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Purification and Properties of γ-Glutamyl Transpeptidase from *Bacillus* sp. KUN-17

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γ-Glutamyl transpeptidase (γ-GTP; EC 2.3.2.2) present in the culture filtrate of *Bacillus* sp. KUN-17 was purified 400-fold through a consecutive procedure including organic precipitation and column chromatography. The enzyme has an estimated molecular weight of 70,000 and consists of hetero-subunits with molecular weights of 42,000 and 22,000. *In vitro* optimal conditions for those transfer and hydrolysis reactions appeared to be pH 7.0 at 50°C and pH 8.4 at 40°C, respectively. The denatured enzyme recovered most of its γ-GTP activity by removing detergents such as sodium dodecyl sulfate (SDS) or urea with dialysis. The enzyme showed higher affinities against a number of amino acids as γ-glutamyl acceptors than glycylglycine in the following order: L-valine, L-methionine, L-glutamic acid or L-asparagine, L-alanine. Also, it was shown that L-glutamine was the most suitable γ-glutamyl donor for the transfer reaction among those tested. Amino acids generally inhibited the enzyme activity for the transfer reaction, but not for the hydrolysis reaction.

γ-Glutamyl transpeptidase [γ-GTP: γ-glutamyl transferase or (5-glutamyl)-peptide: amino acid 5-glutamyl transferase; EC 2.3.2.2] is a plasma membrane-bound enzyme that catalyzes the transfer and hydrolysis reactions at γ-glutamyl moieties of γ-glutamyl compounds (1, 30). In mammals, relatively high levels of γ-GTPs are found among tissues that are active in secretion and absorption (1, 29). γ-GTP is also present in plants or microorganisms (8, 17), but its distribution varies depending on species (16). Studies on enzyme processing, molecular cloning and expression of mammalian γ-GTP genes are mostly up to date (4, 24, 33).

Regarding the physiological function of γ-GTP, it is still controversial. Meister *et al.* proposed a possible role of the enzyme in the γ-glutamyl cycle for amino acid reabsorptions (19). But, related studies have not been carried out in tissues other than brush border membranes. To date considerable interest has been shown in ascertaining the clinical significance of γ-GTP, i.e., the enzyme's role in peripheral uptake of glutathione (GSH) (15, 25, 28, 33). On the contrary, few reports have been made about the presummed function of this enzyme for γ-glutamyl dipeptides which are ubiquitous in living systems (3, 12, 31). The significance of γ-GTPs in microorganisms is still unclear, although there are some indications of relationships to amino acid transports (21).

Microbial γ-GTP can be used as a model enzyme in order to investigate the nature of γ-GTP. Also, it is thought to be suitable as a bioreactor for mass production of γ-glutamyl peptides. Despite its long history after the first discovery from *Proteus vulgaris* in 1954 (27), only a few γ-GTPs have been purified and characterized from microorganisms (8, 9, 17, 26). Studies are therefore scarcely systematized nor progressive in microbial species. In our experience, microbial γ-GTP can not be easily determined by conventional assay method unless the cell wall is disrupted. Recently, a spectrophotometric method of assessing cellular γ-GTP was reported (6). This assay proved to be useful to determine the product concentrations. In our efforts aimed at studying bacterial peptidases (7, 22, 23, 34), a γ-GTP activity was observed using this method from the culture filtrate of *Bacillus* sp. KUN-17 (34). The enzyme was isolated and characterized. In this paper, we describe the taxonomical properties of the bacterium, purification and properties of γ-GTP. And also, characteristics of this enzyme will be compared to those from other sources.

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Key words: γ-Glutamyl transpeptidase, *Bacillus subtilis*, purification, properties

MATERIALS AND METHODS

Reagents

L-γ-Glutamyl-paranitroanilide (L-γ-Glu-pNA) was purchased from Tokyo Kasei Co., Japan. Bovine serum albumin (BSA) was obtained from Organo Technica B.V. Boxtel, Holland, and lysozyme was a product of Worthington, USA. Resins for column chromatography were from Pharmacia, Sweden. Others were from Sigma Chem. Co., USA.

