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Production, purification and properties of γ-glutamyltranspeptidase from a newly isolated *Bacillus subtilis* NX-2

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Abstract

Production, purification and properties of γ-glutamyltranspeptidase from a newly isolated *Bacillus subtilis* NX-2 was investigated. At the optimum conditions for enzyme formation, a high level, 3.2 U/ml of γ-GTP was obtained. The extracellular γ-GTP from this strain was purified 111.15-fold to homogeneity from the culture supernatant by acetone precipitation, hydrophobic interaction chromatography and ion exchange chromatography. The purified enzyme was a heterodimer consisting of one large subunit (43 kDa) and one small subunit (32 kDa), and exhibited high activity at 40–60 °C, pH 8.0. It preferred basic amino acids as γ-glutamyl acceptor in transpeptidation, and the stereochemistry of the γ-glutamyl acceptor had no influence on the enzyme activity, which was different from other γ-GTPs reported. Furthermore, it was proved that γ-GTP of this strain could catalyze the transfer of L-glutamine to glycylglycine to synthesize Gln–Gly–Gly, which was promising for the synthesis of valuable γ-glutamyl peptides.

Keywords: γ-Glutamyltranspeptidase; *B. subtilis* NX-2; Transpeptidation; Purification; Enzymatic characteristic

1. Introduction

γ-Glutamyltranspeptidase (γ-GTP), which is widely distributed in living organisms, is an important enzyme in metabolism [1]. γ-GTP catalyzes the hydrolysis of γ-glutamyl compounds such as glutathione, as well as the hydrolysis of the amide bonds of glutamine to yield glutamic acid and ammonia. γ-GTP can also catalyze the transfer of γ-glutamyl moieties to amino acids or peptides [2]. Recently, quite a few γ-glutamyl compounds, such as glutathione, γ-glutamyl-DOPA, theanine and γ-glutamyltaurine were found to be valuable medicines [3–6]. They were obtained by a one-step enzymatic reaction through γ-GTP transpeptidation, so that the synthesis of them was much simpler than the chemical synthesis. Furthermore, the enzymatic process did not require ATP or any coenzymes. However, the γ-GTPs were all from strain *Escherichia coli* K-12 in these enzymatic reactions [7–10]. The amount of the γ-GTP produced by *E. coli* K-12 was still at a low level, about 0.04–0.25 U/ml [8–10]. In the other hand, γ-GTP is present in the periplasmic space in *E. coli* [11], its purification requires fractionation of the periplasm that is rather troublesome procedure [12]. In contrast, the γ-GTPs of *Bacillus* species are extracellular enzymes, they could be purified from the culture broth easily. But there are also problems in some γ-GTP producers in *Bacillus* species. Some *Bacillus* species produced γ-GTP only after the end of the exponential phase and the γ-GTP activity disappeared in several hours, which is inconvenient for industrial use [13]. The wide type *Bacillus subtilis* NAFM5 only accumulated 28×10^{-3} U/ml in such a long fermentation time for 6 days [14]. And as far as we know the maximum activity of γ-GTP produced by *Bacillus* strain is only 3.0 U/ml [15]. Therefore, it is obviously necessary to isolate *Bacillus* strain capable of producing large quantities of γ-GTP without these problems.

In this work, the production of γ-GTP from a newly isolated *B. subtilis* NX-2 was investigated. The extraction and purification of γ-GTP from the culture broth was carried out. And then the properties of purified γ-GTP were characterized.

2. Experimental

2.1. Strain and culture conditions for the production of γ-GTP

B. subtilis NX-2, a γ-GTP-producing bacterium, was isolated from soil in our laboratory [16]. It was deposited in China General Microbiological Culture Collection Center with the accession number of CGMCC No.0833.

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For the production of γ-GTP by flask culture, a loop of the NX-2 cells was inoculated into 60 ml fermentation medium containing glucose 20 g/l, corn steep liquid 10 g/l, yeast extract 15 g/l, K_2HPO_4 1 g/l, $MgSO_4$ 0.5 g/l in 500 ml flask and aerobically incubated at 32 °C in a rotary shaker at 220 rpm for 38 h.

