THE USE OF α-(N-γ-DL-GLUTAMYL)-AMINONITRILES FOR THE COLORIMETRIC DETERMINATION OF A SPECIFIC PEPTIDASE IN BLOOD SERUM

APOLINARY SZEWCZUK AND MARIAN ORLOWSKI

Department of Biochemistry, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences and Third Medical Clinic, Wroclaw (Poland)

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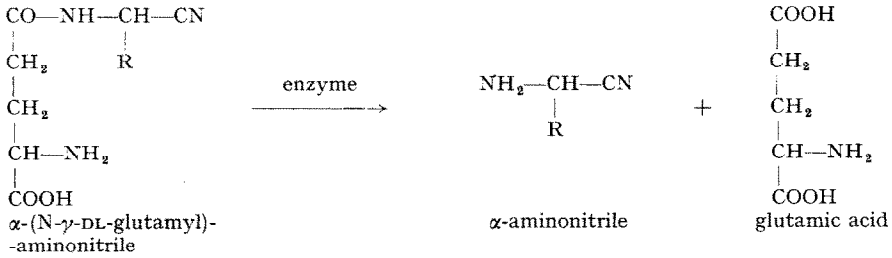
The presence in blood serum of enzymes which hydrolyse peptides has been known for more than 50 years. For the assay of the activity of these enzymes di- and tripeptides are most frequently used, and the products of enzymic hydrolysis are estimated by alkalimetry[1] or by the colorimetric ninhydrin method[2], often after their separation from the substrates by paper chromatography[3]. GOMORI proposed some years ago that chromogenic substrates obtained by combining various amino acids through their carboxyl group with β-naphthylamine, should be used[4].

In this paper we describe the presence in blood serum of an enzyme capable of hydrolysing α-(N-γ-DL-glutamyl)-aminonitriles. We also provide evidence to show that the enzymic reaction proceeds according to the following equation:



The following γ-glutamyl derivatives were tested for enzymic hydrolysis:

N-(γ-DL-glutamyl)-aminoacetonitrile	(R = H)
α-(N-γ-DL-glutamyl)-aminopropionitrile	(R = CH_3)
α-(N-γ-DL-glutamyl)-aminobutyronitrile	(R = C_2H_5)

For enzymic activity measurements α-(N-γ-DL-glutamyl)-aminopropionitrile was used as substrate.

The assay method is based on the determination of the free α-aminopropionitrile which is formed in addition to glutamic acid during the enzymic hydrolysis of α-(N-γ-DL-glutamyl)-aminopropionitrile. On reaction with bromine, α-aminopropionitrile gives an unidentified substance which, in presence of pyridine and benzidine hydrochloride, produces an intensive red color suitable for colorimetric determination[5]. In the described procedure as little as 0.2 μg of α-aminopropionitrile per 1 ml could be estimated.

Some results obtained suggest that the described peptidase differs from other known peptidases in serum. The activity of the enzyme changes in some pathological conditions.

MATERIALS

Synthesis of α-(N-γ-DL-glutamyl)-aminopropionitrile

This compound was obtained by a method similar to that described by KING AND KIDD[6]. The starting materials were phthaloyl-DL-glutamic anhydride (obtained according to KING AND KIDD) and fresh redistilled α-aminopropionitrile (obtained by the method of COOK AND LEVY[7]). To a chilled solution of 0.015 mole of phthaloyl-DL-glutamic anhydride in 8 ml of dry dioxane, 0.03 mole of α-aminopropionitrile was added. The mixture was stirred occasionally for 1 h at room temperature; the oily product was precipitated by the addition of 30 ml of ethyl ether and then dissolved in 10 ml of 10% sodium carbonate. 3 ml of 24% hydrazine were added and the solution was left for two days. It was then acidified with 2 *N* hydrochloric acid using Congo red as indicator, filtered under suction and the precipitate was washed with 10 ml of water. The combined filtrates were shaken with 8 g of silver oxide to remove chlorides. After filtering, the solution was neutralized to pH 7.0 with fresh distilled hydriodic acid, filtered and condensed under reduced pressure at 45°. A product crystallized from the obtained syrup after the addition of 20 ml of 99% ethanol. It was further purified by dissolving in a small amount of water and precipitating with four volumes of ethyl alcohol. The yield was 1.6 g of α-(N-γ-DL-glutamyl)-aminopropionitrile with a melting point at 193–194° (with decomposition). This was used for the assay of enzyme activity. The properties and identification of α-(N-γ-DL-glutamyl)-aminopropionitrile as well as N-(γ-DL-glutamyl)-aminoacetonitrile and α-(N-γ-DL-glutamyl)-aminobutyronitrile have already been described[8]. α-(N-acetyl)-aminopropionitrile was obtained by the method of DELEPINE[9]. Buffers: Tris (tris(hydroxymethyl)-aminomethane), 2-amino-2-methyl-1,3-propanediol, veronal and boric acid–borax buffers were prepared according to GOMORI[10] (all 0.1 *M*). Potassium hydrogen phosphate–borax buffer was prepared according to KOLTHOFF[11].