Microorganism and Culture Conditions

Bacillus sp. KUN-17 was isolated from soil in the Chung-Nam District, Korea (34). Taxonomical identification of this strain was carried out according to the methods of Gordon *et al.* (5) and Krieg *et al.* (13). Cells were grown overnight in a medium containing 0.5% polypeptone and 0.3% yeast extract (pH 7.0) at 30°C using a water bath shaker (90 rpm) as described previously (7).

Enzyme Assay

γ-GTP assay was carried out spectrophotometrically as follows: For hydrolysis reaction, typically 1 ml of the reaction mixture consisted of 2 ng enzyme, 0.1 mM L-γ-Glu-pNA and 50 mM Tris-HCl buffer, pH 8.8 was placed in a test tube, and incubated for 10 min at 30°C. The reaction was terminated by adding 2 ml of 2 N acetic acid, and the amount of liberated para-nitroaniline was determined by measuring the absorbance at 405 nm (ε_{405}, 10,000/M/cm). Transfer reaction was performed by incubating the enzyme solution at 30°C with 10 mM L-phenylalanine and consecutively with 20 mM L-γ-glutamyl ethylester (L-γ-Glu-OEt) for 10 min and 30 min, respectively. The reaction was terminated on ice, followed by the addition of saturated sodium borate solution (pH 9.5) containing 10 mM 2,4,6-trinitrobenzene sulfonate (TNBS) to give a final volume of 1 ml. After 30 min at room temperature, the decreased amount of picryl adducts was monitored at 420 nm as described previously (6).

Protein Determination

The protein concentration was determined by the method of Lowry *et al.* (14) using BSA as standard.

Isolation and Purification of γ-GTP

All of the following steps were performed at 0~4°C unless stated otherwise.

Step 1: Filtration. Culture broth was centrifuged at 10,000 g for 10 min, and the resulting supernatant was passed through a millipore membrane to obtain proteins of molecular weights between 10,000 and 100,000.

Step 2: Precipitation with ammonium sulfate. Ammonium sulfate was added to the filtrate to give 70% saturation, and then stored until proteins were precipitated completely. The precipitate was then harvested by centrifugation at 10,000 g for 10 min, and dialyzed against 50 mM phosphate buffer (PB), pH 7.0.

Step 3: Ion-exchange column chromatography. Anion and cation-exchange resins were subsequently employed at pH 7.0 and 6.0, respectively. The dialysate was loaded onto a column of DEAE-Sephadex A-50, and fractionated the enzyme pool by eluting at 0.3 M of NaCl gradient. After dialysis of the pool against PB, pH 6.0, a column chromatography using CM-Sephadex C-50 was performed, followed by elution of the active fractions at 0.3 M of NaCl gradient. Active fractions were then combined, concentrated by precipitating with ammonium sulfate.

Step 4: Gel filtration. The concentrate was dialyzed against PB (pH 7.0), and applied to gel filtration using a column with Sephadex G-100 (1.2×95 cm; PB, pH 7.0). Elution was performed at a flow rate of 5 ml per hour. Active fractions were pooled and lyophilized.

Step 5: Precipitation with ethanol. The resultant powder was dissolved into a small volume of ice-cooled distilled water as appropriate, diluted 3 times by dropping prechilled conc. ethanol under gentle stirring. After storage for several days in refrigerator, white aggregates were formed. An aliquot of the homogenized suspension of aggregates was collected and centrifuged at 10,000 g for 10 min. The resultant pellet was dissolved in PB, pH 7.0 before use. The enzyme aggregate was stable for several months at 4°C without significant loss of the enzyme activity.

Electrophoresis

Native-polyacrylamide gel electrophoresis (PAGE) was carried out with Tris-glycine buffer at pH 9.5 as described previously (7). The relative Rf for enzyme activity was measured by incubating L-γ-Glu-pNA and gel slices in PB (pH 7.0). 1% SDS-PAGE was performed using the method of Weber and Osborn (32).

Molecular Weight Determination

The molecular weight of intact enzyme was estimated by Sephadex G-100 gel filtration using lysozyme, casein, egg albumin and BSA as marker proteins. Molecular weight analysis of the dissociated enzyme was carried out by SDS-PAGE, and for molecular markers, lactalbumin, trypsinogen, glyceraldehyde 3-phosphate dehydrogenase, egg albumin and BSA were used.

