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A Novel Fluorometric Ultramicro Determination of Serum γ-Glutamyltranspeptidase Activity[1)]

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A simple and highly sensitive rate assay of serum γ-glutamyltranspeptidase activity is described, using a novel fluorogenic substrate, 7-(γ-L-glutamyl)-4-methylcoumarinylamide. The reaction is initiated by adding 20 μl of serum, and fluorescence development in 1 min due to the 7-amino-4-methylcoumarin liberated at 37°C is followed directly on a recorder. A close correlation was found between activities measured by the fluorometric method and by a conventional method using a chromogenic substrate, γ-L-glutamyl-*p*-nitronanilide.

Keywords—7-amino-4-methylcoumarin; 7-(γ-L-glutamyl)-4-methylcoumarinylamide; fluorescent amine; fluorometric enzyme assay; clinical determination; γ-glutamyltranspeptidase

γ-Glutamyltranspeptidase (γ-GTP, EC 2.3.2.2) is an enzyme that catalyzes transfer of the γ-glutamyl group from glutathione or from certain other γ-glutamylpeptides to suitable acceptors. The enzyme is widely distributed in animal tissues and organs and, though its physiological function is not yet clear, a rise in serum γ-GTP activity is observed almost exclusively in diseases of the liver, bile ducts, and pancreas.[2)] Thus, the determination of its activity in human blood serum is of great importance in the diagnosis of hepatobiliary diseases[3)] including alcoholic hepatitis.[4)] Some colorimetric[5)] and, more recently, fluorometric methods[6)] are known for the determination of γ-GTP.

In the course of our broadly based studies of organic fluorescence reagents,[1,7)] we have been aware of the particular usefulness of amino coumarin derivatives as fluorophores and a study has been undertaken to develop a series of fluorescence reagents employing 7-amino-4-methylcoumarin (AMC; **1**) as a key fluorophore. Thus, several AMC amides of appropriate amino acid derivatives have been synthesized[8)] and successfully employed for laboratory and clinical assays of corresponding proteolytic enzymes: *e.g.*, leucine aminopeptidase,[1a,9)] trypsin and papain[10)] and cystine aminopeptidase.[1b)] As an extension of this work, the synthesis of 7-(γ-L-glutamyl)-4-methylcoumarinylamide (GCA) and its use for the microdetermination of human serum γ-GTP activity are described in the present paper.

Materials and Methods

Apparatus

Fluorescence intensity and spectra were measured with a Hitachi model 203 fluorescence spectrophotometer connected to a Hitachi QPD 53 recorder and a Hitachi MPF-3 fluorescence spectrophotometer, respectively.

Materials

The enzyme source was human sera obtained from healthy people, unless otherwise stated. Pathological sera were obtained from patients admitted to the Liver Unit of the Juntendo Hospital. Glycylglycine was purchased from Wako Pure Chemicals. The other chemicals used were of analytical grade.

Synthesis of GCA[8)]

7-Amino-4-methylcoumarin (**1**; **AMC**)——3-Carbethoxyaminophenol[11)] (8.06 g; 50 mmol) and ethyl acetoacetate (7.81 g, 60 mmol) were suspended in 75% H_2SO_4 (40 ml) and the mixture was stirred at room

temperature for 4 h. The whole was poured into ice-water (300 ml) and the precipitate was collected and washed with cold water, and recrystallized from EtOH to give 7-carbethoxy-amino-5-methylcoumarin, 10 g (81%), mp 185—187°C (lit., 186—188°C).[11] The protected aminocoumarin (4.94 g, 20 mmol) was mixed with conc. H_2SO_4 (20 g) and AcOH (20 g) and the mixture was refluxed for 3 h. After cooling, the mixture was poured into ice-water (100 ml) and the solution was made alkaline with 50% NaOH under cooling. The precipitate was collected, washed with water and recrystallized from EtOH to provide colorless prisms of mp 221—224°C (lit., 223°C),[11] 2.87 g (82%).