ASSAY METHOD

Reagents

Sodium arsenite: 2 g of arsenic trioxide were dissolved in 100 ml of hot 0.1 *N* NaOH and cooled to room temperature. Water saturated with bromine. 10% Trichloroacetic acid. Pyridine solution: 120 ml of pyridine were mixed with 80 ml of water and then 20 ml of concentrated hydrochloric acid were added with cooling. Benzidine hydrochloride solution: 1.8 g of pure benzidine were dissolved with heating in 50 ml of 0.5 *N* HCl and then chilled. This solution was prepared fresh daily. Benzidine–pyridine mixture: this was prepared just before use by mixing one volume of benzidine hydrochloride solution with 3 volumes of pyridine solution. Standard cyanide solution: 0.65 g of potassium cyanide were dissolved in 1000 ml of 1 *N* NaOH. The concentration of this solution was determined by the method of LIEBIG AND DÉNIGÈS[12]. The solution was diluted just before use with 0.01 *N* NaOH to obtain exactly a 0.0001 *M* KCN solution. 0.01 *M* Mercuric chloride solution. Substrate solution: 100 mg of α-(N-γ-DL-glutamyl)-aminopropionitrile was dissolved in 10 ml of water. This solution was stored in a refrigerator.

Determination of enzyme activity

0.25 ml of tris buffer pH 8.8 and 0.2 ml of substrate solution were mixed in a centrifuge tube and placed in a water bath at 37°. The reaction was started by the addition of 0.25 ml of serum. After exactly 120 min the reaction was stopped by the addition of 1.3 ml of 10% trichloroacetic acid. A blank was prepared simultaneously in the same way, except that the serum was added after the trichloroacetic acid. The precipitate was removed by centrifugation and 1 ml of the clear supernatant solution was transferred to a glass-stoppered test-tube (10 ml capacity) for the determination of α-aminopropionitrile. Afterwards 0.1 ml of 0.01 *M* mercuric chloride and 0.25 ml of water saturated with bromine were added and the tube was stoppered and placed in a water bath at 37° for 3 h. After this time the tube was cooled and opened, and excess of bromine was decomposed by the addition of 0.25 ml of sodium arsenite, the remaining bromine vapours being removed by a stream of air. 3.4 ml of benzidine–pyridine mixture were then added and the tube was left after mixing for 15 min; this was required for full colour development. The optical density of the solution was measured at 530 mμ with a green filter in a Pulfrich photometer against the blank. The colour is stable for about 30 min.

Calibration curve and calculation of activity

It has been shown[5] that the estimated amount of pure α-aminopropionitrile (like other α-aminonitriles) has the same optical density as an equivalent amount of potassium cyanide. Therefore it is possible to calculate the amount of α-aminopropionitrile formed during the enzymic hydrolysis from a calibration curve prepared with the readily available potassium cyanide instead of the α-aminopropionitrile which is unstable in the pure state.

To a series of test-tubes 0.1 to 0.5 ml portions of 0.0001 *M* potassium cyanide were pipetted and each solution was diluted to 0.5 ml with water. 0.5 ml 10% trichloroacetic acid and 0.25 ml of water saturated with bromine were then added and mixed, and excess bromine was decomposed by the addition of 0.25 ml of sodium arsenite. The remaining bromine vapours were removed with air and the red colour was developed by the addition of 3.5 ml of benzidine–pyridine mixture. The optical density was determined as described above for α-aminopropionitrile.