2.2. Purification of γ-GTP

2.2.1. Acetone precipitation

Culture broth of *B. subtilis* NX-2 was centrifuged at 10,000 rpm and 4 °C for 20 min. The obtained supernatant was precipitated by adding the same volume of cold acetone and kept for 10 min at 0 °C to allow complete precipitation. The resulting precipitate was collected by centrifugation at 8000 rpm and 4 °C for 30 min, and dissolved in 0.05 mol/l Tris–HCl buffer containing 1 mol/l $(NH_4)_2SO_4$ at pH 8.0. The insoluble materials were removed by centrifugation.

2.2.2. Hydrophobic interaction chromatography

The enzyme solution was applied to a CL4B-Sepharose column (HiPrep™ 16/10, Amersham Pharmacia) pre-equilibrated with buffer containing 0.05 mol/l Tris–HCl, 1 mol/l $(NH_4)_2SO_4$ at pH 8.0. The column was then washed with the same buffer at the flow rate of 2 ml/min for 20 min. The bound proteins were then eluted with a linear gradient of $(NH_4)_2SO_4$ in the range of 0.7–0 mol/l in the equilibrating buffer. The active fraction with corresponding γ-glutamyl transpeptidation activity was collected.

2.2.3. Ion exchange chromatography

The enzyme solution was further purified by ion exchange chromatography on a DEAE-Sepharose column (DEAE fast-flow XK16/10, Amersham Pharmacia), which had been equilibrated with buffer comprising 0.05 mol/l Tris–HCl at pH 8.0. After loading the sample, the column was washed at 2 ml/min with the same buffer for 20 min. A step gradient was generated from Milli-Q water (A) and elution buffer comprising 0.05 mol/l Tris–HCl (pH 8.0) and 0.5 mol/l NaCl (B). The initial condition was 100% A and 0% B. The fraction of B was increased in a non-linear fashion to 20% in 30 min, followed by a linear increase of B from 20% to 80% in 30 min. Cleaning of the column was achieved by further increasing B from 80% to 100% in 30 min and maintaining this concentration of B for 30 min. And the fraction with γ-glutamyl transpeptidation activity was collected.

2.3. Assay of activity and stability of γ-GTP

γ-GTP activity was determined as described previously [1]. The reaction mixture contained 50 mmol/l Tris–HCl (pH 8.0), 5 mmol/l γ-glutamyl-*p*-nitroanilide (γ-G_pNA), 20 mmol/l glycylglycine and the enzyme solution. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 0.1 mol/l HCl. The absorbance of the sample at 410 nm was measured and the enzyme activity was calculated. One unit of enzyme was defined as the amount of enzyme that released 1 μmol of *p*-nitroaniline per minute from γ-glutamyl-*p*-nitroanilide through the transpeptidation reaction.

2.4. Substrate specificity for γ-glutamyl acceptors

The substrate specificity for γ-glutamyl acceptors was measured by the γ-GTP activity assay method, except that various amino acids were used as γ-glutamyl acceptors instead of glycylglycine.

2.5. Transpeptidation with L-glutamine as γ-glutamyl donor

The transpeptidation activity of γ-GTP utilizing L-glutamine as γ-glutamyl donor was measured by the γ-GTP activity assay method, except that the donor L-glutamine, at 20 mmol/l, was used instead of γ-G_pNA.

2.6. Analytical methods

2.6.1. Subunit composition of γ-GTP

The purified enzyme was analyzed by SDS-PAGE, following the method described previously [17].

2.6.2. Measurement of the protein concentrations

Total protein concentrations were determined by means of Bradford [18], using bovine serum albumin as a standard.

2.6.3. Thin layer chromatogram

The reaction mixtures of L-glutamine and glycylglycine were determined by thin layer chromatogram. The glass plate was coated by cellulose and silica gel. The components were developed with a mixture of *n*-butanol/acetate/water (4:1:1, v/v/v), followed by being blotted with 0.2% ninhydrin.

