GLYCYL PEPTIDASE IN HUMAN SERUM AND TISSUES

Apolinary SZEWCZUK, Marian MULCZYK and Andrzej SZCZEKLIK
Institute of Immunology and Experimental Therapy, Polish Academy of Sciences and Third Department of Internal Medicine, School of Medicine, Wrocław, Poland

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1. Introduction

Since Gomori in 1954 described the enzymatic hydrolysis of glycyl-α- and -β-naphthylamides [1], many amino acid as well as peptide naphthylamides have been obtained and used as chromogenic substrates for peptidases. Later it was demonstrated that the naphthylamide substrates are not hydrolysed by known classical peptidases but by enzymes named arylamidases [2,3].

In this paper a colorimetric method for determination of glycyl peptidase activity using new N-glycyl-γ-L-glutamyl-β-naphthyl amide is described. The enzyme was purified from human plasma and evidence is provided that it is different from that hydrolysing glycyl-β-naphthylamide.

2. Experimental

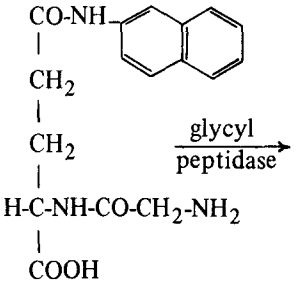
N-Glycyl-γ-L-glutamyl-β-naphthylamide was obtained in two-step synthesis starting from γ-L-glutamyl-β-naphthylamide. To this substrate (0.05 mole) dissolved in N sodium hydroxide (200 ml) and cooled with ice bath chloroacetyl chloride (0.1 mole) was slowly dropped. The obtained N-chloroacetyl-γ-L-glutamyl-β-naphthylamide was precipitated by acidification of the solution with hydrochloric acid and purified by recrystallization from methanol-water mixture (2:1). This chloroacetyl product was dissolved in concentrated ammonia (200 ml) at 37° and after 20 hr the excess of ammonia was removed in vacuum. Then pH of the mixture was brought to 1 with hydrochloric acid and after filtration the final product was precipitated with addition of the sodium acetate excess. It was further purified by dissolving in hot 0.1 N sodium carbonate (500 ml), filtered, and precipitated by slow acidification with 0.2 N acetic acid. The yield of N-glycyl-γ-L-glutamyl-β-naphthylamide was 7.6 g (46% calculated for γ-L-glutamyl-β-naphthylamide) and the melting temperature 232-35°.

For $C_{17}H_{19}N_3O_4$ calculated:

	C: 61.99%	H: 5.82%	N: 12.76%
found	61.62%	5.78%	12.36%

3. Determination of glycyl peptidase activity

The method is based on the following reaction



N-glycyl-γ-L-glutamyl-β-naphthylamide

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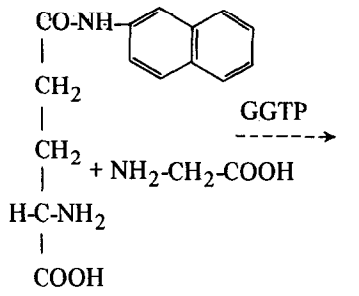
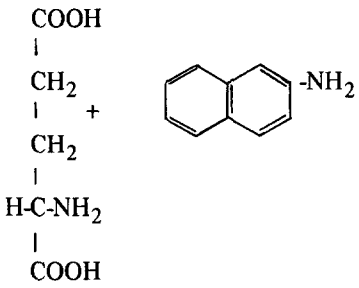


γ-L-glutamyl-β-naphthylamide



β-naphthylamine

Glycyl peptidase hydrolyses one peptide bond in N-glycyl-γ-L-glutamyl-β-naphthylamide liberating glycine and γ-L-glutamyl-β-naphthylamide. The latter product was completely hydrolysed by an excess of γ-glutamyl transpeptidase (GGTP) and the liberated β-naphthylamine was determined colorimetrically.