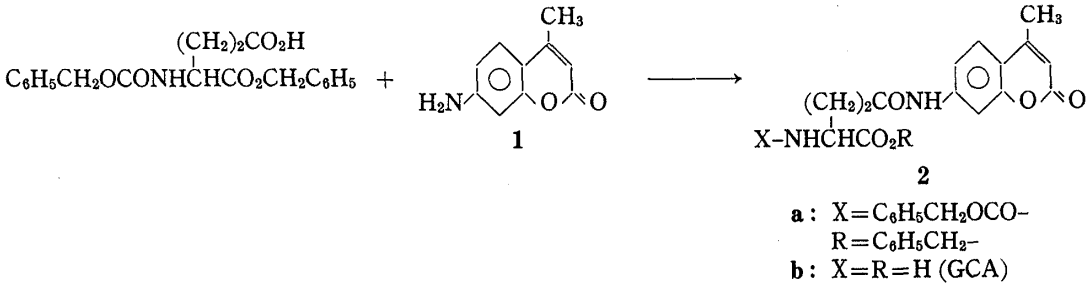

Chart 1

7-(N^{α}-Benzyloxycarbonyl-α-benzyl-γ-L-glutaminyl)-4-methylcoumarinylamide (2a)——Isobutyl chloroformate (411 mg, 3 mmol) was added to a solution of N^{α}-benzyloxycarbonyl-α-benzyl-glutamic acid (1.1 g, 3 mmol) and triethylamine (303 mg, 3 mmol) in tetrahydrofuran (5 ml) at −5°C under stirring. The mixture was stirred for 10 min at −5°C, then a solution of 7-amino-4-methylcoumarin **1** (525 mg, 3 mmol) in dimethylformamide (5 ml) was added and the whole was stirred under cooling for 1 h then at room temp. for 4 h. The solvent was evaporated off *in vacuo* and the residue was dissolved in $CHCl_3$ (15 ml). The solution was washed successively with 10% HCl, saturated $NaHCO_3$, and dried (Na_2SO_4), and the solvent was evaporated off. Recrystallization of the residue from AcOEt gave colorless fine needles, mp 153—156°C, 984 mg (62%). *Anal.* Calcd for $C_{23}H_{22}N_2O_7$: C, 63.01; H, 5.06; N, 6.39. Found: C, 62.86; H, 5.17; N, 6.20.

7-γ-L-Glutamyl-4-methylcoumarinylamide (GCA; 3b)——**2a** (1058 mg, 2 mmol) was hydrogenated in 70% AcOH (50 ml) with 10% Pd-C (500 mg) for 4 h. The catalyst was filtered off, and the filtrate was evaporated to dryness *in vacuo* and the residue was recrystallized from H_2O to yield GCA (**3b**) as colorless fibers of mp 212—215°C (dec.), 413 mg (68%). $[\alpha]_D^{20}=+12°$ ($c=0.8$ in 0.2 M Na_2CO_3 containing 20% of DMSO), $\lambda_{max}^{H_2O}$ 326 nm ($\varepsilon=15200$). *Anal.* Calcd for $C_{15}H_{16}N_2O_5$: C, 59.20; H, 5.30; N, 9.21. Found: C, 58.97; H, 5.42; N, 9.05.

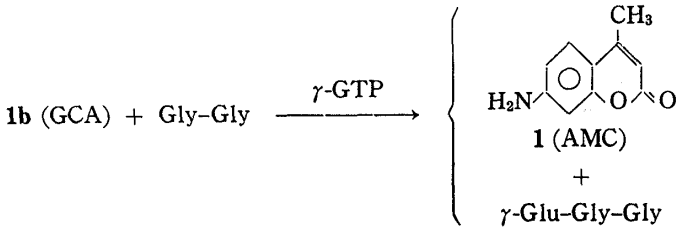

Chart 2

Method

Fluorometric assay of γ-GTP was carried out at 37°C as follows: the reaction was started by adding 20 μl of human serum to 2.0 ml of the reaction mixture containing 0.08 mM GCA, 20 mM glycylglycine, and 50 mM phosphate buffer, pH 8.0, in a 10×10 mm quartz cuvette kept at 37°C. Increase in fluorescence due to the formation of the product, AMC, was directly recorded on a chart for 60 s at a chart speed of 50 mm/min using a Hitachi 203 fluorescence spectrophotometer, excited with the 365 nm line of a mercury lamp and measured at 460 nm.

