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A Spectrophotometric Assay for γ-Glutamyl Transpeptidase Activity

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A colorimetric assay for γ-glutamyl transpeptidase (γ-GTP; E.C 2.3.2.2) employing 2,4,6-trinitrobenzene sulfonate (TNBS) to detect the amount of disappeared acceptor *via* transpeptidation, has been developed. Under the experimental conditions using L-γ-glutamyl ethylester and L-phenylalanine as γ-glutamyl donor and acceptor, respectively, it was found that the decreased absorbance of yellow color at 420 nm was strictly related to the amount of L-γ-glutamyl-L-phenylalanine (L-γ-Glu-L-Phe) formed, which was determined by DEAE-cellulose column chromatography. Concentrations of the enzyme and γ-glutamyl products were able to be determined in the nanogram and nanomoles per milliliter range, respectively, with high precision and reliability. This novel assay system may therefore be a useful means for understanding of catalytic function of the γ-GTP spectrophotometrically without any usage of sophisticated instruments.

γ-Glutamyl transpeptidase, also called γ-glutamyl transferase (γ-GTP; E.C 2.3.2.2) has been extensively studied *in vitro*. The nucleotide sequence and the molecular mechanism of expression of the mammalian γ-GTPs have also been recently reported (2, 10). The enzyme is found in cell membrane fragments, and is known to catalyze either the hydrolysis or transfer reaction on the γ-glutamyl carboxamide moieties both in the absence and presence of γ-glutamyl acceptors, respectively (1, 11, 14). However, the physiological function of the γ-GTP is still uncertain due to its complicated catalytic behavior, especially its transpeptidation reaction (12). To ascertain the transpeptidase activity, automatic amino acid analyzer or high performance liquid chromatography should be used. But, these methods are cumbersome and require inconvenient efforts. Furthermore, it may only be possible to assay its hydrolytic activity even in the presence of suitable acceptors. In a sense, one may assume that the rate of the hydrolysis depends on the acceptor specificity. Unfortunately, the increase in the rate of hydrolysis cannot provide any significant information about the yield of the γ-glutamyl products (5, 6). To date, the most accessible method to synthesize valuable γ-glutamyl compounds (4) has been accomplished by the use of γ-GTP. In this regard, it is important to know precisely the substrate specificities of this enzyme. For this reason, a novel spectrophotometric recording method was devised to assay the γ-GTP activity. This paper describes the sensitivity of the assay, which employs the determination of the decrease in absorbance at 420 nm to monitor the incorporation of the acceptors.

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MATERIALS AND METHODS

Reagents

A lyophilized powder of γ-GTP (type II: bovine kidney), DEAE-cellulose resin, amino acids, peptides, and 2, 4, 6,-trinitrobenzene sulfonate (TNBS) were obtained from Sigma Chem. Co., U.S.A. L-γ-Glutamyl mono- and di-ethylesters were purchased from Tokyo Kasei Co., Japan. All other chemicals were commercial preparations of analytical reagent grade.

Preparation of Microbial γ-GTP

Cells of *Bacillus* sp. KUN-17 (7) grown in a medium containing 0.5% polypeptone and 0.3% yeast extract (pH 7.0) were discarded by centrifugation (10,000 rpm× 10 min), and the γ-GTP was purified from the culture filtrate as described previously (15).

Determination of γ-GTP Activity

Hydrolase activity: The activity was determined by using L-γ-glutamyl-*p*-nitroanilide (L-γ-Glu-pNA) as a substrate. 10 μl of the enzyme solution was added to 990 μl of the reaction mixture (50 mM phosphate buffer, pH 7.0) containing 0.1 μmole of the above substrate. The amount of *p*-nitroaniline liberated after hydrolysis was determined by measuring the absorbance at 405 nm.

