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Efficient expression and purification of recombinant glutaminase from *Bacillus licheniformis* (GlsA) in *Escherichia coli*

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ABSTRACT

Glutaminase or L-glutamine aminohydrolase (EC 3.5.1.2) is an enzyme that catalyzes the formation of glutamic acid and ammonium ion from glutamine. This enzyme functions in cellular metabolism of every organism by supplying nitrogen required for the biosynthesis of a variety of metabolic intermediates, while glutamic acid plays a role in both sensory and nutritional properties of food. So far there have been only a few reports on cloning, expression and characterization of purified glutaminases. Microbial glutaminases are enzymes with emerging potential in both the food and the pharmaceutical industries. In this research a recombinant glutaminase from *Bacillus licheniformis* (GlsA) was expressed in *Escherichia coli*, under the control of a p*tac* promoter. The recombinant enzyme was tagged with decahistidine tag at its C-terminus and could be conveniently purified by one-step immobilized metal affinity chromatography (IMAC) to apparent homogeneity. The enzyme could be induced for efficient expression with IPTG, yielding approximately 26,000 units from 1-l shake flask cultures. The enzyme was stable at 30 °C and pH 7.5 for up to 6 h, and could be used efficiently to increase glutamic acid content when protein hydrolysates from soy and anchovy were used as substrates. The study demonstrates an efficient expression system for the production and purification of bacterial glutaminase. In addition, its potential application for bioconversion of glutamine to flavor-enhancing glutamic acid has been demonstrated.

Introduction

Glutaminase (GlsA)[2] or L-glutamine aminohydrolase (EC 3.5.1.2) is a member of the beta-lactamase superfamily that catalyzes the hydrolytic degradation of L-glutamine to L-glutamic acid and ammonia [1]. This enzyme is highly specific to L-glutamine and diverges from glutaminase–asparaginase (EC 3.5.1.1), which is less specific and can catalyze the hydrolysis of both glutamine and asparagine to glutamic and aspartic acid, respectively [2,3]. The enzyme functions in cellular metabolism of every organism by supplying the nitrogen required for the biosynthesis of a variety of metabolic intermediates, whereas glutamic acid plays a role in both the sensory and the nutritional properties of food [2]. Microbial glutaminases are enzymes with emerging potential in both the food and the pharmaceutical industries [2]. Even though many native glutaminases from microorganisms including bacteria, yeast and fungi have been purified and characterized [4–14], there have been only a few reports on the cloning, expression, purification, and characterization of recombinant glutaminases [3,15].

Bacillus licheniformis is a Gram-positive endospore-forming bacterium from which glutaminase is induced when grown on glutamine as the sole nitrogen source [4]. It is generally non-pathogenic and has been used extensively for industrial production of various enzymes and metabolites [16]. In addition, previous studies in our laboratory have shown that it is a good source of several enzymes that are appropriate for biotechnological applications such as, β-mannanase [17], endo-chitinase [18], and β-galactosidase [19].

This research reports the cloning, expression, and characterization of glutaminase from this bacterium. In addition, its potential application for bioconversion of glutamine in protein hydrolysates is demonstrated.

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[2] *Abbreviations used:* GlsA, Glutaminase; MCS, multiple cloning site; IPTG, isopropyl β-d-1-thiogalactopyranoside; **PMSF,** phenylmethylsulfonyl fluoride; Ni–NTA, nickel–nitrilotriacetic acid; **Vo,** Void volume; **Ve,** elution volume; **GDH,** glutamate dehydrogenase; NAD, nicotinamide adenine dinucleotide; **PMS,** phenazine methosulphate; **DCPIP,** 2,6-dichlorophenol-indophenol; **ADP,** adenosine diphosphate; **SPI,** Soy protein isolate; **TNBS,** trinitrobenzenesulfonic acid.

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Materials and methods

Bacterial stains, culture conditions, plasmids, and enzymes

B. licheniformis DSM13 (ATCC 14580) was obtained from DSM German Culture Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Escherichia coli* TOP 10 was purchased from Invitrogen Corporation (San Diego, CA, USA). *Pfu* DNA polymerase was purchased from Promega Corporation (Madison, WI, USA). All enzymes for molecular cloning were purchased from New England Biolabs Inc. (Ipswich, MA, USA). All chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Construction of pMY202 expression vector

A vector for the expression of *B. licheniformis* glutaminase was constructed by replacing the multiple cloning site (MCS) of pFLAG-CTS expression vector (Sigma Chemical Co., St. Louis, MO, USA) with an appropriate 5′ overhang DNA fragment, flanking with *Hind* III and *Xho* I restriction sites, according to a previously published technique [20]. This vector was designated pMY202 and its map is depicted in Fig. 1. The vector allows the expression of recombinant enzyme fused with C-terminal 10×His, followed by FLAG tags, under the control of p*tac* promoter.