RESULTS

Identification of the Isolated Strain KUN-17

Results of the electron micrography reveal that the cell has a characteristic morphology of flagellated rod, and can form spore (Fig. 1). According to the proposed strategies for classification of the Genus *Bacillus* in literatures (5, 13), this strain belongs to *Bacillus subtilis* (Table 1).

Enzyme Purification

The adsorptive property of γ-GTP against DEAE- (pH 7.0) and CM-Sephadex resins (pH 6.0) indicates the pI

of this protein is near neutral. The enzyme treatment with ethanol shown in the final step was necessary to remove relatively small proteins. The levels of enzyme recovery and their specific activities at individual purification steps are summarized in Table 2. As indicated, the enzyme was purified 400-fold with 8% recovery. The enzyme yield was markedly lowered during CM-Sephadex chromatography. Later, it was found the enzyme loss was due to the expression of latent protease activities. These enzymes were separately purified, and reported elsewhere (7, 22).

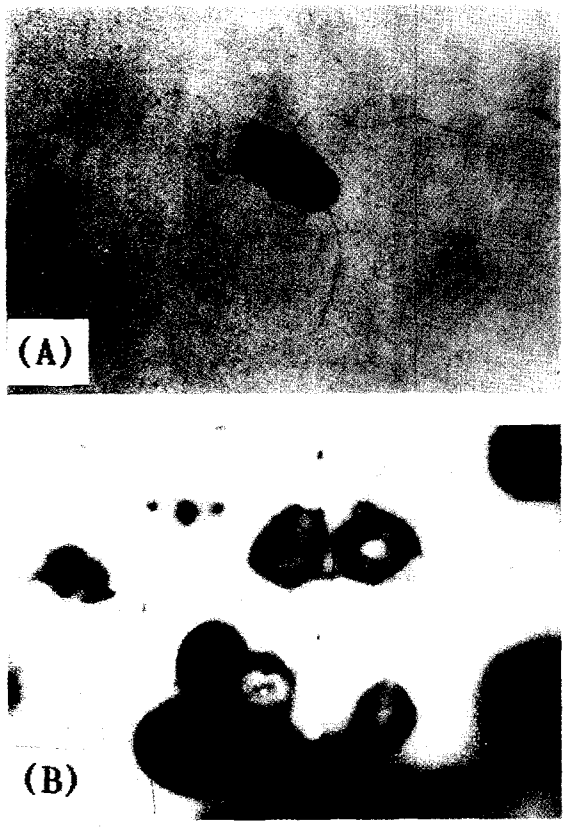

Fig. 1. Electron micrographs of *Bacillus* sp. KUN-17.
Cells were negatively stained with phosphotungsteic acid, and the resultant photographs magnified (1.95×10,000) were presented. (A) A typical vegitative cell. Note that the cell was surrounded by well-marked flagella. (B) Spores. Cells were grown for 2 days at 30°C.

Molecular Structure of γ-GTP

An experiment to determine the molecular structure of γ-GTP was undertaken by measuring its molecular weight in the following ways: The intact enzyme showing a single band in native-PAGE was dissociated into subunits by SDS treatment. Result from SDS-PAGE suggests that this enzyme is composed of two subunits with molecular weights of 42,000 and 22,000, respectively (Fig. 2). By gel filtration using Sephadex G-100, the approximate molecular weight of the enzyme was estimated to be 70,000. These observations illustrate that the purified γ-GTP is a hetero-dimeric protein, among the typical structure of γ-GTP in general. Molecular weights of these subunits were compared with those cited. Interestingly, most of light subunits were identically 22,000, whereas heavy subunits varied individually (Table 3). Unfortunately, γ-GTP activity could not be restored after SDS-PAGE. But, the enzyme activity was retained to some extent under dissociated state. Under this condition, the enzyme was unstable. An experiment was therefore

Table 1. Taxonomical properties of *Bacillus* sp. KUN-17.