Transpeptidase activity: For a typical assay, 10 μl

of the enzyme solution and 50 μl of 20 mM L-phenylalanine were added to 100 μl of 5 mM phosphate buffer, pH 7.0, and incubated at 30°C for 10 min, followed by the addition of 50 μl of 50 mM L-γ-glutamyl ethylester (L-γ-Glu-OEt). Portions (10 μl) of the initial reaction mixture or at 1-min time-intervals were immediately transferred into a boiling water-bath for 3 min, and then 5% sodium borate buffer containing 1 mM of TNBS was added to each to give a final volume of 1 ml. After incubation at 30°C for 30 min, the decreased absorbance (A_{420}) indicating the equivalent amount of the L-γ-glutamyl-L-phenylalanine (L-γ-Glu-L-Phe) formed was monitored ($\varepsilon_{420}=1.0\times10^4$ M^{-1} cm^{-1}) (3). Alternatively, the above dipeptide was purified by DEAE-cellulose column chromatography (0.7×1.5 cm; 5 mM phosphate buffer, pH 7.0), and quantitated spectrophotometrically at 230 and 255 nm.

RESULTS AND DISCUSSION

γ-Glutamyl transpeptidase (γ-GTP) activity was able to be determined spectrophotometrically by measuring the amount of free γ-glutamyl acceptor disappeared. As shown in Fig. 1, the intact progress curves indicate directly the amounts of the γ-glutamyl acceptor, sulfanilic acid, incorporated into each γ-glutamyl donors by the enzyme catalysis, where L-glutamine was suggested to be a most suitable substrate for the peptide generation. The equilibrium of the transfer reaction was observed after 20 min. Alternatively, a column chromatography using DEAE-cellulose resin was carried out to find out whether the above assay was correct. An equilibrated reaction mixture from the standard condition(see 'Materials and Methods') was passed through the column, and eluted successively with water and 0.01 M NaCl. The resulted chromatographic profile is presented in Fig. 2. L-γ-Glu-L-Phe eluted by the addition of 0.01 M NaCl was purified by a preparative TLC (Silica gel), showing a relevant yield as the colorimetric means. The compound was composed of L-Glu and L-Phe as an equal ratio after its acid hydrolysis (data not shown). A time course of transpeptidation was carried out as

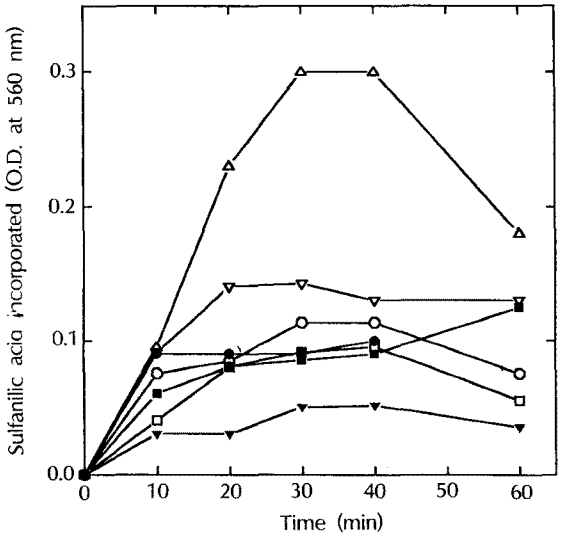

Fig. 1. Time course of γ-glutamyl transpeptidation catalyzed by γ-GTP.

0.01 μg of the purified enzyme from *Bacillus* sp. KUN-17 was preincubated with 1 μmoles of sulfanilic acid in 5 mM phosphate buffer, pH 7.0, at 30°C for 10 min (refer 'Materials and Methods'), followed by the addition of 2.5 μmoles each of the γ-glutamyl donors, and then time course determination of the amount of residual free sulfanilic acid was carried out as follows: 10 μl of the reaction mixture was treated with 1N HCl, and diazotized by 0.1% sodium nitrate, 0.5% ammonium sulfamate and 0.05% *N*-(1-naphtyl)ethylenediamine·2HCl ($\varepsilon_{560}=1.35\times10^4$ M^{-1} cm^{-1}). γ-Glutamyl donors used were glutathione: reduced (○), L-γ-Glu-OEt (●), L-γ-Glu $(OEt)_2$ (▽), L-γ-Glu-L-Phe (▼), L-γ-Glu-L-Glu (□), L-γ-Glu-Gly (■) and L-Gln (△).

