J. Microbiol. Biotechnol. (2001), **11**(6), 946–950

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY
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Purification and Characterization of Extracellular and Intracellular Glutamine Synthetases from *Mycobacterium bovis* BCG

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Received: May 24, 2001
Accepted: September 10, 2001

Abstract Slow-growing pathogenic mycobacterium species, including *Mycobacterium bovis* BCG, secrete a large amount of glutamine synthetase into culture media. Extracellular and intracellular glutamine synthetases were purified from *M. bovis* BCG. While the native molecular weights of both glutamine synthetases were estimated to be 370.2 kDa, those of the subunits were 61.7 kDa, indicating that the native forms were composed of 6 subunits. The enzymes showed a high thermal stability and high degree of sequence similarity with the glutamine synthetase from *M. tuberculosis* in the N-terminal amino acid sequence. Western blotting analysis indicated that the antibodies prepared against both the extracellular and intracellular enzymes exhibited common antigen determinants.

Key words: *Mycobacterium bovis* BCG, glutamine synthetase, secretion

Tuberculosis is one of the leading causes of death from a single infectious agent, accounting for approximately 26% of all preventable adult deaths in the world. It is estimated that approximately 0.6 billion people are infected with the causative agent, *Mycobacterium tuberculosis*, while 8–10 million new cases and 3 million deaths occur annually [3]. Furthermore, the emergence of drug-resistant strains [17], together with the problems associated with tuberculosis in human immunodeficiency virus-infected populations [5], have recently brought tuberculosis research to the forefront.

M. tuberculosis is a facultative intracellular parasite that resides and multiplies within a membrane-bound phagosome in human mononuclear phagocytes, especially lung macrophages [16]. One remarkable finding was that glutamine synthetase [L-glutamate: ammonia ligase (ADP-forming); EC 6.3.1.2] is abundantly secreted by pathogenic mycobacteria, and yet not by nonpathogenic mycobacteria or nonmycobacterial organisms, and released into its phagosome in infected human macrophages [6]. Therefore, glutamine synthetase has been identified as one of several potentially important determinants of *M. tuberculosis* pathogenesis. Glutamine synthetase would appear to influence the ammonia level within the phagosome containing the pathogen in host cells, thereby profoundly altering the physiological conditions in the phagosome, while the enzyme seems to be directly involved in the synthesis of poly-L-glutamic acid/glutamine found abundantly in the cell wall of pathogenic mycobacteria, yet not in nonpathogenic mycobacteria [7].

M. bovis BCG (bacille Calmette-Guerin), an attenuated strain of virulent cow tuberculosis (*M. bovis*), has been developed as a vaccine against tuberculosis, and is widely used in many countries throughout the world [9]. In addition, a renaissance of *M. bovis* BCG utility as a cancer agent has occurred in the intercavitary treatment of superficial bladder cancer [14].

In the present study, we purified both the extracellular and intracellular glutamine synthetases from *M. bovis* BCG and then compared their enzyme properties.

MATERIALS AND METHODS

Bacterial Cultures

The *M. bovis* BCG (Pasteur 1173-P-2), *M. tuberculosis* (ATCC 27294), *M. kansasii* (ATCC 12478), *M. avium* (ATCC 25291), *M. smegmatis* (ATCC 19420), *M. fortuitum* (ATCC 6841), and *M. vaccae* (ATCC 15483) cultures were all grown in a 7H9 medium (Difco, Detroit, U.S.A.). *Escherichia coli* DH5α was grown on an LB medium. *M. bovis* BCG was cultured in Sauton's medium for glutamine synthetase purification.