α-Aminopropionitrile is unstable and undergoes a partial decomposition during the enzymic incubation. Therefore it was necessary to introduce a correction factor to account for the whole amount of α-aminopropionitrile formed. The correction factor was found to be 1.14 (see under EXPERIMENTAL RESULTS). The enzyme activity was expressed as the number of μmoles of α-aminopropionitrile liberated per 100 ml of serum under the described conditions. Thus:

$$\text{Enzyme units} = 800 \times \mu M \text{ KCN (found from calibration curve)} \times 1.14$$

EXPERIMENTAL RESULTS

It was established experimentally that the addition of mercuric chloride to the sample after deproteinisation is necessary to remove the interference of non-protein substances (probable SH compounds) present in serum on the determination of α-aminopropionitrile.

Calculation of the correction factor

The incubation of pure samples of α-aminopropionitrile (before reaction with bromine) under the conditions described for the enzyme assay causes a non-enzymic loss of this substance. This loss depends on the initial concentration of α-aminopropionitrile and proceeds according to a first order reaction. The velocity constant of this reaction (k_1) calculated from the time required for the loss of 50% of α-aminopropionitrile ($t_{1/2}$ = 300 min) is $3.85 \cdot 10^{-5}$ sec^{-1} (Fig. 1)

The formation of α-aminopropionitrile from the enzymic hydrolysis of α-(N-γ-DL-glutamyl)-aminopropionitrile occurs in a situation of "zero-order" kinetics (see under *Substrate concentration*). When both these simultaneous reactions are considered,

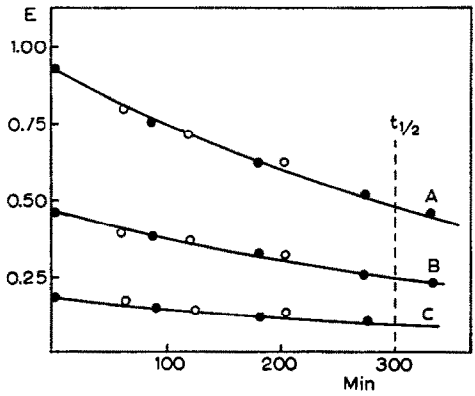


Fig. 1. The loss of α-aminopropionitrile (expressed as optical density) as a function of incubation time at 37°: ● incubated with serum and tris buffer pH 8.8; ○ incubated without serum. A = 0.1 μM, B = 0.05 μM, C = 0.02 μM of α-aminopropionitrile per 0.7 ml incubation mixture.

the velocity of formation (v) of the measured amount of α-aminopropionitrile (p) may be expressed by the following equation:

$$v = \frac{dp}{dt} = k_0 - k_1 p \qquad (1)$$

where k_0 is the velocity constant of the enzymic reaction.

After integration and substitution of p_1/t for k_0 the following relation is obtained:

$$\frac{p_1}{p} = \frac{e^{k_1 t}\, k_1 t}{e^{k_1 t} - 1} \qquad (2)$$

where p_1 is the amount of α-aminopropionitrile formed in the enzymic reaction, t is the enzymatic incubation time and e = 2.718.

p_1/p is the correction factor by which the measured amount of α-aminopropionitrile must be multiplied in order to obtain the amount of α-aminopropionitrile formed in the enzymic reaction. Substitution in eqn. (2) of t = 120 min = 7200 sec and $k_1 = 3.85 \cdot 10^{-5}$ sec^{-1} gives p_1/p = 1.14. This is the correction factor introduced for calculation of enzyme activity.

The identification of products of enzymic hydrolysis of α-(N-γ-DL-glutamyl)-aminopropionitrile

α-Aminopropionitrile. The identification of α-aminopropionitrile as a product of enzymic hydrolysis of α-(N-γ-DL-glutamyl)-aminopionitrile is based on the following

observations: (*a*) The absorption spectrum of the dye obtained from the product after reaction with bromine is identical with that obtained from pure α-aminopropionitrile or cyanide treated in the same way. (*b*) The red dye can be produced from cyanide already after a several seconds incubation time with bromine[13]. α-Aminopropionitrile as well as the product of enzymic reaction must react with bromine for a longer period to give this dye. This indicates that only α-aminopropionitrile is liberated during the enzymic hydrolysis of the substrate.

