Journal of Chromatography, 357 (1986) 191–198
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 451

HIGHLY SENSITIVE ASSAY FOR γ-GLUTAMYLTRANSPEPTIDASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

KAZUTOSHI KIUCHI, KIYOMI KIUCHI and TOSHIHARU NAGATSU*
Department of Biochemistry, Nagoya University School of Medicine, Nagoya 466 (Japan)
AKIFUMI TOGARI*
Department of Life Chemistry, Graduate School at Nagatsuta, Tokyo Institute of Technology, Yokohama 227 (Japan)
and
HIDEHIKO KUMAGAI
Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606 (Japan)
(Received December 4th, 1985)

SUMMARY

A highly sensitive assay for γ-glutamyltranspeptidase activity involving high-performance liquid chromatography (HPLC) with electrochemical detection was devised. γ-Glutamyl-DOPA, a new synthetic dipeptide, which consists of naturally occurring amino acids, was found to be a good substrate for γ-glutamyltranspeptidase purified from *Proteus mirabilis*. Enzymatically formed DOPA was adsorbed on an aluminium oxide column, eluted with 0.5 *M* hydrochloric acid and determined by HPLC with electrochemical detection. The sensitivity limit of this method was 0.5 pmol of DOPA formed. Some properties of γ-glutamyltranspeptidase purified from *P. mirabilis* were investigated using γ-glutamyl-DOPA as a substrate. In the presence of 0.15 *M* glycylglycine, the K_M value of the enzyme for γ-glutamyl-DOPA was 0.013 m*M*, and the maximum velocity was 247 nmol/min per mg protein. This method was applied to the assay of the enzymatic activity in human serum.

INTRODUCTION

γ-Glutamyltranspeptidase [(5-glutamyl)peptide: amino acid 5-glutamyltransferase, E.C. 2.3.2.2; γ-GTP] is an enzyme which catalyzes transfer of the γ-glutamyl group from glutathione (GSH) or from other γ-glutamylpeptides to suitable acceptors, amino acids or peptides[1,2]. Assay of γ-GTP is required clinically and several synthetic substrates have been proposed: α-(γ-D,L-glutamylamino)propionitrile[3], N-(D,L-γ-glutamyl)aniline[4], γ-L-glutamyl-α- or -β-naphthylamides[5–7], γ-L-glutamyl-*p*-ni-

* Present address: Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, Nagoya 464, Japan.

0021-9673/86/$03.50

troanilide (γ-Glu-PNA)[8], γ-glutamyl-3-carboxy-4-nitroanilide[9], L-γ-glutamyl-(S-4-nitrobenz-2-oxa-1,3-diazolyl)-L-cysteinylglycine[10], γ-glutamyl-*p*-aminobenzoic acid[11] and 7-(γ-L-glutamyl)-4-methylcoumarylamide[12]. However, no method using a synthetic substrate composed of naturally occurring amino acids has been reported for routine work.

In this work, newly synthesized γ-glutamyl-DOPA (γ-Glu-DOPA) was used for the determination of γ-GTP activity, and we attempted to develop a highly sensitive electrochemical method using high-performance liquid chromatography (HPLC). DOPA is an intermetabolite in the biosynthesis of catecholamine and melanin, and is electrochemically active. Enzymatically formed DOPA from γ-Glu-DOPA can be measured by electrochemical detection with high sensitivity.

EXPERIMENTAL

Materials

DOPA, α-methyl-DOPA (MDOPA) and glycylglycine (GlyGly) were obtained from Sigma (St. Louis, MO, U.S.A.), and acid-washed aluminium oxide was from Merck (Darmstadt, F.R.G.). Nucleosil 7 C_{18} (particle size, 7 μm) was obtained from Macherey-Nagel (Düren, F.R.G.), and Develosil ODS-15/30 (particle size, 15–30 μm) from Nomura Chemical (Seto, Japan). All other chemicals used were of analytical grade.