GGTP was purified from *Pseudomonas aeruginosa* using the following procedure: Twenty hour cultures of *Pseudomonas aeruginosa* PCM*2 on agar plates were washed with saline; the cells were washed twice, suspended in 3 volume of saline and homogenised in a Brown's disintegrator. After centrifugation at 16,000 RPM the supernatant was fractionated with solid ammonium sulphate and material precipitated between 60 and 90% of sulphate saturation was collected. It was dissolved in 0.005 M potassium phosphate buffer pH 6.8 and dialysed against this buffer overnight. Then it was purified on DEAE-cellulose column and fractions eluted with 0.05 M phosphate buffer were collected. The obtained enzyme preparation showed 135-fold higher GGTP activity than that of crude homogenate – determined using γ-L-glutamyl-α-naphthylamide as substrate [4] at pH 8.0. This preparation did not hydrolyse N-glycyl-γ-L-glutamyl-β-naphthylamide or glycyl-β-naphthylamide. Method of glycyl peptidase activity determination: To the solution of 0.25 ml of 0.01 M N-glycyl-γ-L-glutamyl-β-naphthylamide (33 mg was dissolved in 10 ml of hot 0.006 M sodium carbonate) and 0.1 ml of 0.25 M Tris/HCl buffer pH 8.0, 0.05 ml of GGTP (0.4 units/ml) was added. The enzyme reaction was started by the addition of 0.1 ml of serum and the tube was incubated at 37° for 30 min. After the addition of 1.5 ml of 10% acetic acid and 1 ml of 0.05% Fast Blue B, the colour was developed by incubation at 37° for 1 hr and measured at 520 nm. A blank was prepared simultaneously with serum added after acetic acid. With tissue homogenates, the liberated β-naphthylamine was determined using modified Bratton and Marshall procedure [5]. The enzyme activity was expressed in units as the number of micromoles of naphthylamine liberated per 1 min. The determination of glycyl arylamidase activity was performed at pH 7.5 in a similar way as described above with 0.01 M glycyl-β-naphthylamide as substrate and without GGTP.

* Polish Collection of Microorganisms.

4. Glycyl peptidase in human blood serum

A linear relationship between the amount of liberated naphthylamine and time or volume of serum was observed. The higher enzyme activity was noted to be at pH 8.0–8.3. No activity was detected in normal human serum without addition of GGTP from *Pseudomonas aeruginosa*, it was found to rise with increasing amount of GGTP and show its maximum when the activity of GGTP reached 0.0025 unit per sample.

It was demonstrated that the concentration of N-glycyl-γ-L-glutamyl-β-naphthylamide used in the described method (0.005 M) is not sufficient to saturate the enzyme. For this substrate K_m calculated by graphical method of Lineweaver and Burk [6] was found to be 0.008 M.

During storage of serum at 4° for one week a small decrease of glycyl peptidase activity was observed. Heating at 56° for 10 min destroys about 75% of its

activity. This treatment, however, does not affect the glycyl arylamidase activity.

Glycyl peptidase and glycyl arylamidase activity was determined in blood sera of 36 healthy men and women. The mean activity of peptidase was 23 units/1000 ml, SD ± 4.4, and that of arylamidase 21 units/1000 ml, SD ± 5.4. Both enzyme activities were determined in sera of 174 patients treated at the department of internal medicine. A marked elevation of activity amounting in individual cases up to 10-fold the mean value of healthy subjects was found in obstructive jaundice and cancer of the liver. In liver cirrhosis, viral hepatitis and cholecystitis there was a moderate elevation of activity amounting to a 2–3-fold increase. As a rule a similarity was noted to exist between two investigated activities. In some cases, however, a marked elevation of glycyl peptidase activity and normal arylamidase activity was found (fig. 1).