2.6.4. Mass spectrum

The reaction mixtures of L-glutamine and glycylglycine were also determined by mass spectrum. Detection was performed by a Finnigan Mat LCQ MS detector (Finnigan Mat, San Jose, CA, USA) using electrospray ionisation (ESI) for ion production. The operating parameters were as follows: the spray needle voltage was set at 4.47 kV, capillary voltage was 17.64 V, and capillary temperature was 200.10 °C.

3. Results and discussion

3.1. Time course of γ-GTP production by B. subtilis NX-2

The time course of γ-GTP production by *B. subtilis* NX-2 was investigated, as shown in Fig. 1. After 6 h of short lag phase, the cells started to grow and entered into exponential phase. At the same time, γ-GTP was synthesized rapidly and excreted into the culture broth. The enzyme activity remained at a high level even though the growth of cells slowed down at 20–30 h of cultivation. The γ-GTP activity increased up to 3.2 U/ml at 30 h. After this point, the concentration of cells was decreased as glucose was depleted out, but the activity of γ-GTP was still stable and kept at a high level. This was advantageous for the industrial use of γ-GTP from this strain. While some other *Bacillus* species

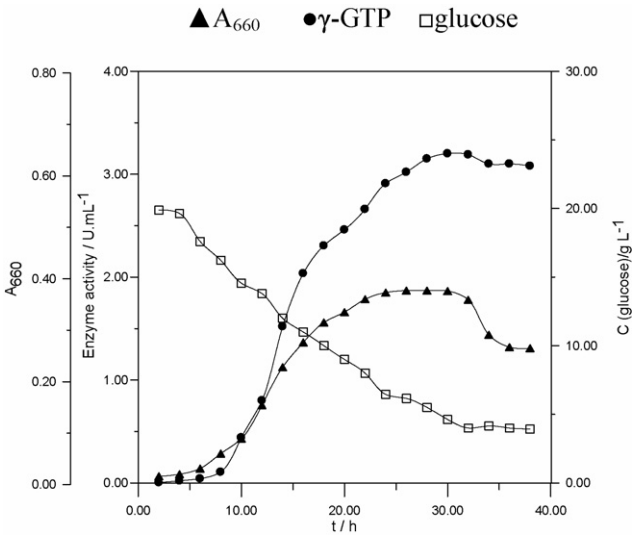


Fig. 1. Time course of γ-GTP production by *B. subtilis* NX-2: (▲) A_{660}; (●) γ-GTP; (□) glucose.

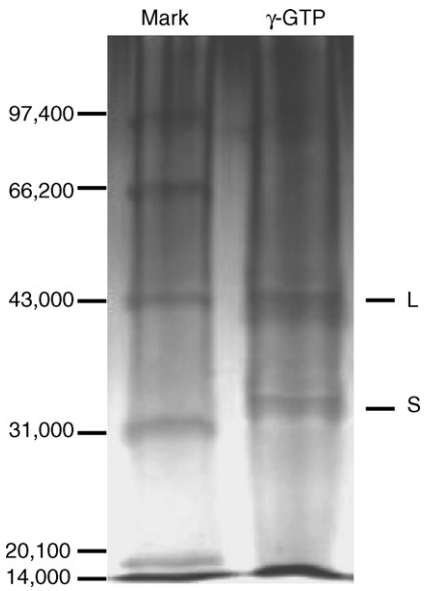


Fig. 2. SDS-PAGE of purified γ-GTP from *B. subtilis* NX-2: Lane 1, molecular weight marker; Lane 2, protein sample. L and S indicated large and small subunits of γ-GTP, respectively.

produced γ-GTP only after the end of the exponential phase and the γ-GTP activity disappeared in several hours [13].