Molecular cloning of the glutaminase gene from Bacillus licheniformis

The glutaminase gene (*glsA*) from *B. licheniformis* was cloned by a PCR-based method as previously described [21]. The oligonucleotide primers were designed from the genomic database of *B. licheniformis* DSM13 (ATCC 14580), according to the DNA sequence of *glsA* gene (NCBI Accession No. NC_006270 REGION: 247283–248266; GeneID: 3027975). The two primers, B.li13GtmNdeIFw (CTG TGC CAT ATG AAT GAA GTA TTG GAA GAA CGC TAT GAC) and B.li13GtmBamHIRv (GCA CAG GGA TCC AAA AAT ACT GAG ATC CCA TTC ACG GGC TAT ATG), carried *Nde* I and *BamH* I restriction sites (underlined bases) for cloning into the corresponding sites (*Nde* I and *Bgl* II) on pMY202. Note that *BamH* I and *Bgl* II restriction sites are compatible. *Pfu* DNA polymerase was used in the PCR reaction, which was performed as follows: an initial DNA denaturation at 95 °C for 1 min, 30 cycles of denaturation (95 °C for 30s), annealing (52 °C for 45 s), extension (72 °C for 2 min), and a final extension at 72 °C for 10 min. The integrity of the construct was confirmed by automated DNA sequencing (Macrogen, Korea). The recombinant plasmid was designated pB.liGlsA1.

Enzyme production and purification

E. coli Top10 harboring the recombinant plasmid pB.liGlsA1was grown on Luria–Bertani (LB) agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar) containing 100 μg/ml of ampicillin at 37 °C, for 12 h. Then, a single colony was inoculated into 5 ml of LB broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 100 μg/ml of ampicillin and incubated at 37 °C, with shaking (200 rpm). After incubation for 12 h, 2 ml of the culture was transferred into 200 ml of the same medium and cultivated at 37 °C, 200 rpm, until the optical density at 600 nm reached 0.6–1.0. Subsequently, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture broth at a final concentration of 1 mM and the culture was continually cultivated at 30 °C, 180 rpm, for 20 h.

To collect the crude enzyme, a cell pellet was collected by centrifugation at 8000*g* for 15 min at 4 °C (RC 28S, Sorvall Co., Newtown, CT, USA). Cells were resuspended in lysis buffer (10 mM imidazole, 10 mM Tris–HCl, 0.3 M NaCl, pH 8.0) containing 1 mg/ml lysozyme and 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated on ice for 30 min. Incubated cells were disrupted by sonication for 4 min (60 amplitude and pulser 6 s) with a High Intensity Ultrasonic Processor (100-W Model, Sonics and Materials Inc., Newtown, CT, USA). Supernatant was collected by centrifugation at 8000*g* for 30 min at 4 °C, and clarified by centrifugation at 12,000*g* for 30 min at 4 °C.

To purify the recombinant enzyme, the clarified sample was filtered through a 0.45-μm cellulose acetate membrane. The filtered sample was applied to nickel–nitrilotriacetic acid (Ni–NTA) agarose (Qiagen, QIAGEN GmbH, Hilden, Germany) equilibrated with three bed volumes of lysis buffer, using the Biologic LP system (Bio-Rad Laboratories, Hercules, CA, USA). The column (0.9 × 4.5 cm) was washed with 10 bed volumes of the lysis buffer, followed by 10 bed volumes of wash buffer (20 mM imidazole, 30 mM Tris–HCl, 300 mM NaCl, pH 8.0). The bound protein was eluted by a linear gradient of 20–300 mM imidazole in 30 mM Tris–HCl, 0.3 M NaCl, pH 8.0 for 20 bed volumes. Fractions of 2 ml were collected at a flow rate of 1 ml/min. All fractions were tested for GlsA activity. Fractions containing GlsA activity were pooled and dialyzed against 50 mM Tris–HCl (pH 8.0), using a membrane with 10 kDa molecular weight cut-off (MWCO) at 4 °C, for 12 h.

Determination of enzyme molecular weight (MW)

MW of the denatured form of the recombinant *B. licheniformis* glutaminase was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [22]. The protein band was visualized by staining with Coomassie brilliant blue R-250. MW of the native, non-denatured, recombinant GlsA was determined using a Superose 6 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) column (1.6 × 90 cm), equilibrated and eluted with 0.15 M NaCl, 50 mM Tris–HCl, pH 7.0. Fractions of 0.85 ml were collected at a flow rate of 0.5 ml/min. Void volume (Vo) was determined using blue dextran (Sigma Chemical Co., St. Louis, MO, USA). The ratio of elution volume (Ve) to Vo was calculated. The column was calibrated using a protein standard consisting of β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (13 kDa) (Sigma Chemical Co., St. Louis, MO, USA).

