Histochemie 7, 197—201 (1966)

A NEW DIPEPTIDE NAPHTHYLAMIDASE HYDROLYZING GLYCYL-PROLYL-β-NAPHTHYLAMIDE

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Received June 10, 1966

A new chromogenic substrate, glycyl-DL-prolyl-β-naphthylamide (Gly-Pro-β-NA) was synthesized for evaluation in studies on the biochemistry and histochemistry of mammalian peptidases. The initial results with this substrate are presented in this note.

Material and Methods

N^{α}-Cbz-Glycyl-DL-Prolyl-β-Naphthylamide. N^{α}-Cbz-Gly-DL-Pro (2 g) and 2-naphthylamine (1 g) were dissolved in 50 ml of tetrahydrofuran at —10° C. To this solution was added 1.35 g N,N_1-dicyclohexylcarbodiimide and with mechanical stirring the solution was brought slowly to 25° C with stirring continued for 16 hours. The precipitated urea was removed by filtration and the filtrate evaporated to dryness *in vacuo*. The amorphous residue was extracted with dichloromethane and crystallization occurred on reduction of solvent *in vacuo*. Recrystallization was performed from dichloromethane on the addition of 60° ligroin and refrigeration. m.p. 142—143° C. Calculation for $C_{25}H_{25}N_3O_4$ (m.w. 431.53): C, 69.60; H, 5.85; N, 9.73. Found: C, 69.83; H, 5.74; N, 9.59.

Glycyl-DL-Prolyl-β-Naphthylamide HBr. The N-protected compound was slowly dissolved in a small volume of glacial acetic acid saturated with hydrogen bromide containing a small quantity of phenol (GLENNER *et al.*, 1965), and agitated for two hours during which time gas evolution ceased. Addition of ten-fold solvent excess of ethyl ether precipitated an amorphous material which was washed, and triturated with further additions of ether. The triturated residue was dissolved in a minimal quantity of methyl alcohol and crystallized on the addition of ether. Recrystallized from methyl alcohol.

Browns, 244° C; m.p. 253—254.5.

Calculated for $C_{17}H_{19}N_3O_2 \cdot HBr$ (m.w. 378.32): C, 53.97; H, 5.34; N, 11.11.

Found: C, 53.98; H, 5.13; N, 11.10; α_D^{20} —119.9 (c, 1 in methanol).

The quantitative assay methods as well as the preparative methods for fractionation of the enzymically active proteins have been described earlier (HOPSU and GLENNER, 1964; HOPSU *et al.*, 1966) and additional details are given in the text.

Results and Conclusions

The hydrolysis of Gly-Pro-β-NA by some commercially available enzymes was tested (Table 1). It appeared that the rate of the hydrolysis by the Acylase I preparation was much greater than that of other enzyme preparations. Therefore, the Acylase I preparation was subjected to gel filtration by Sephadex G 200 and DEAE-cellulose chromatography in order to see whether the enzyme hydrolyzing the present substrate could be separated from a number of other enzymes known to be present in the same preparation (HOPSU and GLENNER, 1964). Sephadex G 200 did not effect significant separation due to the large size of molecule but chromatography on DEAE-cellulose succesfully separated the enzyme (Fig. 1).

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The enzyme hydrolyzing Gly-Pro-β-NA was fractionated from that hydrolyzing leucyl-β-naphthylamide (Leu-β-NA) and from that hydrolyzing chloroacetyl-α-naphthylamide and also from aminoacid acylase I hydrolyzing chloroacetyl-L-leucine.

Fractions no. 9—12 were pooled and subjected to further characterization The optimal pH for the hydrolysis of Gly-Pro-β-NA by the pooled fractions was

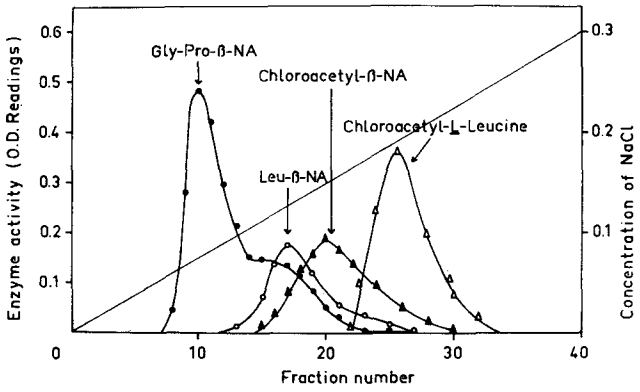

Fig. 1. DEAE-cellulose chromatography of Acylase I preparation. Column 3 × 35 cm, sample 10 mg in 1.0 ml distilled water, linear elution gradient 0 to 0.3 M NaCl in 0.02 M Tris-HCl pH 7.0, fraction volume 10 ml, temperature 4° C. Gly-Pro-β-NA hydrolysis assay: Tris-HCl 0.1 M, pH 7.0, 1.0 ml; enzyme, 0.2 ml; substrate (0.1 mM), 0.5 ml; incubation time, 15 min, readings at 525 mμ. Leu-β-NA hydrolysis assay: buffer above; substrate (0.1 mM), 0.5 ml; enzyme, 0.5 ml; incubation time, 30 min, readings at 525 mμ. Chloroacetyl-α-naphthylamide hydrolysis assay: buffer as above; substrate (1 mM), 1.0 ml; enzyme, 0.2 ml; incubation time 15 min, reading at 540 mμ. Chloracetyl-L-leucine hydrolysis assay: buffer as above; substrate (3 mM), 1.0 ml; enzyme, 0.25; incubation time, 15 min, ninhydrin color at pH 5.0 measured at 600 mμ