Assays of Glutamine Synthetase Activity

The enzyme was assayed both using γ-glutamyl transferase (glutamine + hydroxylamine → γ-glutamylhydroxamate + ammonia), and a biosynthetic reaction (forward) assay

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(glutamate + ATP + ammonia → glutamine + ADP + Pi), as previously described [1]. One unit of glutamine synthetase was defined as the amount of enzyme producing 1 µmol of glutamyl hydroxamate per min. The pH optima of the glutamine synthetases were determined for both assay systems in the pH range of 6.0 to 9.0. The requirement of a cation for the enzyme activities was also examined for both reactions. Cobalt (II) chloride, magnesium chloride, and manganese chloride were added at 50 mM for the biosynthetic reaction and at 3 mM for the transfer reaction.

Measurement of Extracellular and Intracellular Glutamine Synthetases

The activity of the mycobacterial species, including the *M. bovis* BCG glutamine synthetases associated with the cell pellet or released into the culture medium, was determined using the standard transfer reaction for bacteria cultured to the early log phase. The bacterial supernatants were obtained by filtering the cultures through 0.45 µm filters (AVANTEC MFS, Inc., CA, U.S.A.) and concentrating the filtrate 5-fold. To prepare the bacterial cell extracts, the bacteria were first lysed with lysozyme/Triton X-100, vortexed for 40 sec with 0.1 mm diameter glass beads (Biospec Inc., Bartlesville, U.S.A.), then the mixture was centrifuged to obtain the supernatant.

Purification of Glutamine Synthetase

To purify the extracellular glutamine synthetase, the supernatant from *M. bovis* BCG cultured on Sauton's medium was filtered through 3MM paper and 0.45 µm filters, applied to DEAE-Toyopearl (TOSOH, Cop., Tokyo, Japan), and eluted with 1 M NaCl. The eluted sample was precipitated with ammonium sulfate (40–60% saturation), pelleted by centrifugation, and resuspended in the Tris-Cl buffer (50 mM Tris-Cl, pH 7.0/4 mM $MnCl_2$/3 µM Leupeptin/3 mM DTT). The sample was desalted and concentrated by Ultrafree-15 (Millipore, U.S.A.). The proteins were size fractionated on Sephacryl S-300 (Amersham Pharmacia Biotech, U.K.). The enzymatically active fractions were pooled, applied to an adenosine 5'-diphosphate immobilized on an agarose (cross-linked 4%; Sigma, St. Louis, U.S.A.) and eluted with Tris-Cl buffer containing 8 mM ADP. The enzyme was desalted and concentrated by Ultrafree-15.

The intracellular glutamine synthetase was purified as follows: Cells collected from the *M. bovis* BCG culture on Sauton's medium were washed and resuspended in the Tris-Cl buffer. The sample was sonicated by an Sonifier (Branson Ultrasonics, Danbury; output 6.5, Duty cycle 60%) for 6 cycles of 5 min, with a 5 min rest on ice, and then centrifuged at 5,000 ×g for 10 min to remove any cell debris. The recovered supernatant was purified by the same procedure as that used for the supernatant produced after ammonium sulfate precipitation.

Protein Determination, Electrophoresis, and N-Terminal Amino Acid Analysis

The protein concentrations were measured by Bradford's dye binding method [2] using a protein assay kit (Bio-Rad Lab., Richmond, U.S.A.) with bovine serum albumin as the standard.

SDS-PAGE was performed according to Laemmli's method [12] and the gel was stained with Coomassie Brilliant blue R-250. Native PAGE was carried out in the same way as the SDS-PAGE, except without the addition of SDS.

The purified enzyme was processed on 12% SDS-PAGE gel and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Schleicher & Schuell, Dassel, Germany). The glutamine synthetase band was cut out and the N-terminal sequence was analyzed by Edman degradation using a Milligen 6600 (Milligen Applied Biosystems, Bedford, U.S.A) at the Korea Basic Science Institute in Seoul [4].

Immunologic Reactivity of Glutamine Synthetase

Polyvalent antibodies against glutamine synthetase were raised in 6–8-week-old mice (DaeHan Laboratory Animal Research Center Co., Seoul, Korea) through immunization with 2.25 µg of the purified enzyme in an incomplete Freund's adjuvant (GibcoBRL, New York, U.S.A.) containing 50 µg of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Sigma, St. Louis, U.S.A.). Three booster injections were given at 4, 6, and 8 weeks after the primary immunization. The antibody titer was determined by ELISA.