Glutaminase activity assay

Glutaminase activity was assayed according to previous methods [23,24]. The formation of L-glutamic acid generated by the activity of glutaminase was determined using glutamate dehydrogenase (GDH). In the presence of nicotinamide adenine dinucleotide (NAD), GDH reacts with L-glutamic acid to produce α-ketoglutaric acid and NADH. The formation of NADH was detected by two oxidation–reduction reactions. First, phenazine methosulphate (PMS), which is an electron acceptor, is reduced by NADH; then 2,6-dichlorophenol-indophenol (DCPIP) is further reduced by PMSH. The decrease in absorbance at 595 nm, due to the reduction of DCPIP was used to calculate the amount of NADH form, which is proportional to the amount of L-glutamic acid generated. The reaction mixture (200 μl) containing 50 μl of purified recombinant enzyme (1.2 mg/ml), 100 mM Tris–HCl (pH 7.5), and 50 mM glutamine was incubated at 30 °C for 5 min. The reaction was then stopped by heating at 95 °C for 5 min. After that, 100 μl of the terminated reaction mixture was taken for glutamic acid determination by mixing with 100 μl of solution comprising 7.9 mU/μl glutamate dehydrogenase, 500 mM Tris–HCl (pH 9.0), 500 μM adenosine diphosphate (ADP), 1.5 mM nicotinamide adenine dinucleotide (NAD), 350 μM phenazine methosulphate (PMS), and 50 μM DCPIP. Then, the reaction mixture was incubated at room temperature for 8 min before the absorbance was immediately measured at 595 nm. Unit activity was defined as μmole of glutamic acids generated per min.