Gel electrophoresis. Six different sera with high enzyme activity were subjected to horizontal starch gel electrophoresis according to Smithies [7]. After completion of separation the gel was cut horizontally into 3 pieces: one was incubated with N-glycyl-γ-L-glutamyl-β-naphthylamide and GGTP, the second with glycyl-β-naphthylamide and the third one was stained for protein with Amido Black B. After incubation the gel pieces were stained with 0.1% Fast Blue B in 0.2 N

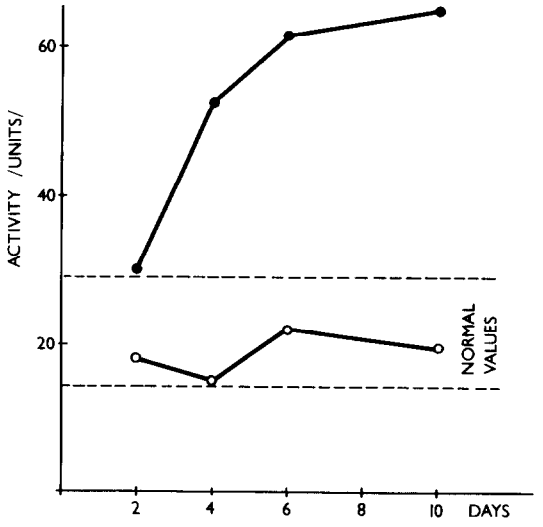


Fig. 1. The course of serum glycyl peptidase –●–●–, and glycyl arylamidase –○–○– activity in a patient with acute cholecystitis.

acetic acid. With the first substrate two glycyl peptidase fractions were visualised. One fraction moved with a mobility corresponding to β-1-globulin, while the second one occupied the region between start and β-lipoprotein. No glycyl arylamidase activity was noted in the region of β-1-globulin, while its activity was observed in fractions corresponding to fast-α-2-globulin, haptoglobins and β-lipoproteins.

Purification of glycyl peptidase from plasma. Fresh human blood was mixed with 0.1 volume of 3.5% sodium citrate and plasma was collected by centrifugation. The enzyme was precipitated from plasma by addition of ammonium sulphate to 35% of saturation at 0°. After centrifugation the precipitate was suspended in water and dialysed against 0.005 M potassium phosphate pH 6.8. After centrifugation the solution was brought on DEAE-cellulose column (1.6 × 22 cm) and the column was washed subsequently with 0.005 M, 0.05 M and finally with 0.15 M phosphate buffer. Fractions containing high enzyme activity were combined and concentrated by ultrafiltration to 4 ml, then the protein solution was filtered through Sephadex G-200 column. Five fractions with high peptidase activity (fig. 2) were combined and used for specificity study. The activity of the obtained preparation was 62 fold higher than that of plasma and yield was 28% (table 1). The activity towards: glycyl-, alanyl-, L-leucyl- and α-L-glutamyl-β-naphthylamide was respectively 1.9, 1.6, 1.5 and 1.7 fold higher as compared with that in plasma.

5. Distribution of glycyl peptidase in human tissues

Glycyl peptidase and glycyl arylamidase activity was found in homogenates of seven different human tissues obtained at the autopsy of two healthy men and a woman killed in street accidents. Marked differences between the two estimated activities were observed in some tissues. For comparison of both enzyme activities the ratio of glycyl peptidase to glycyl arylamidase was calculated. In tissues tested this ratio varied from 11.6 (liver) to 1.3 (skeletal muscle). The obtained data are presented in table 2.