Western Blotting and Immunodiffusion

After SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, U.K.) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Lab., Richmond, U.S.A.) in a transfer buffer (Tris-glycine-methanol) for 2 h at 200 mA. The trans-blotted proteins were detected using a Protein Detector Western Blot Kit (KPL, Gaithersburg, U.S.A.) according to the manufacturer's instruction. Immunodiffusion was carried out on a 1.2% (w/v) agarose gel plate (dissolved in PBS) for 24 h.

RESULTS AND DISCUSSION

Extracellular Release of Glutamine Synthetase from *M. bovis* BCG

It has been previously reported that *M. tuberculosis* and *M. bovis* secrete eight enzymes, including the glutamine synthetase in the culture filtrates, whereas nonpathogenic mycobacteria do not secrete any enzyme, nor are present on the cell surface [14]. It has also been observed that pathogenic mycobacteria species released a

Table 1. Comparison of extracellular release of glutamine synthetase by several mycobacteria and *Escherichia coli.*

Bacteria	Activity		Ratio B/A
	Cell extract (A)	Culture supernatant (B)	
Mycobacteria			
M. tuberculosis	37.46	17.50	1:2
M. bovis BCG	48.08	11.50	1:4
M. avium	44.44	14.40	1:3
M. kansasii	146.61	55.20	1:3
M. smegmatis	6.50	0.06	1:108
M. fortuitum	12.92	0.09	1:147
M. phlei	6.90	0.06	1:115
M. vaccae	0.96	0.01	1: 96
E. coli DH5α	2.20	≤0.01	≤1: 220

substantial amount of the glutamine synthetase into culture media [6].

M. vaccae, a rapidly growing bacillus, has been nominated as the most promising immunotherapeutic agent against tuberculosis [18], and the other rapid-growing mycobacteria species release a lower amount of glutamine synthetase. *M. avium* and *M. kansasii* are slow-growing opportunistic human pathogens, and infections due to these bacteria are the most commonly disseminated bacterial infections in immunocompromised populations, such as patients with AIDS [8, 19].

Table 2. N-terminal amino acid sequencing of various glutamine synthetases.

Species	Sequence
M. bovis BCG (extracellular)	TEKTPDDV FKLAKDE
M. bovis BCG (intracellular)	TEKTPDDV FKLAKDEKVEYVD
M. tuberculosis	T EKTPDDVFKLAKDEKVLYL
M. smegmatis	AEKTSDD I FKL I KDENVEYV
M. phlei	AEKTADD I LKL I RDEDVEYG
B. subtilis	AKYTRED I VKLVKEENVKYI
E. coli	SAEHVLTMLNEHEVKFVDLR

M. tuberculosis glutamine synthetases and other N-terminal amino acid sequences were cited from Ref. [6].

In contrast with rapid-growing nonpathogenic mycobacteria, slow-growing pathogenic mycobacteria release glutamine synthetases in a high ratio. In the current study, as an attenuated vaccine against *M. tuberculosis*, *M. bovis* BCG released a high ratio of glutamine synthetase into the culture medium (Table 1).

Purification, Characterization, and Comparison of Extracellular and Intracellular Glutamine Synthetases from *M. bovis* BCG

The glutamine synthetases from *M. bovis* BCG were purified to elucidate the difference between the extracellular and intracellular glutamine synthetases. The yield of purification was about 30%.