γ-Glu-DOPA was synthesized enzymatically, as reported elsewhere[13]. γ-GTP was purified from *Proteus mirabilis* according to Nakayama *et al.*[14]. It was stored as a crystalline suspension in ammonium sulphate. Before use, this enzyme suspension (250 μl) was dialyzed against 1 l of 0.05 *M* Tris–HCl buffer (pH 7.4) containing 0.01 *M* magnesium chloride. The protein concentration was determined by the method of Lowry *et al.*[15].

Assay of γ-GTP activity

The assay for the enzymatic activity of γ-GTP purified from *P. mirabilis* was performed as follows. The standard incubation mixture (200 μl) contained 30 μmol GlyGly, 40 nmol γ-Glu-DOPA and enzyme in 0.05 *M* Tris–HCl buffer (pH 8.0) containing 0.075 *M* sodium chloride. After incubation at 37°C for 15 min, the reaction was terminated in an ice-bath by adding 250 μl of 0.8 *M* perchloric acid containing 0.04 *M* EDTA and 0.8 μ*M* MDOPA as an internal standard. After 10 min, 150 μl of 1 *M* potassium carbonate and 1 ml of 0.2 *M* Tris–HCl buffer (pH 8.5) containing 1% EDTA were added, and the mixture was placed on a column of acid-washed aluminium oxide (approximately 40 mg). The column was washed with 1 ml of 0.02 *M* Tris–HCl buffer (pH 8.5) and with 1 ml of distilled water. The adsorbed DOPA was eluted with 400 μl of 0.5 *M* hydrochloric acid. An aliquot (5 μl) of the eluate was injected into the HPLC system.

The procedure for assay of the activity of γ-GTP in human serum was almost the same as that in purified enzyme from *P. mirabilis*, but the reaction mixture (200 μl) contained 30 μmol GlyGly, 1 μmol γ-Glu-DOPA and human serum (10–50 μl) in 0.05 *M* Tris–HCl buffer (pH 8.0) containing 0.3 m*M* EDTA, and the incubation time was 15–60 min.

Chromatographic conditions

The HPLC system consisted of a Gilson Model 302 liquid delivery module and a Model 802C manometric module (Gilson, Villiers Le Bel, France), an LC amperometric detector E-502 (Irika, Kyoto, Japan) and a Nucleosil 7 C_{18} analytical column (250 × 4.0 mm). A 1-μm filter and a Develosil ODS-15/30 guard column (10 × 4.0 mm) were placed between the injector and the analytical column.

The mobile phase was a degassed mixture of 0.1 *M* sodium phosphate buffer (pH 3.5) containing 10 μ*M* EDTA and methanol (94:6, v/v). The flow-rate was 0.9 ml/min. For the detection of DOPA, the electrode potential was set to +0.55 V against a silver–silver chloride reference electrode.

RESULTS

Using a reversed-phase column, DOPA, MDOPA and γ-Glu-DOPA in a standard mixture were completely separated from each other. The retention times were: solvent front, 2.6 min; DOPA, 4.2 min; MDOPA, 6.0 min; γ-Glu-DOPA, 8.0 min. A typical chromatogram is shown in Fig. 1A. A linear response of the electrochemical detector to the amount of DOPA injected was observed from 0.5 pmol to 5 nmol. Fig. 1B shows the chromatogram of an eluate from an aluminium oxide column when the amount of γ-Glu-DOPA was 200 times larger in the mixture than those of DOPA and MDOPA. The separation of standards was also complete, and the recovery of DOPA and MDOPA was not changed. As a control, the incubation with γ-GTP from *p. mirabilis* (0.2 μg of protein) was carried out without γ-Glu-DOPA, and γ-Glu-DOPA was added after incubation; no DOPA peak was observed (Fig.