Glutamic acid. The second product of the enzymic reaction was identified as glutamic acid by chromatography on Whatman No. 1 paper in two different solvent mixtures: (*a*) phenol saturated with water, and (*b*) butanol–acetic acid–water (4 : 1 : 5). The incubation of sera with L-glutamine as substrate did not yield appreciable amounts of glutamic acid.

Buffers

The rates of enzymic hydrolysis were investigated in 5 different buffers at pH 8.6. The highest activity was noted in tris and 2-amino-2-methyl-1,3-propanediol buffers. Lower activities were found in veronal, boric acid–borax and potassium hydrogen phosphate–borax buffers.

pH

The optimum pH for hydrolysis of α-(N-γ-DL-glutamyl)-aminopropionitrile was examined with tris buffer over the pH range 7.3 to 8.85. A rather sharp optimum was found between pH 8.5 and 8.7. The same pH optimum was also found in potassium hydrogen phosphate–borax buffer.

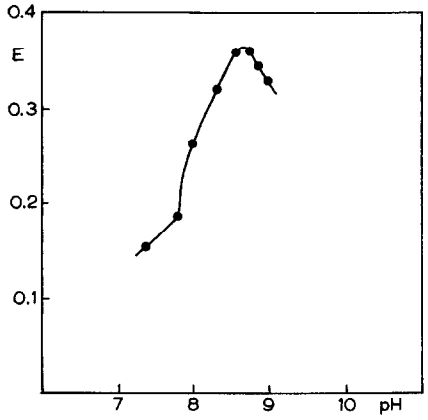


Fig. 2. The effect of pH on enzyme activity (expressed as optical density).

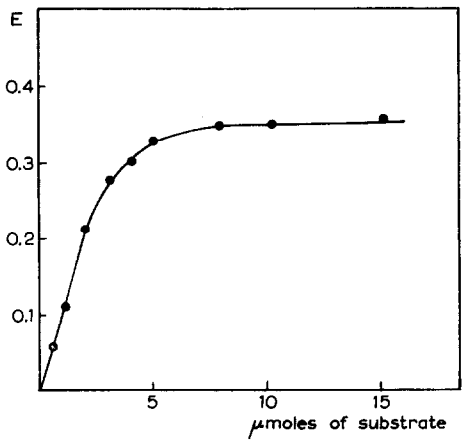


Fig. 3. Rate of hydrolysis of α-(N-γ-DL-glutamyl)-aminopropionitrile as a function of concentration of substrate.

Temperature

The influence of temperature on the rate of enzymatic hydrolysis was examined by the described method on two different sera at 21, 30, 37, 45 and 50°. Maximal activity was observed at 30 and 37°.

Time of incubation

With constant amounts of serum, the quantity of liberated α-aminopropionitrile was proportional to the incubation time if corrected according to eqn. (2) for different incubation times.

Amount of serum

The hydrolysis of the substrate as a function of the concentration of enzyme was investigated with 0.05 to 0.25 ml of serum and a linear relationship was found. If the activity of an unknown sample was too great to give correct readings in the photometer the assay was repeated using an appropriate dilution of the serum.

Substrate concentration

The rate of enzymic hydrolysis was measured with various concentrations of α-(N-γ-DL-glutamyl)-aminopropionitrile from 0.5 to 15.0 μmoles per 0.7 ml of incubation mixture. With increasing amounts of substrate the rate of reaction increased progressively. With substrate concentrations higher than 8 μmoles per 0.7 ml the rate of reaction was practically independent of the substrate concentration and proceeded according to zero-order kinetics. All determinations of activity were therefore carried out with 10 μmoles of substrate.

Stability of enzyme

Heating the serum for 60 min at 45° before the determination did not change the activity. 70–80% of the activity was lost on heating the sample at 55° for 1 h. Heating the serum for 5 min at 65° destroyed the activity. No change in activity was observed after storage of the serum for two days at room temperature or for two weeks at 4°. No change in activity was found on dialysis of the serum against 0.9% NaCl for several hours, nor after lyophilisation.