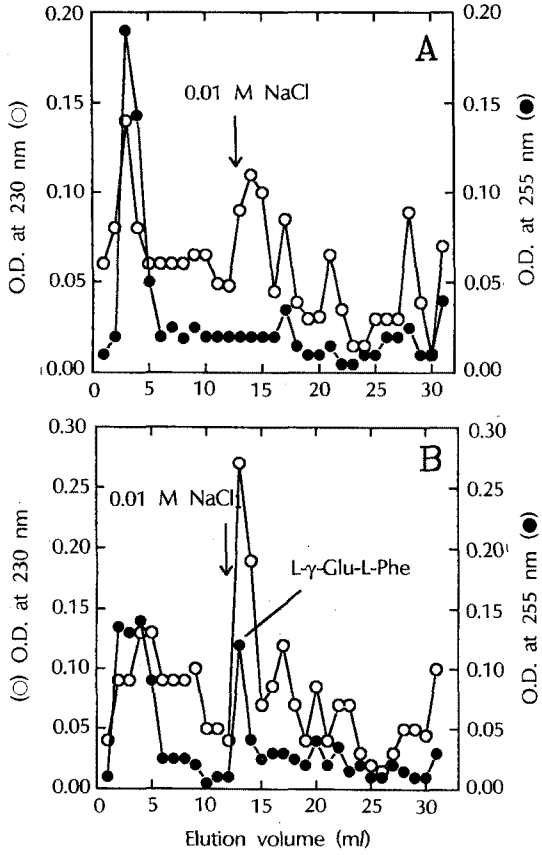

Fig. 2. Chromatographic determination of γ-glutamyl peptide synthesized by the enzyme catalysis.

The reaction mixture (200 μl) containing 1 μmoles of L-phenylalanine and 2.5 μmoles of L-γ-Glu-OEt was incubated at 30°C for 20 min in the absence (A), or presence (B) of γ-GTP. 10 μl-aliquots were separately passed on the DEAE-Cellulose column and eluted successively with distilled water and 0.01 M NaCl. For details, see 'Materials and Methods'.

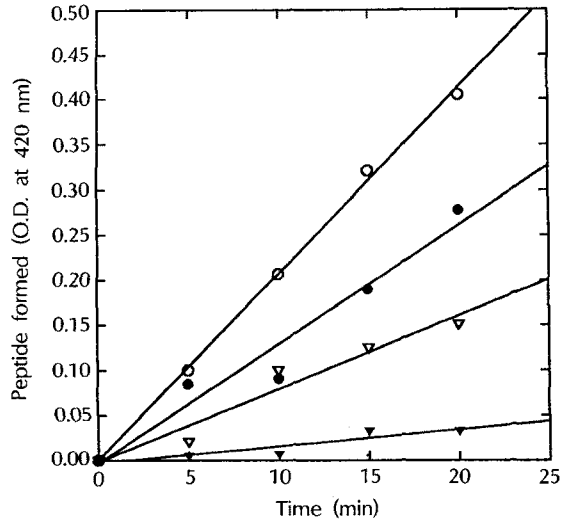

Fig. 3. Time course of γ-glutamyl transpeptidation under different concentrations of the γ-GTP.
The reaction mixture containing L-phenylalanine and L-γ-Glu-OEt was incubated at 30°C with each of the following concentrations of the enzyme per milliliter: ▼, 0.01 μg; ▽, 0.02 μg; ●, 0.03 μg; ○, 0.04 μg. See 'Materials and Methods' for conditions.