3.2. Purification of γ-GTP

γ-GTP from *B. subtilis* NX-2 was purified to electrophoretic homogeneity by a simple purification procedure: acetone precipitation, hydrophobic interaction chromatography, ion exchange chromatography. Four peaks were observed in the profile of hydrophobic interaction chromatography with the corresponding volume of 42, 38, 26 and 40 ml, respectively, peak II showed transpeptidation activity and was collected for further purification via ion exchange chromatography. Four peaks were obtained on ion exchange chromatography with the corresponding volume of 20, 32, 32 and 44 ml, respectively. The second peak showed a high transpeptidation activity. So it was chosen for further properties research. Table 1 showed the changes of transpeptidation activity of γ-GTP during its purification steps. The specific activity of the purified γ-GTP increased from 0.66 to 73.36 U/mg. The γ-GTP was purified 111.15-fold with a recovery of 15.44%.

3.3. The subunit composition of γ-GTP

SDS-PAGE analysis of the enzyme gave two bands corresponding to molecular weight of 43 and 32 kDa in Fig. 2, indicating that the γ-GTP of *B. subtilis* NX-2 was a heterodimer consisting of one large and one small subunit. This subunit composition was similar to those from mammalian and bacterial species [19–22]. But the size of the small subunit was slightly larger than that of most of the γ-GTPs reported, which might lead to the differences in enzyme structure and the properties.

3.4. The optimum temperature and pH of γ-GTP

The effect of temperature on enzymatic activity of purified γ-GTP of *B. subtilis* NX-2 was studied by carrying out the enzyme reaction at different temperatures in the range of 10–80 °C. γ-GTP exhibited maximal activity at 40 °C (Fig. 3), and the enzyme activity remained high in a wide range of 40–60 °C.

Another important factor significantly affecting the activity of γ-GTP of this strain was the pH value. The experimental results showed the optimum pH for the transfer activity of the purified γ-GTP was pH 8.0 (Fig. 4), which was similar to most of the γ-GTPs reported.

3.5. Substrate specificity of γ-GTP for γ-glutamyl acceptors

The transfer of γ-glutamyl from γ-GpNA to various amino acids by the catalysis of γ-GTP of this strain was studied, and the results were shown in Table 2. The transfer activity with gly-

Table 1
Purification of γ-GTP from *B. subtilis* NX-2

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Supernatant	8572	5700	0.66	100.00	1.00
Acetone precipitation	1329	3298	2.48	57.86	3.76
CL4B-Sepharose	53	1631	30.77	28.61	46.62
DEAE-Sephodax	12	880	73.36	15.44	111.15

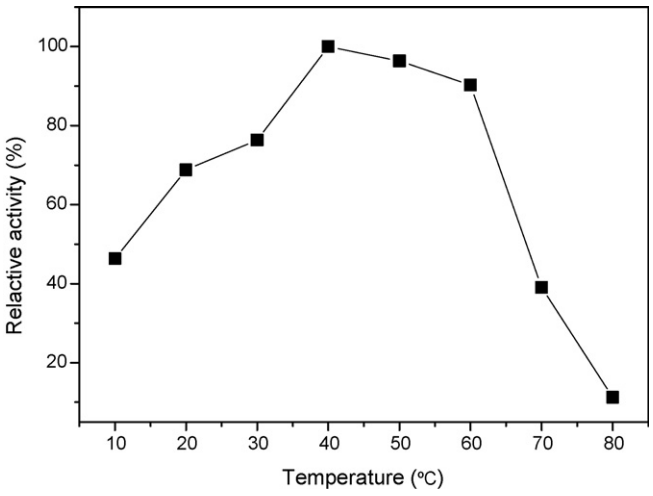


Fig. 3. Effect of temperature on γ-GTP activity.