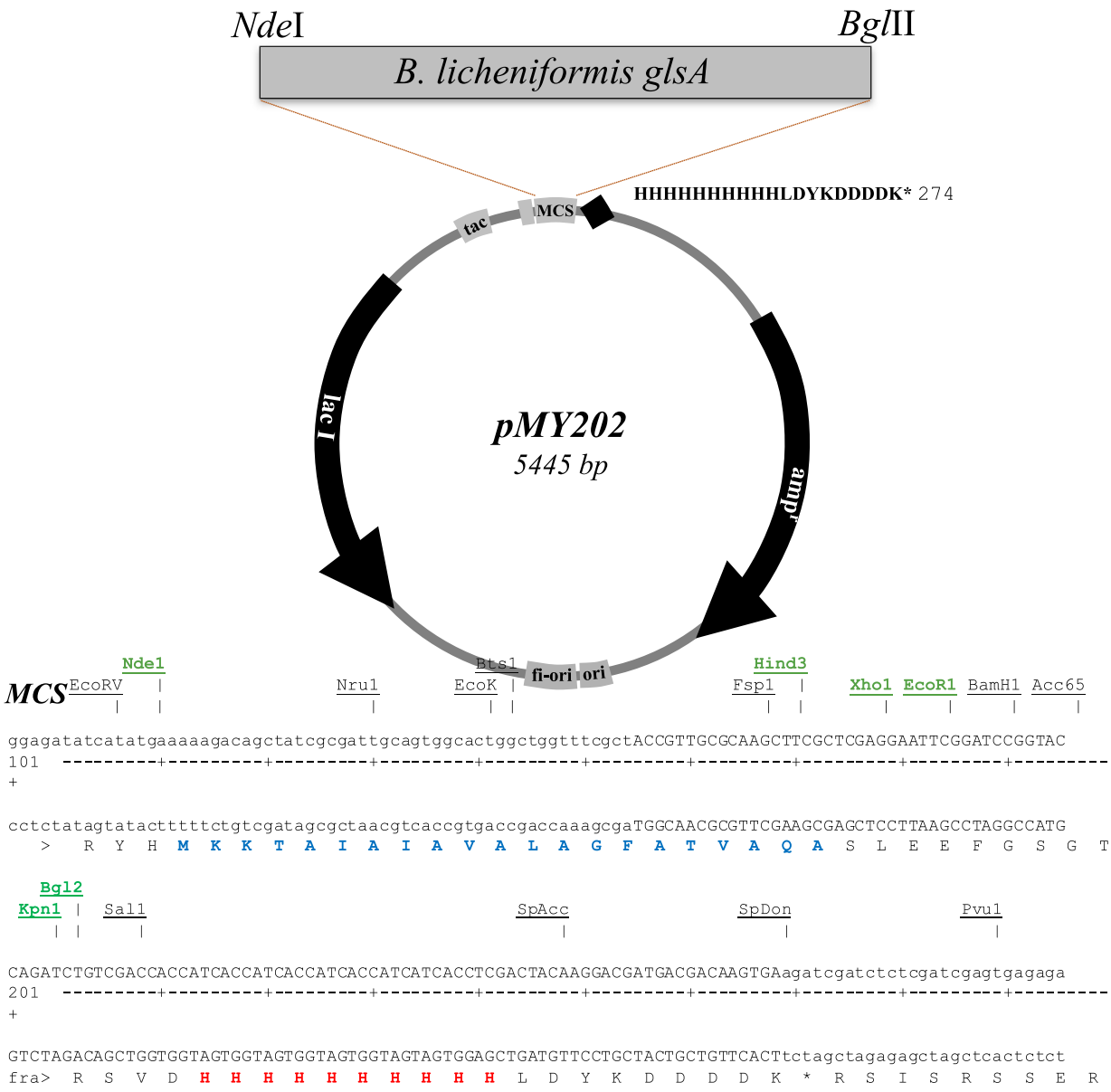

Fig. 1. Map of the pMY202 expression vector and the recombinant *B. licheniformis* glutaminase construct (pB.liGlsA1). The expression vector pMY202 was modified from pFLAG-CTS. The sequence of the multiple cloning site (MCS) is shown at the bottom of the figure. The MCS was located behind OmpA signal peptide for the secretion of the fusion protein into periplasmic space. Useful cloning sites are *Hind* III, *Xho* I, *Eco* RI, *Kpn* I, and *Bgl* II. This vector allows C-terminal fusion of recombinant protein with decahistide tag, followed by FLAG eptiope (DYKDDDDK), as depicted. *Nde* I restriction site is located at the 5′ end of the DNA fragment encoding OmpA sequence, allowing the cloning of recombinant protein without *E. coli* signal peptide. *B. licheniformis glsA* gene was cloned between *Nde* I and *Bgl* II restriction sites, in frame with 10×His and FLAG tags. The gene is under the control of a p*tac* promoter, which is a hybrid of *lac* and *trp* promoters. The plasmid contains ampicillin-resistant gene for selection and maintenance of the recombinant construct.

Kinetic analysis

The reaction was carried out using L-glutamine as a substrate at various concentrations ranging from 10 to 150 mM, at 30 °C and 100 mM Tris–HCl (pH 7.5). The Kinetic constants (K_m and V_{max}) were calculated from the Michaelis–Menten plot of experimentally obtained data, using a non-linear regression analysis (Sigma plot; version 2.0, SPSS, Chicago, IL, USA).

Protein determination

The protein concentration was determined by Bradford's method [25] using bovine serum albumin as a standard.

Effect of temperature and pH on enzyme activity

The optimal temperature for catalytic activity of recombinant *B. licheniformis* glutaminase was measured at 25, 30, 35, 40, 45, 55, and 60 °C in 100 mM Tris–HCl, pH 7.5. Optimal pH was measured using 100 mM sodium acetate buffer for pH 3, 4, 5, and 6; 100 mM Tris–melate for pH 6, 6.5, and 7; 100 mM Tris–HCl for pH 7, 7.5, 8, 8.5, and 9; and 100 mM glycine–NaOH for pH 9, 10, and 11.

Thermal stability

Temperature stability was investigated by pre-incubating the purified enzyme in 100 mM Tris–HCl (pH 7.5) at 25, 30, 35, 40, 50, 60, and 70 °C for 30 min, without substrate. In addition, the