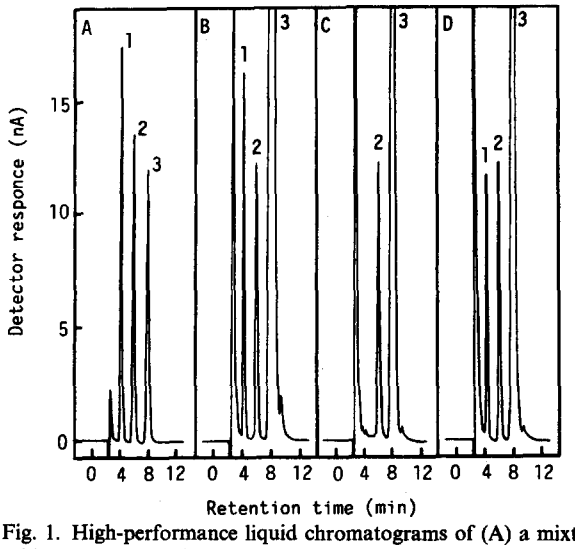

Fig. 1. High-performance liquid chromatograms of (A) a mixture of standards in 0.01 *M* hydrochloric acid (12.5 pmol each of DOPA, MDOPA and γ-Glu-DOPA); (B) the eluate from an aluminium oxide column, when 12.5 pmol each of DOPA and MDOPA and 2.5 nmol of γ-Glu-DOPA were applied; (C) control: incubation with *Proteus* γ-GTP in the absence of γ-Glu-DOPA, and γ-Glu-DOPA added after the incubation; (D) γ-Glu-DOPA reacted with *Proteus* γ-GTP; the reaction mixture was treated as described in Experimental. Peaks: 1 = DOPA; 2 = MDOPA; 3 = γ-Glu-DOPA.

1C). In contrast, the experimental incubation with γ-Glu-DOPA shows a significant formation of DOPA at 37°C after 15 min (Fig. 1D).

The variation of the transpeptidation or hydrolysis activity of this enzyme toward γ-Glu-DOPA as a function of pH is shown in Fig. 2. In the case of transpeptidation, the optimum pH was around pH 8.8 with 0.15 *M* GlyGly. Without GlyGly, no activity was observed in the range pH 8.0–9.2 using Tris buffer. The optimum pH for the hydrolysis reaction without GlyGly was around pH 6.0, however, the enzymatic activity in hydrolysis was about a quarter of that in transpeptidation at pH 6.0. In the present method, we adopted pH 8.0 for the standard assay because DOPA is unstable at pH > 8.4.

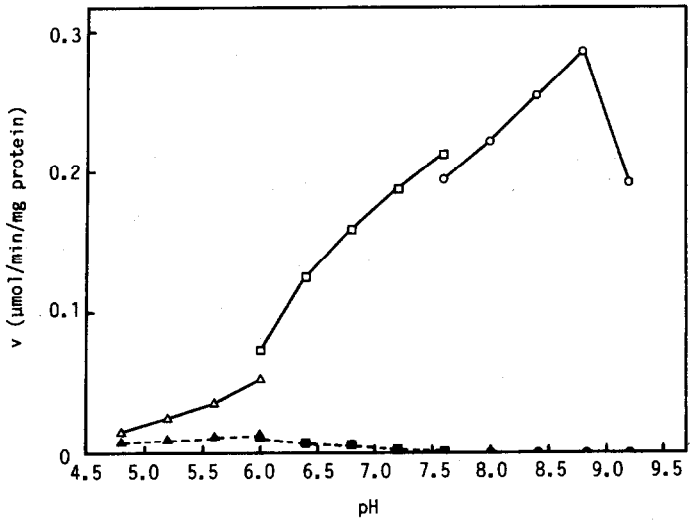

Fig. 2. Effect of pH on transpeptidation or hydrolysis of γ-Glu-DOPA by *Proteus* γ-GTP. Incubation was carried out for 15 min at 37°C, in the presence of 0.15 *M* GlyGly (○, □, △), or in the absence of GlyGly (●, ■, ▲). The incubation mixture (200 μl) contained 40 nmol of γ-Glu-DOPA and 0.08 μg of *Proteus* γ-GTP in 0.05 *M* buffer solution containing 0.075 *M* sodium chloride. The DOPA formed was measured as described in Experimental. The following buffers were used: (○, ●) Tris–HCl; (□, ■) sodium phosphate; (△,▲) sodium acetate.