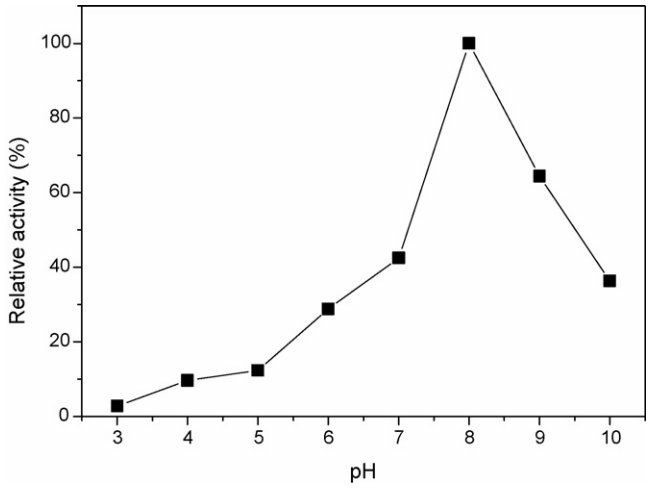


Fig. 4. Effect of pH on γ-GTP activity.

cylglycine as substrate was taken as 100%. γ-GTP of *B. sutilis* NX-2 exhibited much higher activity with the basic amino acids than the acidic amino acids as γ-glutamyl acceptors. The activity decreased when the side chain length of amino acid acceptor was prolonged, which implied the effect of steric hindrance for the catalysis of γ-GTP of this strain. Therefore, it could be deduced from the results that basic amino acids and amino acids with simple structure were better γ-glutamyl acceptors in the transpeptidation reaction catalyzed by the γ-GTP of this strain. Besides, γ-GTP of this strain exhibited the same activities in catalyzing L-amino acid and its racemic form, which indicated no influence of the stereochemistry on the enzyme activity of the γ-glutamyl transfer. All the results obtained as above indicated the γ-GTP of this strain is of different catalytic characteristic, comparing with most γ-GTPs reported [13,19,23–25].

Table 2
Substrate specificity of γ-GTP for γ-glutamyl acceptors

Amino acid	Relative rate (%)	Amino acid	Relative rate (%)
L-Arg	113.58	L-Val	30.22
Gly–Gly	100.00	L-Thr	27.33
L-His	82.85	DL-Thr	26.97
L-Lys	79.39	L-Try	24.11
L-Met	48.76	L-leu	21.32
DL-Met	48.41	L-Ile	20.68
L-Cys	46.91	L-Pro	13.82
DL-Phe	39.94	DL-Ser	13.25
L-Asn	39.33	L-Ser	12.75
L-Tyr	34.01	L-Asp	4.50
L-Gly	33.19	L-Glu	4.40

3.6. The reaction of L-glutamine and glycylglycine catalyzed by γ-GTP

Compared with γ-G_pNA, L-glutamine is less expensive, with better water-solubility. Therefore, it was more suitable to be used as γ-glutamyl donor in the transpeptidation reactions in industrial applications.

Fig. 5 shows the thin layer chromatogram of L-glutamine and glycylglycine reaction system. After reaction, the amounts of the substrates L-glutamine and glycylglycine decreased remarkably, simultaneously, L-glutamic acid and a new matter were produced. The mass spectrum of the reaction system indicated that the new matter was Gln–Gly–Gly (Fig. 6). It implied that γ-GTP from *B. subtilis* NX-2 could not only catalyze the transfer of γ-glutamyl moieties to the glycylglycine, but also catalyze the hydrolysis of the γ-glutamyl linkage. L-Glutamine functioned as a substrate for both the transpeptidation and hydrolysis reactions, which was identical to the properties of γ-GTP in *E. coli*. The reactions in this system were shown in Scheme 1.

The yield of transpeptidation reaction would be reduced due to the hydrolytic activity of γ-GTP. Fortunately, it was reported