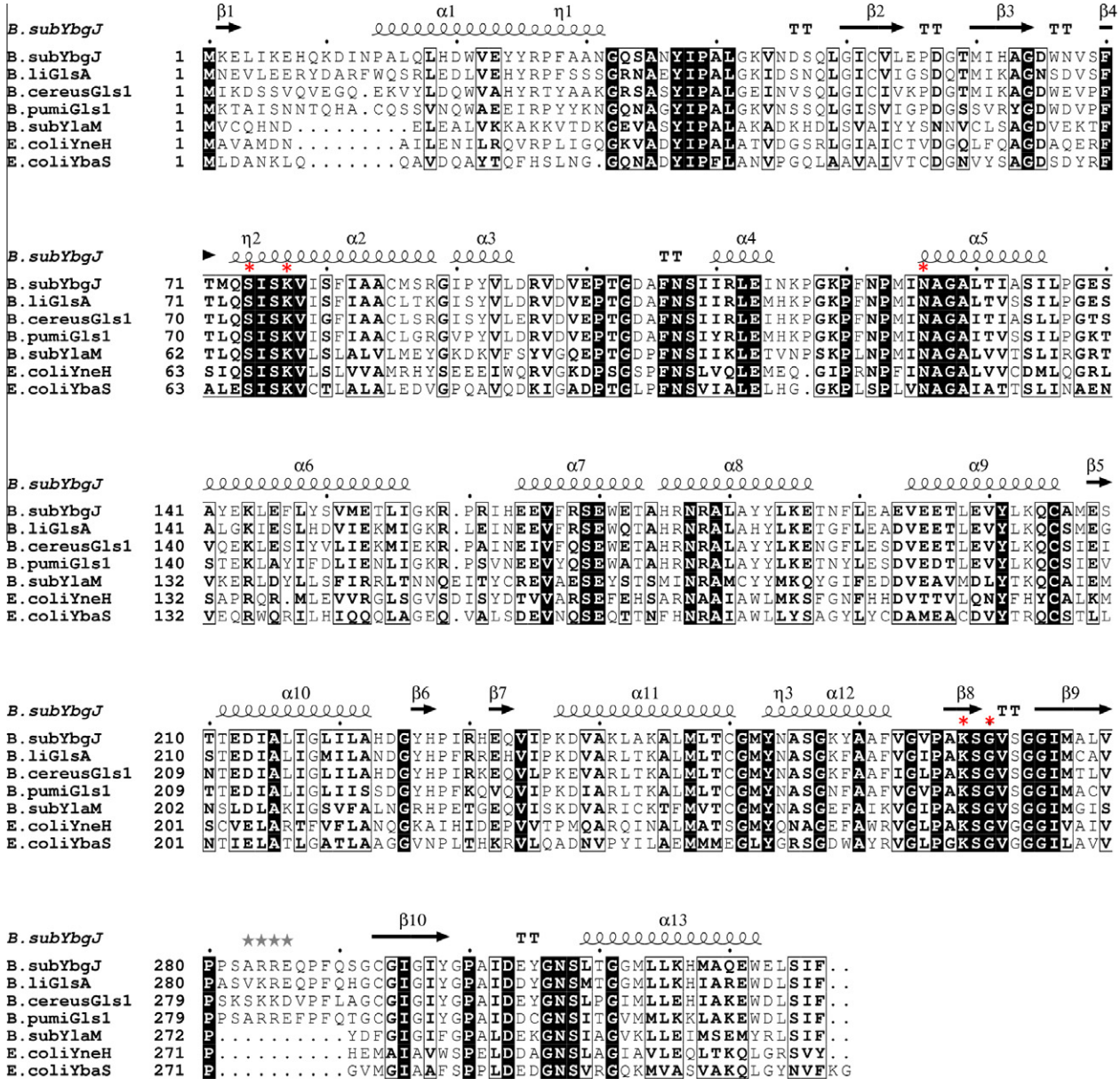

Fig. 2. Amino acid sequence alignment of glutaminase from *B. licheniformis* and other related bacterial glutaminases The secondary structure elements are shown above the aligned sequences. α-Helices are displayed as squiggles; β-strands are rendered as arrows. A white character in a box indicates strict identity, whereas a black character in a frame indicates similarity across a group. Key catalytic residues of β-lactamases are marked with asterisks. Multiple sequence alignment was done by CLUSTAL W followed by ESPript to display the secondary structure of the *B. subtilis* YbgJ (1MKI). The glutaminases compared are *B. subtilis* YbgJ (O31465), *B. licheniformis* GlsA (YP_077519.1), *B. cereus* Gls1 (YP_002452114.1), *B. pumi* Gls1 (ZP_03056239.1), *B. subtilis* YlaM (O07637), *E. coli* YneH (P0A6W0), and *E. coli* YbaS (P77454).

thermal inactivation kinetics at 30 °C was determined in 100 mM Tris–HCl (pH 7.5) by measuring the residual enzyme activity at various time points under standard assay conditions. Relative activity was calculated using the sample showing the highest activity as 100%.

Glutamic acid production in a reaction using protein hydrolysate as a substrate

Soy protein isolate (SPI) and minced whole anchovy (*Stolephorus* spp.) were mixed with cold 100 mM Tris–HCl (pH 8.0) at a final concentration of 1% and 10% (w/w), respectively. After stirring at 4 °C for 6 h, 0.25% (v/v) of enzyme Alcalase (Novozymes A/S, Bagsvaerd, Denmark) was added, and the mixture was incubated at 60 °C, with shaking (100 rpm), for 2 h. After that, 0.05% (w/v) of flavozyme (Novozymes A/S, Bagsvaerd, Denmark) was added, and the incubation was continued at 50 °C, with shaking (100 rpm), for another 4 h. Then, the activity of the proteinases was stopped by heating at 90 °C for 10 min, and the samples were collected by centrifugation at 20,000*g* at 4 °C, for 30 min. Supernatants were collected and used as substrates for the bioconversion reactions. For fish hydrolysate, the sample was filtrated through No.1 filter paper (Whatman, 12.5 cm diameter) before use. α-Amino content was determined using trinitrobenzenesulfonic acid (TNBS), according to Fields's method [26]. An absorbance at 420 nm was measured, using L-leucine as a standard.

The bioconversion reaction mixture (1 ml) contained 80 mM α-amino content of protein hydrolysate, 200 mM Tris–HCl (pH 7.5), and 100 μl (118 μg) of recombinant *B. licheniformis* glutaminase. The mixtures were incubated at 30 °C and stopped by heating at 90 °C for 5 min at various time intervals. Glutamic acid content was determined as described above. Blank (0 min) was run in the

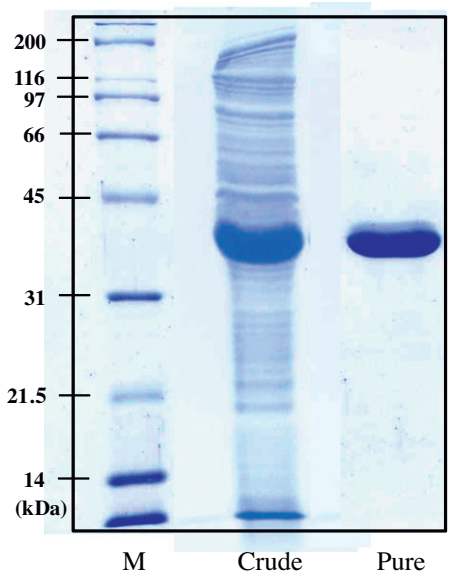

Fig. 3. Purified recombinant glutaminase from *B. licheniformis* DSM13 Coomassie staining of SDS–PAGE analysis of crude extract (crude) and purified enzyme (pure) using one-step IMAC is shown. M: molecular weight marker.