Fig. 3 shows the Lineweaver–Burk plot of the γ-GTP activity toward GlyGly as an acceptor for the liberated γ-glutamyl residue. The Michaelis constant of this enzyme for GlyGly was 50 m*M*. A stimulating effect of sodium chloride on the enzymatic activity was observed at concentrations from 25 to 87.5 m*M*. The rate of enzymatic transpeptidation of γ-Glu-DOPA was proportional to the amount of the enzyme in the reaction mixture up to 0.4 μg (Fig. 4), and the reaction proceeded linearly up to 30 min at 37°C (Fig. 5). The effect of the substrate concentration on the enzymatic activity is shown in Fig. 6. The Michaelis constant of *Proteus* γ-GTP for γ-Glu-DOPA was 0.013 m*M*.

The enzymatic activity in human serum from normal controls was also measured under the optimum conditions as described in Experimental. The mean value and standard deviation of the enzymatic activity was 1.26 ± 0.49 nmol/min · ml

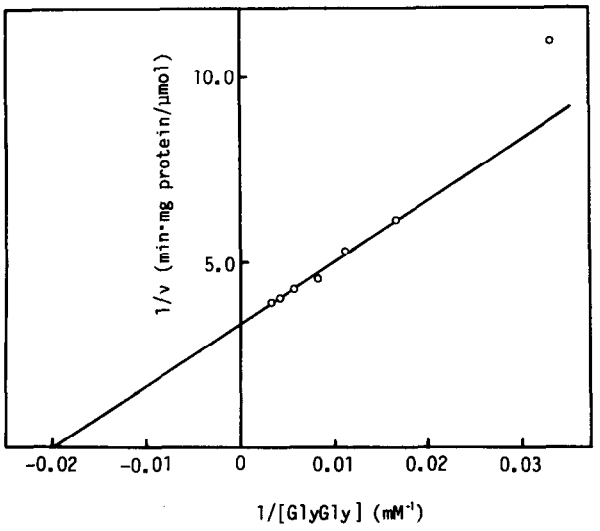

Fig. 3. Lineweaver–Burk plot of the effect of the GlyGly concentration on the formation of DOPA. The incubation mixture (200 μl) contained 40 nmol of γ-Glu-DOPA, 0.08 μg *Proteus* γ-GTP and various concentrations of GlyGly in 0.05 *M* Tris–HCl buffer (pH 8.0) containing 0.075 *M* sodium chloride. The GlyGly concentration was varied from 0.03 to 0.3 *M*.

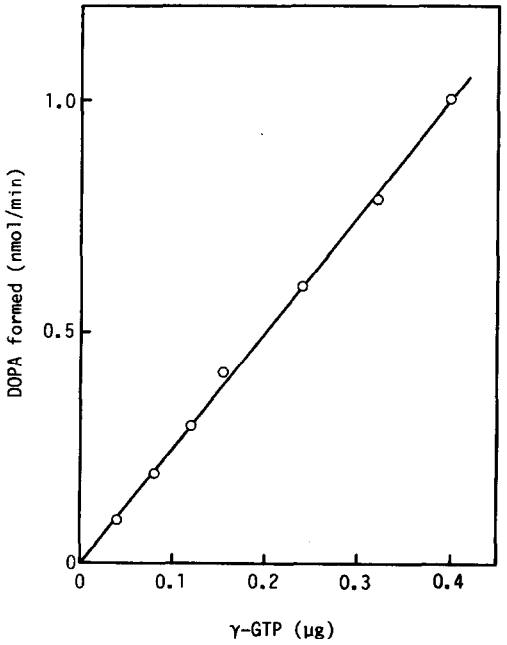

Fig. 4. Relationship between the amount of *Proteus* γ-GTP and the rate of formation of DOPA. The incubation mixture (200 μl) contained 30 μmol of GlyGly, 40 nmol of γ-Glu-DOPA and enzyme in 0.05 *M* Tris–HCl buffer (pH 8.0) containing 0.075 *M* sodium chloride. The enzyme amount was varied from 0.04 to 0.4 μg.