Table 1. *Hydrolysis of Gly-Pro-β-NA by several commercial enzyme preparations*

Incubation solution: 0.1 M phosphate buffer pH 7.0, 2 ml; enzyme solution (1 mg/40 ml water), 0.5 ml; substrate solution (0.4 mg/ml water), 0.5 ml. Incubation time, 30 min. After incubation 1 ml fast garnet GBC solution was added. O.D. reading at 525 mμ.

Enzyme	O.D. reading
Trypsin (Sigma)	.016
Chymotrypsin (Sigma)	.015
Lipase 448 (Nutr. Bioch.)	.025
Acylase I (Nutr. Bioch.)	.720

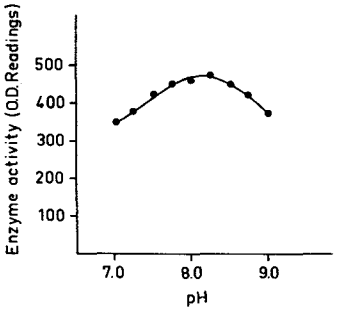

Fig. 2. Dependence of the hydrolysis of Gly-Pro-β-NA on pH. Tested in 0.1 M Tris-HCl buffer as described in Fig. 1

found to be 8.0—8.2 (Fig. 2). The hydrolysis rate was not affected by the presence of $CaCl_2$, $CoCl_2$, EDTA (1 mM), E 600 or pCMB (0.1 mM). The result of the hydrolysis of several other aminoacid and dipeptide naphthylamides by the same preparation is given in Table 2. Gly-Pro-β-NA is the only one of the tested aminoacid naphthylamide derivatives measurably hydrolysed. It is to be especially noted that Pro-β-NA and Cbz-Gly-Pro-β-NA were not hydrolyzed. Human hemoglobin also was not hydrolyzed even during a 12 hour incubation period. Ascending paper chromatography demontrated that Gly-Pro was liberated from the sub-

strate with no release of free glycine or proline. Also, it was shown that the hydrolysis curve of Gly-Pro-β-NA was linear in time. These results suggest that the enzyme is an aminopeptidase with a fairly strict substrate specificity and alkaline pH optimum.

An effort was made to determine whether the presence of a similar enzyme could be demonstrated in other mammalian tissues. The liver of the rat was chosen as test object.

A rat liver homogenate prepared in 0.2 M sucrose using a Potter-Elvehjem homogenizer hydrolyzed Gly-Pro-β-NA very rapidly; approximately twice as fast as Leu-β-NA. The hydrolysis rate of Gly-Pro-β-NA was ten times higher (while that toward Leu-β-NA was only two times higher) in the particle fraction

Table 2. *Hydrolysis of various aminoacid and dipeptide naphthylamides by the preparation pooled from fractions 9—12 of the chromatography of Fig. 1*

Buffer 0.1 M Tris-HCl pH 8.0, 2.0 ml; enzyme preparation, 0.5 ml; and substrate (0.1 mM), 0.5 ml. Incubation time 30 min. O.D. reading at 525 mμ.

Substrate	O.D. reading
Gly-DL-Pro-β-NA	.910
L-Ala-DL-Ala-β-NA	.060
γ-L-Glu-β-NA	.015
Gly-Gly-β-NA	.010
L-Phe-β-NA	.007
L-Pro-β-NA	.006
α-L-Glu-β-NA	.005
L-His-β-NA	.004
L-Try-β-NA	.003
L-Lys-β-NA	.003
L-Thr-β-NA	.003
L-Leu-DL-Leu-β-NA	.003
Gly-DL-Phe-β-NA	.003
L-norLeu-β-NA	.000
L-Tyr-β-NA	.000
L-Val-β-Na	.000
Cbz-L-Ala-DL-Ala-β-NA	.000

Table 3. *Hydrolysis of various aminoacid and dipeptide naphthylamides by the rat liver preparation*

Tris-HCl buffer 0.1 M, pH 7.5 (1.5 ml). Enzyme preparation, 0.5 ml; substrate, 0.5 ml (0.1 mM); incubation time, 30 min; O.D. readings at 525 mμ.