Table 1
One-step purification of recombinant glutaminase from *B. licheniformis* (GlsA).

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell lysate	58	6960	120	1.0	100
Ni–NTA	13	4225	325	2.7	62

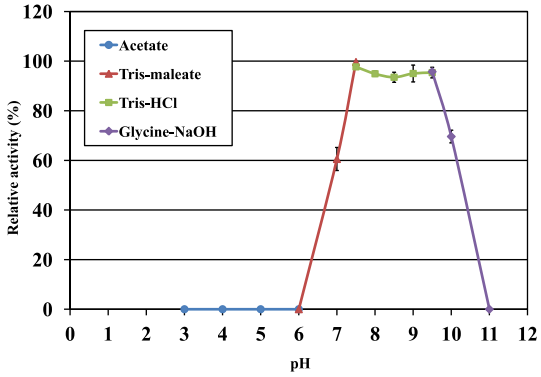

Fig. 4. Effect of pH on the activity of recombinant glutaminase from *B. licheniformis* DSM13 The optimal pH was determined at 30 °C using 50 mM L-glutamine as substrate by an enzyme-couple reaction as described in the Materials and methods section. The buffers used were 100 mM sodium acetate buffer (circle) for pH 3, 4, 5, and 6; 100 mM Tris–melate from (triangle) for pH 6, 6.5, and 7; 100 mM Tris–HCl (square) for pH 7, 7.5, 8, 8.5, and 9; and 100 mM glycine–NaOH (diamond) for pH 9, 10, and 11. Average values are shown for three replicates, along with standard errors.

same manner except that recombinant glutaminase was heated at 90 °C for 5 min before being added to the reaction mixture.

Results and discussion

Cloning and expression of glutaminase from Bacillus licheniformis

A gene encoding *B. licheniformis* ATCC 14580 (DSM13) glutaminase, *glsA* (NCBI Gene ID: 3027975) was cloned by a PCR-based method into the pMY202 vector such that its C-terminus was fused with deca-histidine to facilitate affinity purification by immobilized metal affinity chromatography (IMAC) as shown in Fig. 1. An OmpA signal peptide from *E. coli* outer membrane protein was removed by digesting the plasmid with *NdeI* restriction enzyme because it was found to interfere with the expression of *glsA* gene (data not shown). This finding was in accordance with the previous observation that OmpA signal peptide could interfere with the expression of certain intracellular enzymes such as β-galactosidase [19].

Amino acid sequence analysis revealed that the glutaminase GlsA from *B. licheniformis* belongs to the glutaminase family (EC 3.5.1.2). Sequence alignment with closely related glutaminases from *E. coli* and other bacilli is shown in Fig. 2. The classical β-lactamase-like fold of the enzyme in this family and the key conserved catalytic residues (Ser 74, Lys 77, Asn 126, Lys 268, and Ser 270, according to *B. licheniformis* GlsA numbering) are indicated. The similarities of different enzymes (% identity) based on the sequence of glutaminase from *B. licheniformis* DSM13 are 74% for *Bacillus subtilis* YbgJ, 70% for *Bacillus cereus* Gls1, 69% for *Bacillus pumi* Gls1, 45% for *B. subtilis* YlaM, 36% for *E. coli* YneH, and 32% for *E. coli* YbaS.

Based on the 3-dimentional structure of the active site of YbgJ [3], an essential amino acid residue for the catalytic reaction of glutaminase from *B. licheniformis* consisted of serine 74, lysine 77, and tyrosine 253. Serine74 acted as a catalytic nucleophile, whereas lysine 77 accepted the proton from serine 74 and transferred it to tyrosine 253. In addition, six amino acid residues, i.e., tyrosine 37, glutamine 73, glutamic acid 170, asparagine 177, asparagine 126, and valine 271 also interacted with the substrate (L-glutamine).