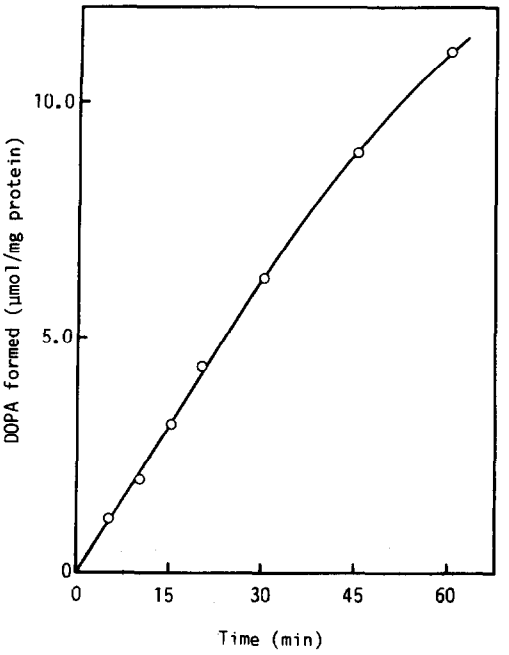

Fig. 5. Relationship between the reaction time and the rate of formation of DOPA. The standard incubation mixture was used, and at various intervals the reaction was terminated and the amount of DOPA formed was assayed as described in Experimental.

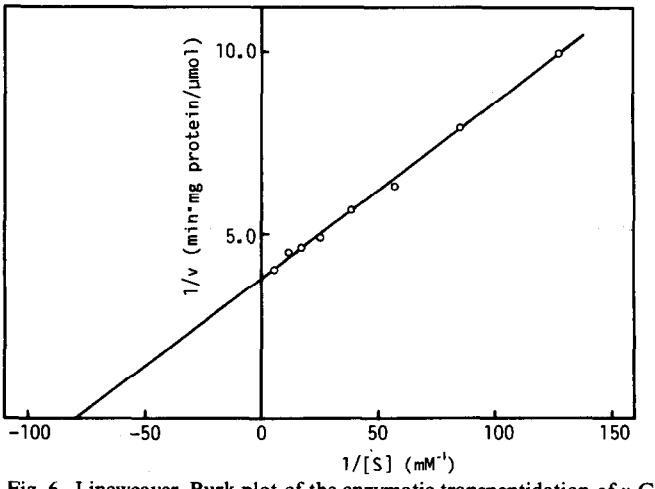

Fig. 6. Lineweaver–Burk plot of the enzymatic transpeptidation of γ-Glu-DOPA by *Proteus* γ-GTP. The incubation mixture (200 μl) contained 30 μmol of GlyGly, 0.08 μg of γ-GTP and various concentrations of γ-Glu-DOPA in 0.05 *M* Tris–HCl buffer (pH 8.0) containing 0.075 *M* sodium chloride. The substrate concentration was varied from 7.8 to 200 μ*M*.

(number of samples = 7). The apparent K_M value of the human serum enzyme for γ-Glu-DOPA was 2.2 m*M*. An aromatic L-amino acid decarboxylase inhibitor, O-(4-bromo-3-hydroxybenzyl)oxylamine (NSD-1055), was not necessary for the assay of the activity in serum, but should be added for homogenates of tissues with relatively high aromatic L-amino acid decarboxylase activity.

DISCUSSION

This new assay of γ-GTP activity using γ-Glu-DOPA as a substrate has several advantages.

First, it is highly sensitive. The sensitivity limit was about 0.5 pmol of DOPA formed enzymatically. The enzymatic activity of *Proteus* γ-GTP using γ-Glu-DOPA as a substrate was about six times that using γ-Glu-PNA (37.4 nmol/min per mg protein), and was almost the same as that using GSH[14]. Our procedure requires only small amounts of enzyme samples so that it is possible to reduce the volume of the reaction mixture to 100 μl.