	Bacillus sp. KUN-17	*Bacillus subtilis*	*Bacillus megaterium*
Width of rod (μm)	0.85	0.7-0.8	1.2-1.5
Length of rod (μm)	2.85	2-3	2-5
Gram staining	+	+	+
Spore staining	+	+	+
Catalase test	+	+	+
VP reaction	+	+	-
Growth at 50°C	-	-	-
Acid from glucose	-	+	+
Gas from glucose	-	-	-
Growth at pH 5.7	+	+	+
Starch hydrolysis	+	+	+
Anaerobic growth	-	-	-
Growth in 7% NaCl	+	+	+

Assessments were done under the conditions as follows: For growth at 50°C, after incubation for 3 days; for growth at pH 5.7, after incubation in Sabouraud dextrose agar medium for 3 days at 37°C, and for growth in 7% NaCl, after incubation in nutrient medium as indicated in the text for 2 days at 37°C.

Table 2. Purification summary of γ-GTP from *Bacillus* sp. strain KUN-17.

Step	Volume (ml)	Total protein (mg)	Total activity (u)*	Specific activity (u/mg)	Recovery (%)
Filtration	100	660	764.7	1.16	100
$(NH_4)_2SO_4$ treatment	35	78.8	542.5	6.69	71
DEAE-Sephadex A-50	4	5.4	390	72.2	51
CM-Sephadex C-50	1	0.7	125.5	179.3	16
Sephadex G-100	13	0.21	72.8	346.6	10
Ethanol treatment	1	0.15	63.8	425	8

*One unit(u) of the enzyme activity was defined as the amount of enzyme that released 1 μmole of para-nitroaniline per minute.

carried out to know whether it was due to the latent protease activity (9). The dissociated enzyme showed no discernible activity against protein substrates, suggesting that the enzyme would be destabilized, followed by autoproteolysis (9) (data not shown).

Physicochemical Properties of γ-GTP

The enzyme was stable for 1 h at 40°C, but the enzyme lost all of its activity after 1 h at 65°C (data not shown). Optimal temperature for transfer reaction of this enzyme was 50°C, which was over 10°C higher than that for its hydrolysis reaction. The stability of the enzyme was relatively sensitive against pH in solution. The enzyme was most stable at neutral pH (data not shown). Interestingly, optimal pH of the transfer reaction was found at 7.0, whereas the hydrolysis reaction preferred the alkaline pH (Fig. 3). As a further test of this enzyme protein, an experiment was carried out to determine its resistance against denaturation. For comparison, a mammalian γ-GTP was tested at the same time. Data in Table 4 indicate that the purified γ-GTP is far more resistant against detergent treatment than the mammalian enzyme.

Substrate Specificity

γ-GTP has an unique property recognizing acceptors and donors together via γ-glutamyl carboxamide moieties. When L-γ-Glu-OEt was used as a γ-glutamyl donor, the enzyme transferred a number of amino acids over 2 times more than glycylglycine in the following order: L-valine, L-methionine, L-glutamic acid or L-asparagine, and L-alanine. This result was compared with data cited, and presented in Table 5. As indicated, the specificity of γ-GTP from *Bacillus* sp. KUN-17 was rather similar to that found in a mammalian enzyme than those found in other bacterial enzymes. For certain purposes, sulfanilic acid was used as γ-glutamyl acceptor (6). Since this compound implicates di-

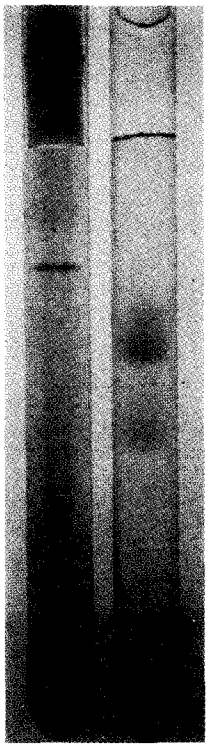

Fig. 2. Electrophoretic profiles of the purified γ-GTP.
30 μg of the enzyme protein was subjected to 7% PAGE at room temperature in the absence (A) or presence of 1% SDS (B). Proteins were stained with coomassie brilliant blue R-250.

Table 3. Comparison of molecular weights of γ-GTP subunits.