The fluorescence development was completely linear for at least 3 min even in the cases of sera with the highest values for γ-GTP activity; nearly 460 I.U./l. The coefficient of variance (C.V.) of the fluorometry was 0.95% using a serum with 304 I.U./l (five measurements).

As for the unit of enzyme activity, the maximum velocity was calculated from the fluorescence increment, f, of the sample in 60 s using the following equation

$$\text{I.U./l} = f/s \times 2 \times 50 \times 4.6 \text{ mol/min/l of serum}$$

The symbol s is the fluorescence of 2.0 ml of 1 μM AMC standard solution with 20 μl of the same serum. The last multiplier, 4.6, is the factor converting the velocity at the substrate concentration used, 0.08 mM, to the maximum velocity, since the value of Km was 0.29 mM (see below).

Results and Discussion

Fluorescence intensity of the substrate, GCA, when excited at 365 nm was negligible (below 0.5%) as compared with that of the product, AMC. Fluorescence excitation and emission spectra of GCA are shown in Fig. 1.

It was found that the concentration of glycylglycine as an acceptor of the γ-glutamyl moiety was saturated at 10—20 mM under the conditions used (Fig. 2). In the presence of 20 mM glycylglycine, K_m of human serum γ-GTP was found to be 0.29 mM, which was the lowest among the values of K_m, as shown in Table I. It is not clear at present whether the low K_m value is due to the structure of GCA or the conditions used for determining K_m, *i.e.*, very low substrate concentrations from 0.01 to 0.08 mM.[14] In the table, the values of K_i are also shown for oxidized glutathione, a potent inhibitor of the enzyme, measured by fluorometry and by one of the colorimetry procedures for comparison. The colorimetry was carried out at pH 8.5 with a "γ-GTP test pack, Sankyo" based on the method of Orlowski,[5] containing 5 mM γ-L-glutamyl-*p*-nitroanilide, 28.3 mM glycylglycine, and polyoxyethylene nonylphenol

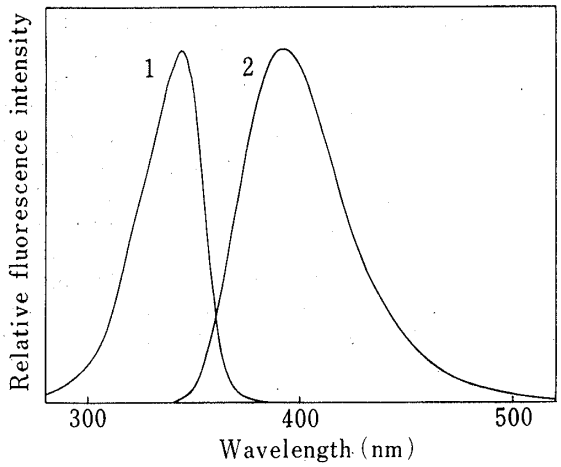

Fig. 1. Fluorescence Excitation (1) and Emission (2) Spectra of GCA in 50 mM Phosphate Buffer (pH 7.0)

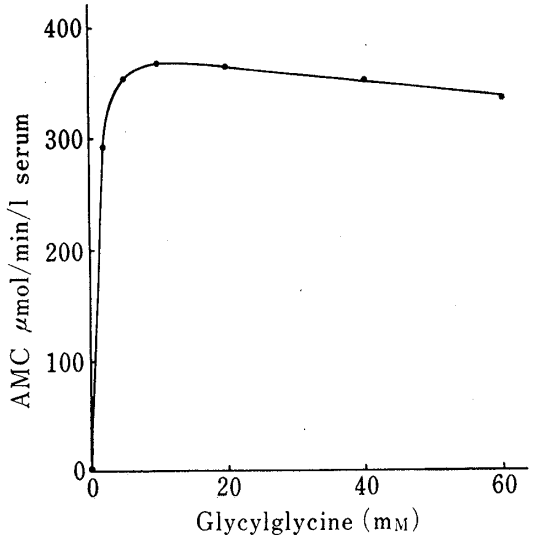

Fig. 2. Dependence of γ-GTP Activity on the Concentration of Glycylglycine

Conditions: 0.08 mM GCA at pH 8.0 and 37°C. Excitation 365 nm and emission 460 nm.