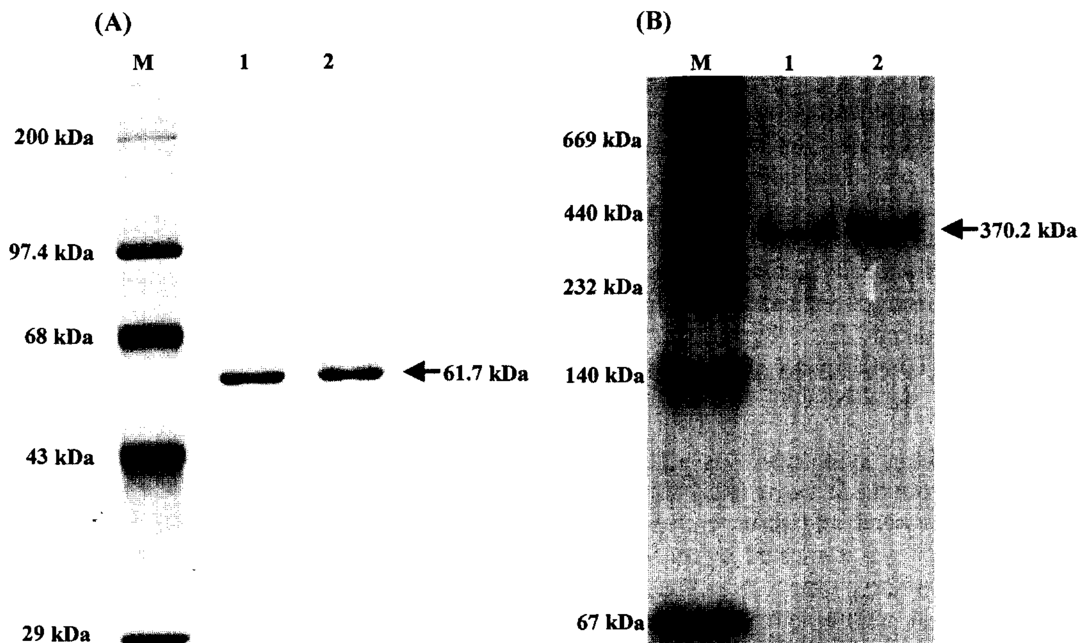

Fig. 1. Ten percent SDS-PAGE and 6% native PAGE of purified glutamine synthetases from *M. bovis* BCG.
Purified glutamine synthetases were electrophoresed on 10% SDS-PAGE (A) and 6% native-PAGE (B) and stained with Coomassie brilliant blue R-250. Lane M: Molecular size marker; lane 1: *M. bovis* BCG extracellular glutamine synthetase; lane 2: *M. bovis* BCG intracellular glutamine synthetase.

Table 3. Optimum pH and relative activity of extracellular and intracellular glutamine synthetases from *M. bovis* BCG in the presence of activating cations.

Glutamine synthetases from *M. bovis* BCG	γ-Glutamyl transferase assay			Biosynthetic assay		
	Mn^{2+}	Mg^{2+}	Co^{2+}	Mn^{2+}	Mg^{2+}	Co^{2+}
Extracellular	7.0(100)	7.5(62)	8.5(23)	6.5(97)	8.0(100)	ND
Intracellular	7.0(100)	7.5(66)	8.5(37)	6.0(41)	8.0(100)	ND

Above data are shown as optimum pH (relative activity (%)).The biosynthetic activities were too low to read in the presence of Co^{2+} (≥5.5% of Mg^{2+}).

On the SDS-PAGE gel, the molecular weights of the subunit for both glutamine synthetases were estimated to be 61.7 kDa (Fig. 1). However, the native molecular weights of both glutamine synthetases were estimated to be 370.2 kDa by the native PAGE gel (Fig. 1) and Sephacryl S-300 chromatography. These results suggested that the native extracellular and intracellular glutamine synthetases from *M. bovis* BCG were both composed of 6 subunits and thereby classified as the GSIII form [13]. The glutamine synthetases from other mycobacteria species, such as *M. tuberculosis*, *M. smegmatis*, and *M. phlei*, have been reported to be composed of 12 subunits (GSI form), with 56–58 kDa for subunit molecular weights and 670–680 kDa for native molecular weights [6, 10, 11]. It is possible that the current results reflect the dissociated form of two layers of a superimposed hexagonal ring of GSI. Yet, there was no other band on the native PAGE gel. Accordingly, the number of subunits suggest that the glutamine synthetases from *M. bovis* BCG are different from the glutamine synthetases from *M. tuberculosis* and other species.