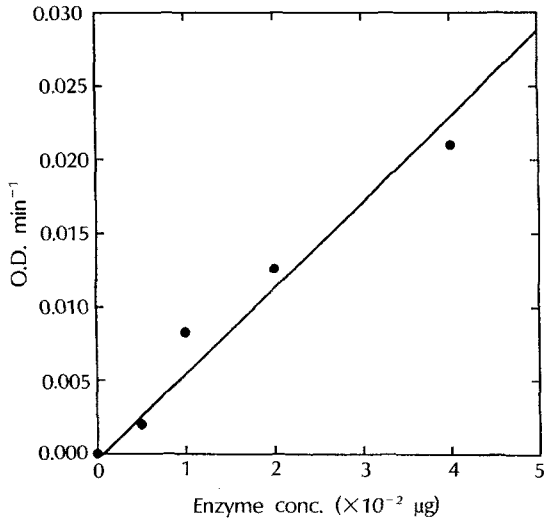

Fig. 4. Linearity of γ-GTP activity with the enzyme concentration.
The rates of transpeptidation indicate the mean values of decreased absorbance (420 nm) per minute. Conditions were in Fig. 3.

Table 1. Comparison of substrate specificities of γ-GTPs from *Bacillus* sp. KUN-17 and bovine kidney

γ-Glutamyl acceptor	Relative activity from	
	Bacillus sp. KUN-17	Bovine kidney
glycine	163	65
L-alanine	206	167
L-leucine	135	65
L-isoleucine	0	0
L-arginine	75	129
L-aspartate	149	129
L-asparagine	225	33
L-glutamate	225	290
L-glutamine	75	65
L-phenylalanine	125	129
L-proline	50	113
L-histidine	0	339
L-lysine	38	167
L-cysteine	63	0
L-methionine	281	400
L-serine	163	0
L-threonine	0	113
L-valine	400	16
glycylglycine	100	100

L-γ-Glu-OEt was used as γ-glutamyl donor. Activity is expressed by percent ratio relative to that found with glycylglycine.

a function of the enzyme concentration. As can be seen in Fig. 3, the increase of absorbances under the different enzyme concentrations proceeded linearly with time. It reveals that the assay can easily quantitate less than 1 nmole, 0.01 μg of γ-glutamyl products and enzyme per 1 ml, respectively. Moreover, the rate of transpeptidation is directly proportional to the enzyme concentration over this range (Fig. 4). An experiment was carried out to compare substrate specificities of the γ-GTP from different sources by using this assay. Table 1 shows clear evidence that the two kinds of γ-GTP employed appeared to have few relationship with each other with respect to their specificities for γ-glutamyl acceptors.

A number of chromogenic γ-glutamyl donors, i.e., L-γ-Glu-*p*-NA, L-γ-Glu-β-NA, etc., in the presence of γ-glutamyl acceptors, i.e., glycylglycine (Gly-Gly), have been used to assay γ-glutamyl transpeptidase (γ-GTP) either in clinical diagnostic or laboratory works (6,13). As noted in the introduction, the transpeptidase activity can not be monitored by the above assay since the effect of γ-glutamyl acceptors on the hydrolysis of the chromogenic substrate is negligible. Accordingly, the assay system has always been dependent upon certain equipments, i.e., an automatic amino acid analyzer (9). The spectrophotometric assay proposed here for γ-GTP determines simply the amount of γ-glutamyl acceptor transferred by measuring the residual absorbance of picryladducts in the micromolar range, and was proved to be useful to monitor the amount of the γ-glutamyl peptide formed. The enzyme catalysis is somewhat complex because, under the equilibrated state, the enzymatic reaction continues to form any probable products (8). Therefore, the initial mode of its catalytic behavior seems to be important in observing the amount of necessary product, for which a progress curve should be obtained by scanning the reaction followed. In this regard, the novel spectrophotometric assay for γ-GTP activity may be useful for various aims although a certain problem exists such as the high value of the initial abso-

rbances when the intact serums are employed to this assay.

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