found in kidney. Pro-2-NNap hydrolyzing activity was present in brain, skeletal muscle and testis, but none of the tissue extracts catalyzed the hydrolysis of Z-Pro-2-NNap. There was no detectable relationship between activities hydrolyzing Z-Gly-Pro-2-NNap, Gly-Pro-2-NNap and Pro-2-NNap, respectively, in different rat tissues. Ala-2-NNap, Leu-2-NNap and Cys-di-2-NNap hydrolyzing activities were shown to be widely distributed.

DEAE-Sephadex A-50 column chromatography

Fig. 2 shows the elution profile of the extracts from kidney, testis and brain from DEAE-Sephadex A-50 column. Z-Gly-Pro-2-NNap hydrolyzing enzyme was eluted at 0.3 M of NaCl concentration and Leu-2-NNap hydrolyzing enzyme appeared in the 0.35 M NaCl fraction. Almost the same pattern of elution was also observed when the extracts from liver, lung and skeletal muscle were subjected to the chromatography under the same condition. Each active fraction was subjected to inhibition experiments. The hydrolysis of Z-Gly-Pro-2-NNap and Gly-Pro-2-NNap was strongly inhibited by incubation of the eluates with 0.1 mM diisopropylphosphofluoridate at 30°C for 30 min at pH 7.0, but no inhibition was observed for Leu-2-NNap hydrolysis. This arylamidase activity was markedly inhibited by incubation with 1 mM EDTA, which did not affect the activities hydrolyzing Z-Gly-Pro-2-NNap and Gly-Pro-2-NNap. Only Z-Gly-Pro-2-NNap hydrolyzing activity was markedly inhibited by incubation with 0.1 mM Z-Gly-Pro-CH_2Cl under the above condition.

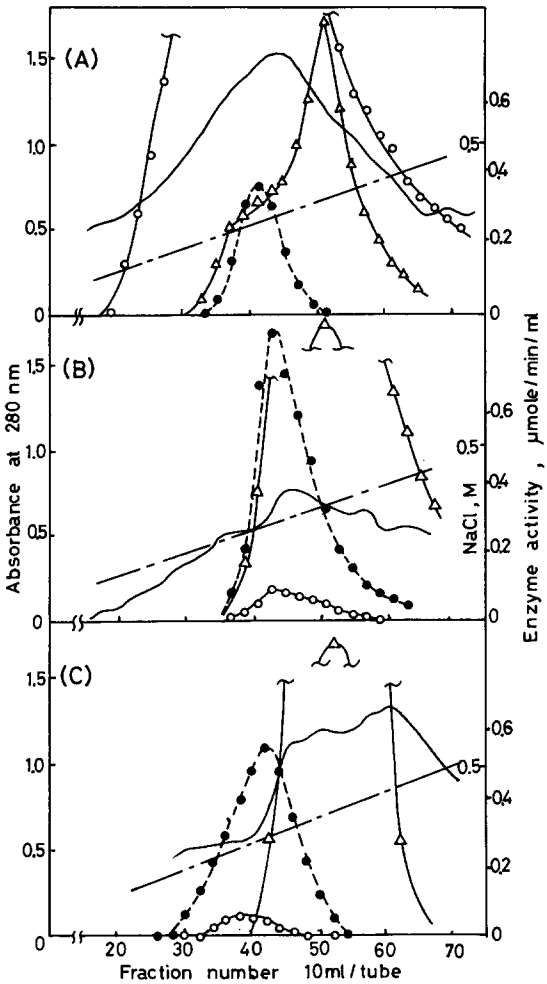

Fig. 2. DEAE-Sephadex A-50 column elution profiles of rat tissue extracts. See text for the experimental details. A, kidney; B, testis, C, brain. ———, Absorbance at 280 nm; · — · — ·, NaCl concentration; ●- - - - -●, Z-Gly-Pro-2-NNap; ○———○, Gly-Pro-2-NNap; △———△, Leu-2-NNap.

Post-proline cleaving enzyme in human body fluids

Post-proline cleaving enzyme activity of human serum, urine, saliva and semen was measured by using Z-Gly-Pro-MeCouNH. The activity was found to be present in all of the fluids tested except in urine. Human semen showed the highest activity (3 nmol substrate hydrolyzed/min per ml), followed by serum and saliva (0.6—0.9 nmol/min per ml).

Discussion

In the early studies on post-proline cleaving enzyme [1,2,15], radio-labeled peptide hormones, (9-[1-^{14}C]glycinamide)arginine-vasopressin and (9-[1-^{14}C]-glycinamide)oxytocin, were powerful markers, since they are very sensitive substrates and since the radio-labeled fragments formed as a result of enzyme-

catalyzed hydrolysis are easy to identify even in crude homogenates. However, as post-proline cleaving enzyme was obtained in more purified form, Z-Gly-Pro-Leu-Gly was adopted as the substrate for the general assay method during the continued studies of purification and characterization [3,4]. Substrate specificity studies with purified post-proline cleaving enzyme revealed that the protease possesses a high specificity for catalyzing the hydrolysis of a proline-containing peptide at the carboxyl group of the proline residue (primary specificity site, S_1 site) [4–7]. The specificity of the enzyme at the S_1 site is, therefore, similar to that of post-proline dipeptidyl aminopeptidase (dipeptidyl aminopeptidase IV, EC 3.4.14.–) [12,16], prolinase (EC 3.4.13.8) [17] and proline aminopeptidase (EC 3.4.11.5) [18]. However, post-proline cleaving enzyme is clearly distinguishable from these three enzymes on the basis of its secondary specificity sites [6,7]. Arylamidases, which catalyze the hydrolysis of certain amino acid derivatives of β-naphthylamide, fail to cleave 2-naphthylamide derivatives of N-substituted amino acids and peptides [19]. Hence, it is concluded that Z-Gly-Pro-MeCouNH, Z-Gly-Pro-2-NNap and Z-Gly-Pro-ONp are specific substrates for post-proline cleaving enzyme.