Enzyme purification and assay

The recombinant *B. licheniformis* GlsA, tagged with decahistidine at its C-terminus, could be conveniently purified to apparent homogeneity by one-step affinity purification using immobilized metal affinity chromatography (IMAC), as shown in Fig. 3, with a final yield of 62% and a purification fold of 2.7, as shown in Table 1. Routinely approx. 80 mg of the purified enzyme could be obtained from a 1-l shake flask culture, which corresponded to approximately 26,000 units. The apparent molecular weight on SDS–PAGE was approximately 39 kDa, which corresponded well to the theoretical molecular weight (MW) of 38.5 kDa, and the MW of the enzyme under the native condition, based on gel filtration chromatography of 37 kDa (see Supplementary data). These results indicated that the recombinant *B. licheniforms* GlsA is a monomer. The quaternary structures of bacterial glutaminases are found in different multimeric forms, ranging from monomers to tetramers. The recombinant *B. licheniformis* glutaminase in this study and those from *Pseudomonas nitroreducens* IFO12694 [10] and *Stenotrophomonas maltophilia* NYW-81 [12] were monomeric. Glutaminases from *Micrococcus luteus* K-3 [7], *Bacillus pasteurii* [5], *E. coli* YneH, and *B. subtilis* 168 YlaM were dimers [3], whereas those from *E. coli* YbaS and *B. subtilis* 168 YbgJ were tetramers [3].

So far, there have been only a few reports on the cloning and expression of recombinant glutaminases [3,15]. Salt-tolerant glutaminase from *M. luteus* K-3 was cloned and expressed under the tac promoter of the pKK223-3 expression vector and was purified using three column chromatography steps with 17% yield [15]. A previous study of the expression of four recombinant glutaminases from *E. coli* and *B. subtilis* used the pET expression system (pET 15b), in which the expression of N-terminal 6×His tagged-recombinant enzymes was under the control of a T7 promoter [3]. All four enzymes could be expressed in soluble form and purified by IMAC with a yield of 2–5 mg/L of culture and over 95% homogeneity. In this study, the yield of the recombinant *B. licheniformis* GlsA

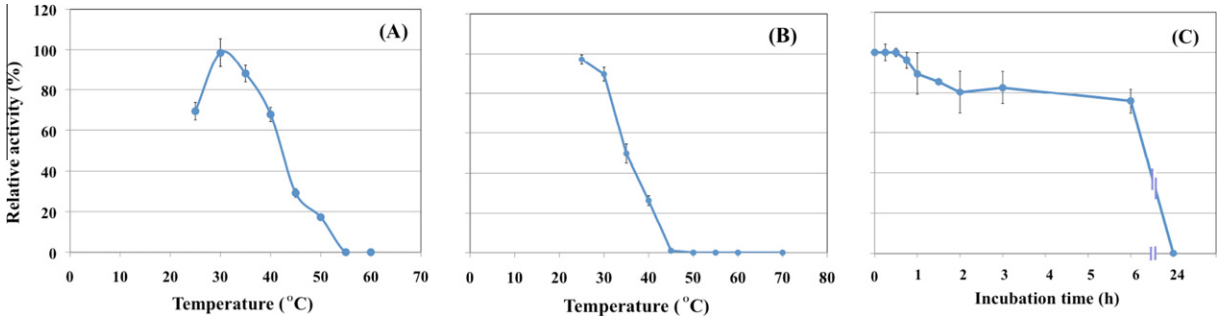

Fig. 5. Effect of temperature on the activity (A) and stability (B and C) of recombinant glutaminase from *B. licheniformis* DSM13 The optimal temperature (A) was determined in 100 mM Tris–HCl (pH 7.5) at various temperatures using 50 mM L-glutamine as substrate. Temperature stability was investigated by pre-incubating the purified enzyme in 100 mM Tris–HCl (pH 7.5) at 25, 30, 35, 40, 50, 60, and 70 °C for 30 min without substrate (B). In addition, the remaining enzyme activity after incubation at 30 °C in 100 mM Tris–HCl, pH 7.5 at various time points was also determined (C). Average values are shown for three replicates, along with standard errors.

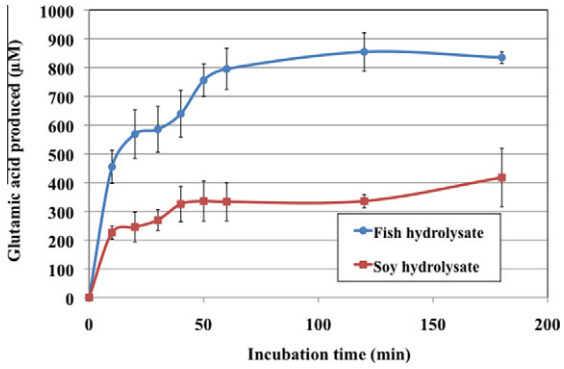

Fig. 6. Bioconversion of glutamine in protein hydrolysates to glutamic acid The amounts of glutamic acid produced at various time points after recombinant *B. licheniformis* glutaminase was incubated with fish protein hydrolysate (circle) or soy protein hydrolysate (square) at 30 °C were reported. The reaction mixture (1 ml) contained 80 mM α-amino content of protein hydrolysate, 200 mM Tris–HCl (pH 7.5), and ~100 μg of purified recombinant glutaminase. Average values are shown for three replicates, along with standard errors.

was approx. 10 times as high as those observed in previous studies [3,15], and the enzyme could be purified to apparent homogeneity (>99%) by one-step purification. The improvement in the yield and purity could be because the expression system in this study was based on a p*tac* promoter, which has previously been used successfully to express a number of extracellular [21,27] and intracellular [19] enzymes. At 0 h of induction, a slight amount of recombinant enzyme could be observed. This result confirms the previous observation that the p*tac* promoter is leaky [15,27]. Nevertheless, since the recombinant enzyme did not appear to be toxic, a slight amount of enzyme before the induction with IPTG did not evidence any detrimental effects on the cells.