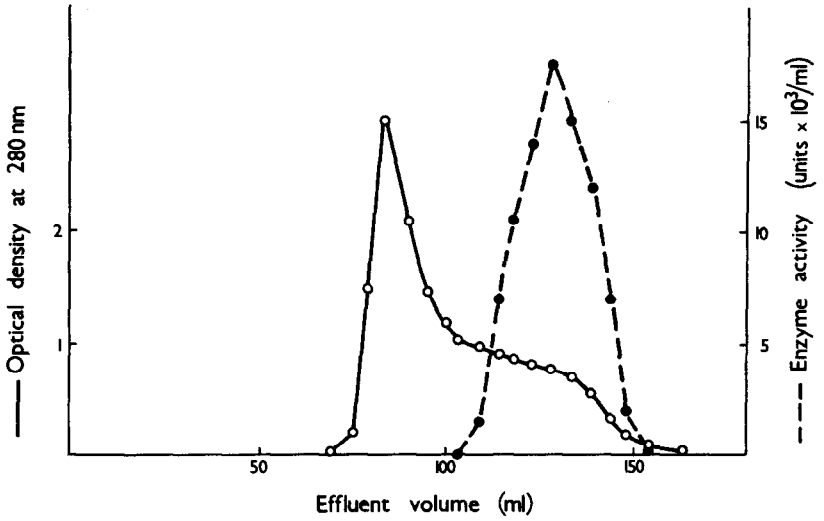


Fig. 2. Purification of glycyl peptidase on Sephadex G-200. Four ml of concentrated preparation were brought on Sephadex column (2.6 X 40 cm) and washed with 0.02 M phosphate buffer with 0.2 M potassium chloride.

Table 1
Purification of glycyl peptidase from human plasma.

	Activity units/g protein	yield (%)
Plasma	0.285	100
I. After ammonium sulphate precipitation	0.74	77
II. After elution from DEAE-cellulose	5.55	52
III. After filtration through Sephadex G-200	17.7	28

Table 2
The activity of glycyl peptidase and glycyl arylamidase in human tissues.

Tissue	Units per 1 g protein		
	glycyl peptidase	glycyl arylamidase	peptidase arylamidase
Kidney	131	37	3.5
Liver	44	3.8	11.6
Lung	9.1	1.3	7.0
Brain	27	7.3	3.7
Pancreas	16.6	2.9	5.7
Skeletal muscle	9.0	6.7	1.3
Heart muscle	11	2.4	4.4

6. Discussion

The problem of existence and specificity of peptidases has been discussed during many years. Recently evidence has accumulated to indicate the existence of highly specific peptidases. Patterson et al. [2] found the differences between the well known leucylamide and leucylglycine splitting-peptidase and the leucyl-β-naphthylamide hydrolysing-enzyme, named by these authors leucyl arylamidase. Similarly, our results indicate the existence of at least two N-glycyl peptide bond hydrolysing-peptidases. The enzyme that splits off glycine from N-glycyl-γ-L-glutamyl-β-naphthylamide, named glycyl peptidase, was found to be different from that hydrolyzing glycyl-β-naphthylamide, named in this paper glycyl arylamidase. These two enzymes present in human blood plasma were separated by precipitation with ammonium sulphate or by starch gel electrophoresis. The activity of glycyl arylamidase as compared with glycyl peptidase was also more stable when heated at 56° or stored at 4°. Clinical studies showed marked differences between both enzyme activities in hepatobiliary diseases. The additional evidence that the activities are connected with different enzymes was obtained in studies on distribution of the enzymes in human tissues.

At present, it is not possible to ascertain whether the described glycyl peptidase, liberating glycine from

N-glycyl-γ-L-glutamyl-β-napthylamide is different from the known peptidases which hydrolyse glycyl-glycine, glycylleucine or other glycyl peptides. Further studies on this subject are in progress in our laboratory.

References

[1] G.Gomori, Proc. Soc. Exper. Biol. Med. 87 (1954) 559.
[2] E.K.Patterson, A.Kappel and S.H.Hsiao, J. Histochem. Cytochem. 9 (1961) 609.
[3] W.O'Connel and R.J.Winzler, Cancer Research 23 (1963) 78.
[4] M.Orfowski and A.Szewczuk, Clin. Chim. Acta 7 (1962) 755.
[5] A.C.Bratton and E.K.Marshall, J. Biol. Chem. 128 (1939) 537.
[6] H.Lineweaver and D.Burk, J. Amer. Chem. Soc. 56 (1934) 658.
[7] O.Smithies, Biochem. J. 74 (1959) 585.