Z-Gly-Pro-MeCouNH showed the highest affinity for post-proline cleaving enzyme and the greatest sensitivity during assays (Fig. 1 and Table I). Detection of the enzyme at levels of 10 ng per ml is possible after 1 min reaction time, indicating that the compound is useful for the detection of very low levels of the enzyme activity.

Using Z-Gly-Pro-MeCouNH, Z-Gly-Pro-2-NNap and Z-Gly-Pro-ONp in addition to several other peptide or amino acid derivatives, the distribution of post-proline cleaving enzyme was assayed in a variety of rat tissues (Table II). Almost all of the activities were confirmed to be extracted under the conditions mentioned in Materials and Methods by checking the activities of resuspended precipitates. High levels of enzymatic activity were observed in testis, liver, lung, skeletal muscle and brain. The proteolytic activities hydrolyzing Z-Gly-Pro-MeCouNH, Z-Gly-Pro-ONp and Z-Gly-Pro-2-NNap were roughly parallel in the tissue extracts tested, except for liver, skin, gland vesiculosa and serum. The reason for these unusual phenomena found in liver and other tissues and fluid is not clear, but preliminary experiments suggested that the liver extract contains a factor(s) that inhibits the color development despite cleavage of the substrate in the assay system.

The highest degree of activity cleaving Gly-Pro-2-NNap was seen in the kidney extract. This activity is due to post-proline dipeptidyl aminopeptidase [12,16], confirming a previous report by McDonald et al. [20]. Hydrolysis of Pro-2-NNap, presumably due to prolinase activity, was observed in extracts of brain, skeletal muscle, kidney, heart and in testis. Z-Pro-2-NNap was completely resistant to extracts of all the tissues tested. Leucine aminopeptidase (or arylamidase) activity was found ubiquitously in all tissues. Human serum, urine, saliva and semen were also tested for the presence of post-proline cleaving enzyme activity using Z-Gly-Pro-MeCouNH as a substrate. By far the highest levels of activity were found in human semen.

In order to compare the chromatographic behavior of post-proline cleaving enzyme obtained from some tissues of rat, the extracts were applied to a DEAE-Sephadex column and the elution profiles of Z-Gly-Pro-2-NNap hydrolyzing

activity were followed. Almost the same elution pattern as that found for post-proline cleaving enzyme isolated from rat kidney was observed with the extracts tested (Fig. 2). Every fraction which contained Z-Gly-Pro-2-NNap hydrolyzing activity was also markedly inhibited by diisopropylphosphofluoridate and by Z-Gly-Pro-CH_2Cl, as in the case of post-proline cleaving enzyme purified from lamb kidney [5,6,12]. These results suggest that Z-Gly-Pro-2-NNap hydrolyzing activity is due only to post-proline cleaving enzyme. Purification of the enzyme from brain, liver, and testis of pig is now in progress and the properties of the purified enzyme will be reported elsewhere.

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References

1 Walter, R., Shlank, R., Glass, J.D., Schwartz, I.L. and Kerenyi, T.D. (1971) Science 173, 827—829
2 Walter, R. (1973) in Peptides 1972 (Hanson, H. and Jakubke, H.D., eds.), pp. 363—378, North-Holland Publ. Co., Amsterdam
3 Walter, R. (1976) Biochim. Biophys. Acta 422, 138—158
4 Koida, M. and Walter, R. (1976) J. Biol. Chem. 251, 7593—7599
5 Yoshimoto, T., Orlowski, R.C. and Walter, R. (1977) Biochemistry 16, 2942—2948
6 Yoshimoto, T., Fischl, M., Orlowski, R.C. and Walter, R. (1978) J. Biol. Chem. 253, 3708—3716
7 Walter, R. and Yoshimoto, T. (1978) Biochemistry 17, 4139—4144
8 Yoshimoto, T. and Tsuru, D. (1978) Agric. Biol. Chem. 42, 3017—3019
9 Nakayama, H. and Kanaoka, Y. (1973) Chem. Pharm. Bull. 21, 2804—2805
10 Zimmerman, M., Yurewicz, E. and Patel, G. (1976) Anal. Biochem. 70, 258—262
11 Fujiwara, K. and Tsuru, D. (1978) J. Biochem. 83, 1145—1149
12 Yoshimoto, T. and Walter, R. (1977) Biochim. Biophys. Acta 485, 391—401
13 Pechman, H.V. and Schwarz, O. (1899) Chem. Ber. 32, 3696—3699
14 Bliss, C.I. and James, A.T. (1966) Biometrics 22, 573—602
15 Walter, R. and Simmons, W. (1977) in Neurohypophysis (Moses, A.M. and Share, L., eds.), pp. 167—188, Karger, Basel
16 Oya, H., Nagatsu, I. and Nagatsu, T. (1972) Biochim. Biophys. Acta 258, 591—599
17 Davis, N.C. and Smith, E.L. (1953) J. Biol. Chem. 200, 373—384
18 Sarid, S., Berger, A. and Katchalski, E. (1959) J. Biol. Chem. 234, 1740—1746
19 Behal, F.J. and Story, M.N. (1969) Arch. Biochem. Biophys. 131, 74—82
20 McDonald, J.K., Callahan, P.X., Ellis, S. and Smith, R.E. (1976) in Tissue Proteinases (Barrett, A.J. and Dingle, J.K., eds.), pp. 69—107, North-Holland Publ. Co., Amsterdam