Source	Heavy subunit	Light subunit
Bacillus sp. KUN-17	42,000	22,000
Escherichia coli K-12	39,000	20,000
Proteus mirabilis	47,000	28,000
Bacillus subtilis (natto)	45,000	22,000
Rat kidney	46,000	22,000
Rabbit kidney	50,000	22,000
Human, Bovine, Sheep and Hog kidney	64,000	22,000

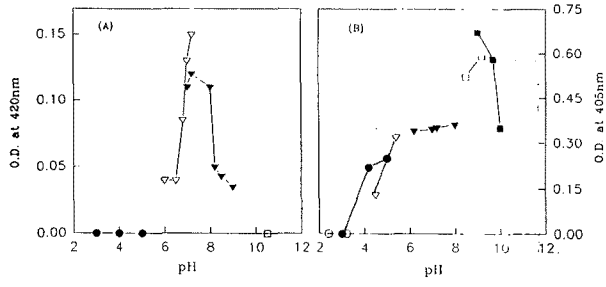

Fig. 3. Determination of optimal pH for γ-GTP activity.
(A) Optimal pH for transfer reaction. 50 mM Buffers were used: Na-citrate (●), phosphate (▽), Tris-HCl (▼) and glycine-NaOH (□). (B) Optimal pH for hydrolysis reaction: glycine-HCl (○), Na-citrate (●). Na-acetate (▽), phosphate (▼), Tris-HCl (□) and glycine-NaOH (■).

Table 4. Effect of detergents on the γ-GTP activity.

Condition	Enzyme recovery (%)	
	Bacillus sp. γ-GTP	Bovine kidney γ-GTP
None	100	100
Urea (0.5 M)	100	48.7
(5 M)	68.1	25
SDS (0.2%)	95.9	18.1
(2%)	49.1	12.5
NaCl (0.1 M)	100	100
(1 M)	100	100

*Enzyme activity is expressed by percent ratio relative to that found in the absence of detergent. Enzymes incubated for 1 h at 30°C under the above conditions were dialyzed before test.

Table 5. Specificities of γ-GTP against γ-glutamyl acceptors.

Amino acid	Enzyme source				
	Bacillus sp. KUN-17	Bovine kidney	*Proteus mirabilis*	*Escherichia coli* K-12	*Bacillus subtilis (natto)*
Glycylglycine	100	100	100	100	100
Glycine	163	65	17	21	21
L-Alanine	206	167	40	0	4
L-Leucine	135	65	21	3	21
L-Isoleucine	0	0	25	14	22
L-Arginine	75	129	21	141	5
L-Aspartic acid	149	129	20	4	18
L-Asparagine	225	33	ND	32	47
L-Glutamic acid	225	290	0	10	5
L-Glutamine	75	65	3	ND	3
L-Phenylalanine	125	129	52	43	43
L-Proline	50	113	13	2	13
L-Histidine	0	339	37	50	52
L-Lysine	38	167	16	121	21
L-Cysteine	63	0	16	14	13
L-Methionine	281	400	47	92	71
L-Serine	163	0	6	10	1
L-Threonine	0	113	16	23	35
L-Valine	400	16	13	11	34

Transfer reaction was monitored as described in Materials and Methods. Activity is expressed by percent ratio relative to that found with glycylglycine. ND: not determined.

Table 6. Specificities of γ-GTP against γ-glutamyl donors.

Substrate	Enzyme source		
	Bacillus sp. KUN-17	*Escherichia coli* K-12	*Bacillus subtilis (natto)*
L-γ-Glu-OEt	100	100	100
GSH	126	114	163
L-γ-Glu-$(OEt)_2$	11	ND	ND
L-γ-Glu-L-Phe	56	85	127
L-γ-Glu-L-Glu	101	ND	ND
L-γ-Glu-Gly	94	ND	ND
L-Glutamine	333	90	114
L-γ-Glu-pNA	144	127	140

Activity for transfer reaction was determined by using the method of diazotization with sulfanilic acid. Activity is expressed by percent ratio relative to the that found with L-γ-Glu-OEt. ND: not determined.

azotization, the transfer reaction can be monitored without interruption by the other amino acids. This technique was particularly useful to determine either the enzyme specificity against γ-glutamyl donors or its inhibitory effect by amino acids. According to this assay, L-glutamine appeared to be the most suitable substrate among those tested (Table 6). And, the enzyme showed poor affinities against those blocked at α-glutamyl termini. This finding suggests that the enzyme may be involved in the metabolism of γ-glutamyl peptides rather than in the cellular process of cross-linkages across γ-glutamyl residues. The effect of amino acids on the enzyme activity was examined, and the data are shown in Table 7. The transfer reaction of γ-GTP was

Table 7. Effect of amino acids on the γ-GTP activity.