Secondly, this method is accurate. The recovery of DOPA during the assay procedure was corrected using MDOPA as an internal standard. Since we use an aluminium oxide column for a preliminary isolation and an HPLC system for the main separation procedure, the interferences due to the contaminants in enzyme samples are eliminated. In the case of icteric serum samples, when γ-Glu-PNA is used for the assay, the yellow colour of the serum interferes with the photometric measurement of enzymatically formed yellow *p*-nitroaniline[11].

Thirdly, the newly synthesized γ-Glu-DOPA consists of naturally occurring amino acids and is apparently non-toxic. α-(γ-D,L-Glutamylamino)propionitrile and γ-L-glutamyl-α- or -β-naphthylamide are toxic, and do not find applications in routine work. Furthermore, it may be possible to use γ-Glu-DOPA for measuring the γ-GTP activity *in vivo* in animals when they are previously treated with an inhibitor of aromatic L-amino acid decarboxylase, such as NSD-1055.

Fourthly, since γ-Glu-DOPA is readily soluble in water, the reaction can be done under optimum conditions at the saturation concentration of the substrate. The fluorometric method using 7-(γ-L-glutamyl)-4-methylcoumarylamide[12] is highly sensitive, however the solubility of this compound is poor, and the substrate concentration in the reaction mixture is one-third that of the K_M value.

In this study, the K_M value of *Proteus* γ-GTP for γ-Glu-DOPA was 0.013 m*M*, and is much lower than those for GSH (0.18 m*M*) and for γ-Glu-PNA (0.40 m*M*)[14]. Thus, the newly synthesized γ-Glu-DOPA is a good substrate for this enzyme. The apparent K_M value of the enzyme in human serum for γ-Glu-DOPA was estimated as 2.2 m*M*, much higher than that obtained from *Proteus* γ-GTP. The K_M value should be determined with pure human serum enzyme, because this enzyme in the serum may be affected by various endogenous compounds such as amino acids. However, the optimum activity can be measured by the present method under the optimum conditions.

In conclusion, the present method using γ-Glu-DOPA as substrate and HPLC with electrochemical detection is highly sensitive and accurate, and may be applied to the assay during the enzyme purification, to *in vivo* studies and to routine work on clinical samples.

REFERENCES

1 C. S. Hanes, F. J. R. Hird and F. A. Isherwood, *Nature (London)*, 166 (1950) 288.
2 C. S. Hanes, F. J. R. Hird and F. A. Isherwood, *Biochem. J.*, 51 (1952) 25.
3 A. Szewczuk and M. Orlowski, *Clin. Chim. Acta*, 5 (1960) 680.
4 J. A. Goldbarg, O. M. Friedman, E. P. Pineda, E. F. Smith, R. Chatterji, E. H. Stein and A. M. Rutenburg, *Arch. Biochem. Biophys.*, 91 (1960) 61.
5 M. Orlowski and A. Szewczuk, *Clin. Chim. Acta*, 7 (1962) 755.
6 V. Kulhanek and D. M. Dimov, *Clin. Chim. Acta*, 14 (1966) 619.
7 B. Rietz and G. G. Guilbault, *Clin. Chem.*, 21 (1975) 715.
8 G. Szasz, *Clin. Chem.*, 15 (1969) 124.
9 L. Theodorsen and J. H. Stromme, *Clin. Chim. Acta*, 72 (1976) 205.
10 A. Persson and I. B. Wilson, *Anal. Biochem.*, 89 (1978) 408.
11 A. Szewczuk and M. Wellman-Bednawska, *Clin. Chim. Acta*, 84 (1978) 19.
12 E. Prusak, M. Siewinski and A. Szewczuk, *Clin. Chim. Acta*, 107 (1980) 21.
13 R. Nakayama, H. Kumagai, S. Akashi, H. Sugiura and T. Tochikura, *Agric. Biol. Chem.*, 49 (1985) 1041.
14 R. Nakayama, H. Kumagai and T. Tochikura, *J. Bacteriol.*, 160 (1984) 341.
15 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.