Inhibitors and activators

The following metals were tested in a final concentration of $1.5 \cdot 10^{-3}$ *M* as sulfates or chlorides: Mg^{+2}, Ca^{+2}, Mn^{+2}, Zn^{+2}, Hg^{+2}, Co^{+2}, Al^{+3} and Fe^{+3}. A 23% inhibition was found with this concentration of Co^{+2}, but all the others were without effect. Final concentrations of 10^{-2} *M* of the amino acids β-alanine, L-leucine, phenyl-DL-alanine and L-arginine were also without effect. Some inhibition was observed with DL-α-alanine and L-glutamine (20% and 22% respectively). An inhibition of about 65% was obtained with N-ethylmaleimide. EDTA did not cause any inhibition. Glycylglycine activated about 57% at the same concentration.

Assay of different substrates

The rate of enzymic hydrolysis of different γ-glutamyl derivatives was tested and compared with the rate of hydrolysis of α-(N-γ-DL-glutamyl)-aminopropionitrile which was taken as 100%. A higher rate was observed with α-(N-γ-DL-glutamyl)-aminobutyronitrile (108%), whereas the hydrolysis of N-(γ-DL-glutamyl)-aminoacetonitrile was very slow (13%). This indicates that the length of the chain in the α-aminonitrile residue is not without influence on the enzyme action. It was found that α-(N-acetyl)-aminopropionitrile is not hydrolyzed by the enzyme.

CLINICAL RESULTS

Normal human subjects

The activity of the enzyme was determined in sera of 41 healthy subjects (25 men and 16 women) between the ages of 3 and 65 years. Blood samples were obtained by puncture of the cubital vein and the serum was collected after formation of the clot. The results obtained are shown in Table I.

TABLE I

ENZYME ACTIVITY IN SERA OF HEALTHY SUBJECTS AND IN PATIENTS WITH OBSTRUCTIVE JAUNDICE AND VIRAL HEPATITIS

Enzyme source	*No. Specimen*	*Range units*	*Mean units*	*Standard deviation*	*Standard error of the mean*
Normal human subjects	41	4.5–26.2	10.9	4.7	0.73
Men	25	5.1–26.2	11.7	5.5	1.1
Women	16	4.5–17.8	9.75	3.76	0.94
Obstructive jaundice	12	150–340	222	65.5	18.9
Viral hepatitis	13	29–86	59	23	6.4

The mean value of activity in the healthy group was 10.9 units. No significant differences were found between men and women. No correlation was found between age and enzyme activity. The same activity was noted in blood samples taken before and after a meal.

Enzyme activity in patients

In Table I are also included results obtained for enzyme activity in patients with obstructive jaundice and viral hepatitis. The activity was higher in both groups than in normal subjects, but significantly higher values were observed with obstructive jaundice than with viral hepatitis. Therefore the assay of the enzyme in serum may be valuable for differential diagnosis of these two diseases. Some other results of activity determinations in sera of patients with miscellaneous diseases are given in Table II.

A detailed report on the application of the enzyme assay for clinical diagnosis will be the subject of another communication.

Enzymic activity was found in human urine and pathological exudations as well as in serum. Of interest is the absence of enzymic activity in human red blood cells (for activity determinations 25% hemolysates of red cells from three healthy subjects and two patients with liver disease were used).

Sera of animals

The activity of the peptidase was also investigated in the sera of some animals. The highest activity was found in sera of sheep (36.5 units). Considerable activity was exhibited by the serum of cow (14 units) and cock (9.4 units). Low activity was found in serum of horse (5.2 units), dog (6 units), rabbit (3.3 units) and guinea pig (3.7 units). The sera of rats and pigeons were practically free of enzymic activity.

The enzyme were also found in extracts of liver and kidney of several animal species. Virtually no activity was found in extracts of skeletal muscles of rat, pigeon and rabbit.