Amino acid	Remained activity	
	Transferase	Hydrolase
None	100	100
Glycine	75	109
L-Alanine	50	70
L-Leucine	75	98
L-Isoleucine	86	101
L-Arginine	87	105
L-Aspartic acid	95	102
L-Asparagine	88	95
L-Glutamic acid	52	90
L-Glutamine	57	77
L-Phenylalanine	69	107
L-Proline	95	98
L-Histidine	95	118
L-Lysine	95	107
L-Cysteine	76	115
L-Methionine	85	122
L-Serine	76	105
L-Threonine	90	100
L-Valine	72	91
Glycylglycine	86	106

Sulfanilic acid was used for monitoring the transfer reaction. Each of remained activities are expressed by percent ratio relative to that found in the absence of amino acid.

mostly inhibited by the addition of amino acids. On the contrary, its hydrolysis reaction was accelerated by some amino acids.

DISCUSSION

Taxonomical data illustrate that the *Bacillus* sp. KUN-17 belongs to *Bacillus subtilis*. However, since the Genus *Bacillus* covers unusally broad branches of family (5), further investigations are necessary to clarify the taxonomical position of this isolate. γ-GTP was isolated from the culture filtrate of *Bacillus* sp. KUN-17, suggesting that this enzyme would be an extracellular protein. Extracellular γ-GTPs were also found from *Bacillus* sp. No.12 (8) or *Bacillus subtilis (natto)* (18). However, because of the distinct life style of *Bacillus*, it is controvertible whether these enzymes are secretory. Otherwise, they would be the resultant products in consequence of cell lysis (23). It is noteworthy that the enzymes are usually found in periplasmic spaces in bacteria other than *Bacillus* (17, 26, 27). Investigations are on going in our laboratory about the cellular localization of the enzyme.

In this study, we employed the spectrophotometric assay for γ-GTP (6) in order to avoid the use of high performance liquid chromatography. This assay was particularly useful to ascertain the γ-glutamyl peptide formed. Using this method, some unique results were achieved. As noted in the text, this enzyme showed considerable affinities against amino acids that are inhibitory to γ-GTP in general (9, 17), such as L-alanine or L-serine as γ-glutamyl acceptors (Table 5). Moreover, this enzyme preferred L-glutamine to GSH as γ-glutamyl donor, suggesting that it would utilize L-glutamine as a physiological substrate (Table 6). However, the kinetic power of GSH appeared to be several-fold higher than L-glutamine (11) from competitive spectrophotometric analysis (10). Since the catalytic mechanism of γ-GTP (1) cannot follow the Michaelis kinetics, requiring single substrate, the latter methodology would not provide true kinetic constants for competitive substrate. Concerning this problem, further studies will be undertaken. An interesting observation was found for enzyme transfer reaction, showing that the optimal pH was neutral. This property distinguished it from those that preferred the alkaline pH (26). The neutral condition was particularly useful in increasing equilibrium constant for the transfer reaction in aqueous solution. In fact, the reaction was advanced to give over 3-fold productivity by increasing the concentrations of organic solvents, i.e., ethanol or dimethylformamide (unpublished data).

γ-GTP is thought to be a complex enzyme, because it catalyzes those transfer and hydrolysis reactions simultaneously with unusual substrate specificity. Theoretically, any product can be reused as either γ-glutamyl donor or γ-glutamyl acceptor because of its poor ability to recognize the structure linked to γ-carboxyl group of L-glutamic acid. Following these confusions, *in vitro* characterization of the γ-GTP are cumbersome. In assessing the nature of this enzyme in living systems, investigations are conjunctionally required with ubiquitous γ-glutamyl compounds.

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