The higher purity of the enzyme after one-step purification could be because the recombinant enzyme was tagged C-terminally with decahistidine instead of hexahistidine. Therefore, it could bind better to nickel, allowing more vigorous washing conditions. The presence of C-terminal decahistidine allows convenient and efficient one-step purification, whereas the FLAG tag could be useful for observation of the physiological role of glutaminase by immunolocalization, using specific monoclonal antibody *in vivo*.

To the best of our knowledge, this is the first report on cloning, expression, and characterization of *B. licheniformis* glutaminase. A previous report on partial characterization of native *B. licheniformis* glutaminase A5 in 1981 indicated that there were two distinct glutaminase activities [4]. Bioinformatic analysis of *B. licheniformis* DSM13 genomic database revealed that there are two glutaminases genes in this bacterium, namely *glsA/ybgJ* and *ylaM*, which are the same as those found in *B. subtilis* 168 [3]. These two genes could be responsible for the two distinct glutaminase activities of the native enzymes. GlsA glutiminase was used in this study, because this enzyme has been characterized more than YlaM glutaminase in other microorganisms [2].

Effects of pH and temperature

The effects of pH and temperature on the enzyme activity and stability are shown in Figs. 4 and 5, respectively. The optimal pH of the recombinant *B. licheniformis* glutaminase was between pH 7 and 9.5 (Fig. 4), whereas its optimal temperature was at 30 °C (Fig. 5A). The enzyme was rapidly inactivated after incubation for 30 min at a temperature of more than 40 °C (Fig. 5B). Nevertheless, the enzyme was stable at 30 °C for up to 6 h at pH 7.5 with approximately 80% remaining activity (Fig. 5C).

Enzyme kinetics

The kinetic parameters of the recombinant *B. licheniformis* glutaminase were measured in an enzyme-coupled assay with glutamate dehydrogenase, which detects the formation of glutamic acid. The specific activity of the purified enzyme was 324.9 ± 37.5 U/mg. The K_m and V_{max} of the enzyme were 111.15 ± 30.6 mM and 579.48 ± 90.7 μmol/min/mg, respectively.

The biochemical characteristics of GlsA glutaminase from *B. licheniformis* are similar to those from *B. subtilis* and various organisms [2,3]. The enzymes in this family exhibit low affinity with L-glutamine (high K_m) but are highly selective only with regard to this substrate with no activity against D-glutamine or L-asparagine [3]. Recombinant glutaminase from *B. licheniformis* was active in a wide range of pH (6.5–9), and specific activity was quite high at 30 °C, compared with that of other bacteria [2]. The optimal temperature and thermostability were much less than those isolated from thermophilic bacteria, however. In contrast to the salt-tolerant glutaminase from *M. luteus* K-3, which retained activity over 90% in the presence of 3.08 M NaCl [15], the activity of the recombinant glutaminase from *B. licheniformis* decreased with increased concentration of NaCl. At >200 mM NaCl (1.17% w/v), the activity of the enzyme decreased by more than 50% (data not shown). This result is in accordance with that of YbgJ from *B. subtilis* 168, which was completely inhibited in 1.3 M NaCl (7.6% w/v) [28]. Therefore, this enzyme is only suitable for bioconversion at ambient temperature in a low-salt condition.

Bioconversion of glutamine in protein hydrolysates

To demonstrate the potential application of recombinant *B. licheniformis* glutaminase for the bioconversion of glutamine to

flavor-enhancing glutamic acid in fermented food products, an experiment was performed with two types of substrates, i.e., soy and fish hydrolysates. The α-amino content in the initial reactions of both substrates was adjusted to 80 mM. As shown in Fig. 6, the recombinant *B. licheniformis* GlsA efficiently increased glutamic acid level when either type of protein hydrolysate was used. The hydrolysate from fish seemed to be a better source of glutamic acid production, however, as higher amounts of glutamic acid could be produced. In both conditions, the glutamic acid levels reached the maximum level after approximately 1 h of incubation. Since α-amino content in the initial reactions of both substrates was equal, the results suggested that the amount of free glutamine in fish hydrolysate was higher than that of soy hydrolysate; therefore more glutamic acid could be converted from the free glutamine. The activity of glutaminases in a model system of a soy sauce fermentation has been investigated [29], such as those from *S. maltophilia* NYW-81 [12] and *Cryptococcus albidus* [30].

Conclusions

This study demonstrated an efficient expression system for the production and purification of bacterial glutaminase (GlsA). In addition, its potential application for bioconversion of glutamine to flavor-enhancing glutamic acid in fermented food products was demonstrated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2012.03.001.

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