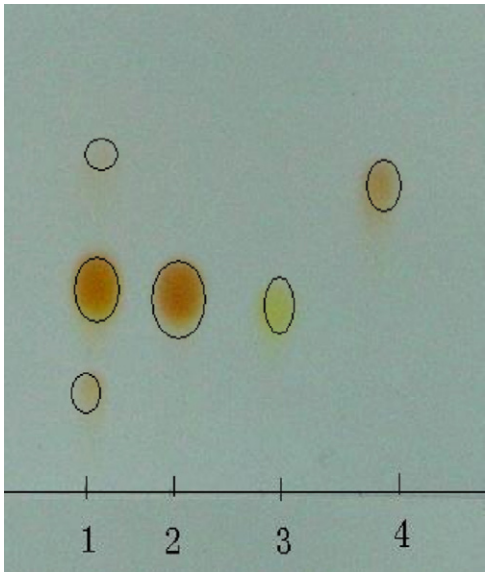


Fig. 5. Thin layer chromatogram of L-glutamine and glycylglycine reaction system: (1) mixtures after reacted; (2) L-glutamine; (3) glycylglycine; (4) L-glutamic acid. The reaction mixtures contained 50 mmol/l Tris–HCl (pH 8.0), 20 mmol/l L-glutamine, 20 mmol/l glycylglycine and the enzyme solution. The concentrations of L-glutamine, glycylglycine and L-glutamic acid spotted were 20 mmol/l, and 10 μl of each sample was spotted on the plate.

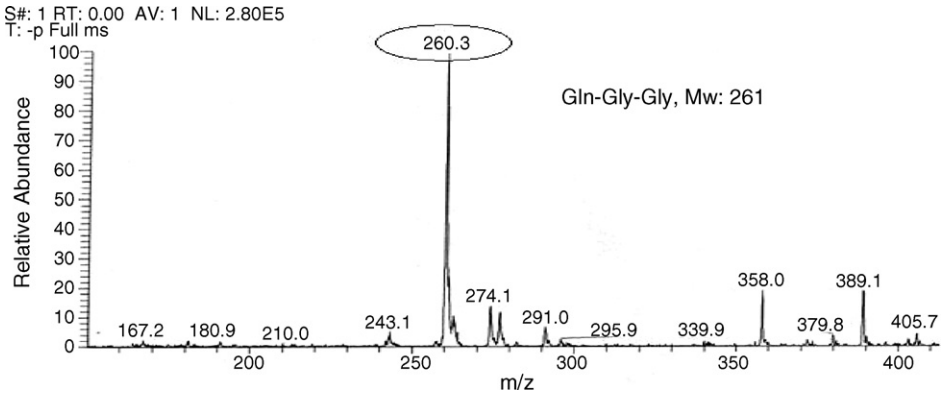
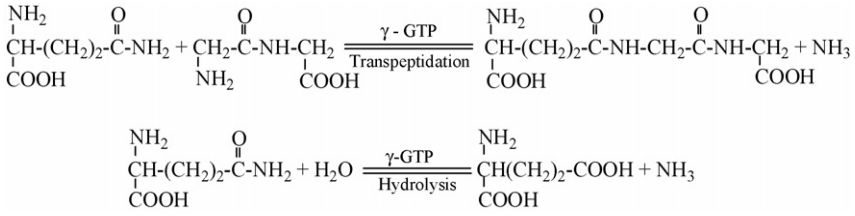


Fig. 6. Mass spectrum of L-glutamine and glycylglycine reaction system.



Scheme 1. Transpeptidation and hydrolysis reactions catalyzed by γ-GTP.

that the optimum conditions of these two reactions were different, such as the reaction pH, the ratio of γ-glutamyl acceptor to donor [13]. Taking advantage of this characteristic, we could make γ-GTP catalyze the transpeptidation reaction selectively by adjusting the reaction conditions.

4. Conclusions

The production of γ-GTP from a newly isolated *B. subtilis* NX-2 was carried out. 3.2 U/ml of high yield was obtained from inexpensive materials. The purified enzyme was gained after a series of extraction and purification steps. γ-GTP of this strain exhibited high activity at 40–60 °C and pH 8.0. It preferred basic amino acids as γ-glutamyl acceptors in the γ-glutamyl transfer reactions, and the stereochemistry of the γ-glutamyl acceptor had no influence on the enzyme activity. These all made it different from other γ-GTPs ever reported. Furthermore, we found it could catalyze the transfer of L-glutamine to glycylglycine to synthesize Gln–Gly–Gly, which was promising for the synthesis of valuable γ-glutamyl peptides.

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