Substrate	O.D. reading
L-Gly-Pro-β-NA	1180
L-Leu-β-NA	250
L-Try-β-NA	240
L-norLeu-β-NA	172
L-Phe-β-NA	144
L-Ala-DL-Ala-β-NA	130
γ-L-Glu-β-NA	090
L-Tyr-β-NA	075
α-L-Glu-β-NA	075
L-His-β-NA	073
L-Leu-DL-Leu-β-NA	060
Gly-DL-Phe-β-NA	035
Gly-Gly-β-NA	028
L-Thr-β-NA	023
L-Lys-β-NA	022
L-Val-β-NA	017
Cbz-L-Ala-DL-Ala-β-NA	010

than in the supernatant obtained by adjusting the above homogenate at pH 5.2 with 0.1 N HCl in ice water followed by centrifugation (30 min, 17.000 rpm). The sediment was twice frozen and thawed, Triton X-100 was added to a concentration of 0.1% and the preparation was centrifuged (17.000 rpm, one hour) and the activity in the supernatant and sediment was again assayed. Now the activity was distributed equally between the supernatant and the homogenate.

The supernatant was subjected to chromatography with Sephadex G 200 and one peak was obtained using Gly-Pro-β-NA as substrate. Gly-Pro-β-NA was the most rapidly hydrolyzed substrate of those tested with this preparation (Table 3). The pH optimum of the hydrolysis of Gly-Pro-β-NA by this peak was found to lie between 7.0—8.0. Activity was clearly higher in 0.05 M than in 0.2 M Tris-HCl buffer at pH 7.5. The hydrolysis of Gly-Pro-β-NA was not affected by

EDTA, Ca^{++}, Mg^{++} and Mn^{++} ions (0.5 mM) nor by pCMB or E 600 (0.1 mM). Paper chromatography revealed that only the dipeptide, Gly-Pro, was liberated from the substrate. The time course of hydrolysis of Gly-Pro-β-NA was found to be linear.

These results are sufficient to demonstrate the presence of an enzyme in rat liver similar to that found in the hog kidney Acylase I preparation.

Preliminary attempts were made to demonstrate the enzyme histochemically using diazonium salt coupling of the liberated naphthylamine. Initial experiments were promising since the enzyme appeared to be particle bound and fairly resistant

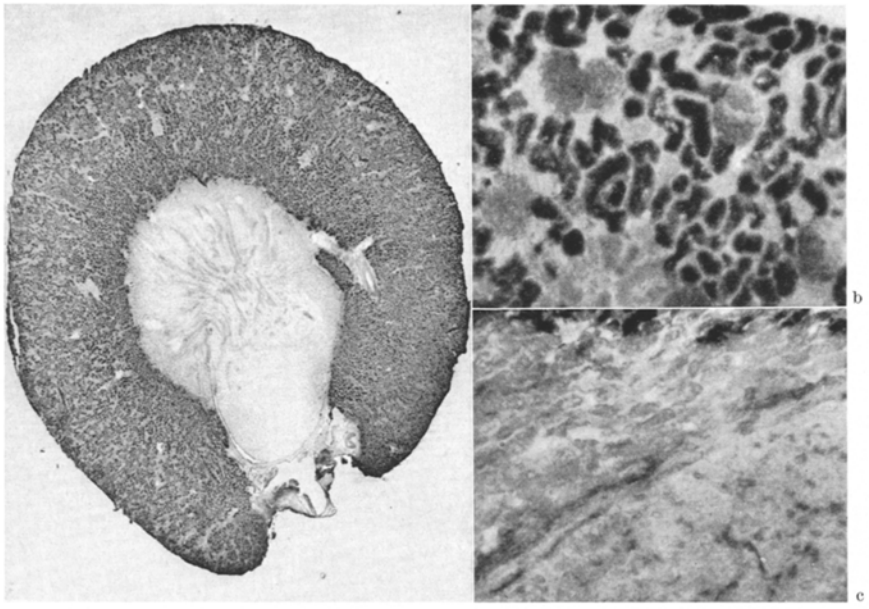

Fig. 3a—c. Localization of the reaction product in rat kidney sections. a An overall view. Magn. ×10. b Kidney cortex with reaction in the glomeruli and tubuli. Magn. ×120. c Kidney medullary rays with a less intense reaction. Magn. ×120

to various affector substances. Fig. 3 demonstrates the localization of the reaction product in defatted rat kidney sections incubated for 5 min in 0.1 M Tris-HCl buffer pH 7.0 containing Gly-Pro-β-NA (1 mg/ml) and fast garnet GBC (0.5 mg/ml). The result is different from the obtained with Leu-β-NA as substrate in so far as the reaction is more uniform throughout the cortical zone, and glomeruli as well as medullary cords show a fairly intense reaction. An intense, even staining throughout the parenchyma is obtained in the rat liver while only a minimal staining can be obtained with Leu-β-NA under identical conditions.

These results give clear chemical and histochemical evidence that the enzyme mainly hydrolyzing Gly-Pro-β-NA in liver and kidney tissue is different from those hydrolyzing mono-aminoacid naphthylamides, e.g., Leu-β-NA. It is also different from the dipeptide naphthylamidase described earlier by us (Vanha-Perttula *et al.*, 1965). The enzyme in question represents a new aminopeptidase with unique substrate characteristics. Work is in progress to obtain a highly purified enzyme preparation for further characterization.

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