The optimal temperatures for the γ-glutamyl transferase and biosynthetic assay were determined. The activities of the intracellular and extracellular glutamine synthetases were highest at 46°C and 40°C for the γ-glutamyl transferase assay, and 48°C and 50°C for the biosynthetic assay, respectively.

Heat treatment of the glutamine synthetases from *M. bovis* BCG at 50°C, 55°C, 60°C, and 65°C for 240 min showed that both enzymes had a high thermal stability in the γ-glutamyl transferase and biosynthetic activity assays. Considering the fact that 87% activities remained at 65°C for 240 min, the thermal stability of the biosynthetic activity of the intracellular glutamine synthetase was considerably higher than those of the glutamine synthetases from *M. smegmatis* and *M. phlei* (data not shown) [11].

The N-terminal amino acid sequences of both glutamine synthetases showed a high degree of homology with the glutamine synthetase from *M. tuberculosis* (Table 2).

The specificity of the glutamine synthetases from *M. bovis* BCG was determined at varying pHs with the addition of different divalent cations (Table 3). The results demonstrated different effects relative to the specificity for the biosynthetic activity of the extracellular glutamine synthetase of *M. tuberculosis*: in the presence of Mg^{2+} and Co^{2+}, the optimum was pH 7.5, while in the presence of Mn^{2+}, it was pH 7.0.

The K_m values of the extracellular and intracellular glutamine synthetases from *M. bovis* BCG were 126.8 and 173.75 mM, respectively, for glutamic acid in the biosynthetic activity, and 28.6 and 26.28 mM, respectively, for glutamine in the γ-glutamyl transferase assay. These values were much higher than those for the extracellular glutamine synthetase from *M. tuberculosis*, which showed K_m values of 2.7 mM for glutamic acid and 2.9 mM for glutamine.

The Western blotting and immunodiffusion analyses using the antibodies of the extracellular and intracellular glutamine synthetases from *M. bovis* BCG showed that the antigen determinants of both enzymes appeared to be in common (Fig. 2).

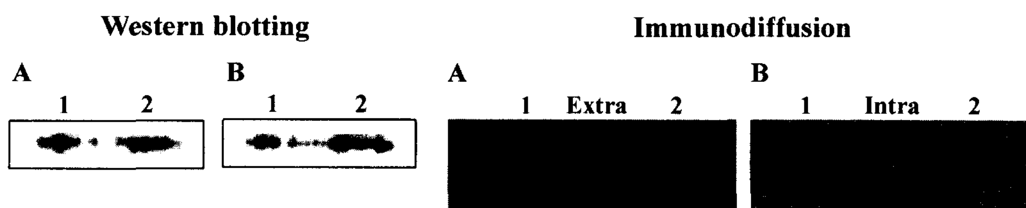

Fig. 2. Analysis of extracellular and intracellular glutamine synthetases by Western blotting and immunodiffusion.
Western blotting: The extracellular (lane 1) and intracellular (lane 2) glutamine synthetases were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies for the extracellular glutamine synthetase (A) and intracellular glutamine synthetase (B). Immunodiffusion: The extracellular (lane 1) and intracellular (lane 2) glutamine synthetases, antibody for the extracellular glutamine synthetase (Ext), and antibody for intracellular glutamine synthetase (Int) were loaded in the hole in a 1.2% agarose plate and diffused for 24 h. The precipitated antibody and glutamine synthetase complex was stained in 0.1% Ponceau S/5% acetic acid.

Acknowledgment

This research was supported by a Brain Korea 21 project grant from the Ministry of Education, Republic of Korea.

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