TABLE I. K_m Values of Human Serum γ-Glutamyltranspeptidase and K_i Values of Glutathione

Substrate	K_m (mM)	pH	temp.	GG (mM)	reference
GCA	0.293±0.079(16)	8.0	37	20	This paper
GNA	0.96	8.2	25	20	Szasz[2]
GNA	1.05	7.6	37	50	Stromme *et al.*[12]
GNA	1.83±0.06	8.25	30	40	Shaw *et al.*[13]
GCNA	1.19±0.04	8.25	30	40	Shaw *et al.*[13]
	K_i (mM) for oxidized glutathione				
GCA	0.638±0.053(12)	8.0	37	20	This paper
GNA	0.918±0.074(4)	8.5	37	28.3	This paper
DL-GNA	0.31	8.7	38	0	Goldbarg *et al.*[14]

GG, glycylglycine; GNA, γ-L-glutamyl-*p*-nitroanilide; GCNA, γ-L-glutamyl-3-carboxy-4-nitroanilide. K_m values were determined from s/v *vs.* s plots (Woolf) at substrate concentrations from 0.01 mM to 0.08 mM GCA in the presence of 20 mM glycylglycine at pH 8.0 and 37°C. K_i values of oxidized glutathione were obtained from Dixon plots in the presence of 0.04 mM and 0.08 mM GCA or 0.4 mM and 0.8 mM GNA under the conditions described above.

ether as a solubilizer for the substrate. As can be seen in the table, the K_i value measured by fluorometry was smaller than that obtained by colorimetry.

Figure 3 shows the correlation between the serum γ-GTP activities of 58 patients with hepatobiliary diseases measured by fluorometry and by colorimetry as described above (Orlowski). A fairly good correlation was observed, its coefficient being 0.978.

It can be concluded from the data described above that the fluorometric γ-GTP assay using GCA is a simple and highly sensitive method, which is especially suitable for clinical use. Adsorption of the dye on serum albumin scarcely influenced this method, when the amount of serum added was restricted to within 50 μl. In addition, the method is free from error due to quenching by hemoglobin or bilirubin due to the use of the standard solution supplemented with the same volume of the same serum. This control run, already described in our previous paper on the determination of leucine aminopeptidase,[1a] was found to be essential, since sometimes sera from patients showed fluorescence due to the metabolites of drugs.

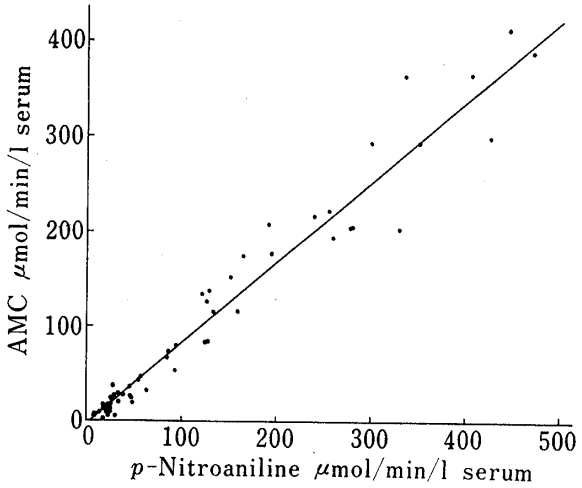

Fig. 3. Correlation between Serum γ-GTP Activities of Patients with Hepatobiliary Diseases as determined by Fluorometry and by Orlowski's Colorimetry.[5] 58 Samples (45 Males and 13 Females)

$Y=-4.018+0.860X$
$R=0.978$

We now have several fluorogenic protease substrates which give rise to one and the same product, AMC.[1,8,10] It should be possible, therefore, to determine the activities of several enzymes in one serum sample at the same time. Such a simultaneous determination might be achieved by adding suitable substrates in a regular sequence to the solution containing the serum at intervals. These studies will be reported elsewhere.

References and Notes

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