TABLE II

ENZYME ACTIVITY IN SERA OF PATIENTS WITH MISCELLANEOUS DISEASES

Case No.	*Age*	*Sex*	*Diagnosis*	*Activity units*
1	50	M	Cholelithiasis. Cirrhosis biliaris	1400
2	46	F	Cholelithiasis. Cirrhosis biliaris	700
3	48	M	Cholelithiasis. Cirrhosis biliaris	550
4	55	M	Carcinoma bronchi. Metastases ad hepar. Subicterus	1100
5	62	F	Carcinoma hepatis primarium cum icteru	1000
6	52	F	Carcinoma hepatis primarium sine icteru	465
7	59	F	Carcinoma hepatis primarium sine icteru	380
8	57	M	Carcinoma capitis pancreatis	382
9	55	M	Carcinoma renis	140
10	40	F	Carcinoma colli uteri	11
11	56	M	Carcinoma ventriculi	16
12	24	F	Myelosis chronica	28.5
13	66	F	Lymphadenosis chronica	19
14	55	M	Infarctus myocardii	28
15	45	M	Cirrhosis hepatis atrophica	22
16	24	M	Vitium mitrale decompensatum	38
17	22	F	Thyreotoxicosis	48
18	53	F	Haemorrhagia cerebri	50
19	44	M	Schizophrenia	20
20	62	M	Asthma bronchiale	14

DISCUSSION

It is not likely that α-aminonitrile derivatives of glutamic acid are natural substrates for the investigated enzyme, since these compounds have not hitherto been found to occur in animals or plants. The enzymic hydrolysis of such compounds should be therefore rather interpreted as an indication of their structural resemblance to natural substrates. The γ-glutamyl peptidic bonds are known to occur in such peptides as glutathione, ophthalmic acid and many γ-glutamyl-amino acids, and the probability must be considered that the ability to hydrolyse γ-glutamyl derivatives of α-aminonitriles is associated with an enzyme capable of splitting off glutamic acid from γ-glutamyl peptides or amino acids. Support for this concept is provided by the finding that the γ-glutamyl linkage is resistant to the action of various ordinary proteolytic enzymes[14–16]. Some of our findings support the view that the investigated enzyme is a specific peptidase, which is different from the known peptidases present in serum. The described peptidase is not present in human red blood cells, while other known peptidases are present in these cells and exhibit an activity about 40 times higher than in serum[17]. The plasma of rats is known to contain a peptidase activity toward various peptides with an α-peptide linkage, while the α-(N-γ-DL-glutamyl)-aminopropionitrile is not split by rat serum. The majority of known peptidases present in serum have a pH optimum about 7.4 and are activated by Mg^{+2}, Mn^{+2} or Co^{+2}, while the described enzyme exhibits a pH optimum between 8.5 and 8.7 and is not activated by these metals; on the contrary some inhibition with Co^{+2} was found. The possible identity of the enzyme with glutaminase was excluded by paper chromatography.

The activating effect of glycylglycine may be interpreted as an indication of a transpeptidation function of the described enzyme, or by the presence in serum of γ-glutamyl transpeptidase which was described in animal tissues by HANES *et al.*[18].

Further work on the purification and isolation of the enzyme is in progress; it will enable a more detailed definition of the described enzyme and its specificity. Work is also going on with substrates obtained by combining other amino acids and peptides with α-aminonitriles and their use as chromogenic substrates for a more general and convenient method for colorimetric determination of different peptidases in serum and tissues.

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SUMMARY

The occurrence of a specific peptidase in human serum is described. The enzyme catalyses the hydrolysis of α-(N-γ-DL-glutamyl)-aminopropionitrile as well as of γ-glutamyl derivatives of other α-aminonitriles. A method is described for assay of the enzyme activity, based on the colorimetric determination of α-aminopropionitrile liberated during the enzymatic incubation; as little as 0.2 μg of α-aminopropionitrile can be estimated. Some properties of the enzyme were investigated and its specificity is discussed. The activity in sera of normal human subjects was 10.9 units (1 unit = 1 μmole α-aminopropionitrile liberated from the substrate per 100 ml serum after a 2-h incubation at pH 8.6 and 37°). Changes in the activity of the peptidase were found in some pathological conditions. Besides human serum, the enzyme activity was found in sera of many animals, as well as in human urine and pathological exudations. No activity was found in human red blood cells.

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