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ORIGINAL ARTICLE

Characteristics of a Cold-Adapted L-glutaminase with Potential Applications in the Food Industry

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Abstract

L-glutaminases are enzymes that catalyze the hydrolysis of L-glutamine, producing L-glutamate and ammonium, and they have promising applications in pharmaceutical and food industries. Several investigations have focused on thermo-tolerant L-glutaminases; however, studies on cold-adapted L-glutaminases have not been reported. These enzymes could be useful in the food industry because they display high catalytic activity at low and room temperatures, a valuable feature in processes aimed to save energy. Besides, they can be easily inactivated by warming and are suitable to prevent decomposition of thermo-labile compounds. The objectives of this work were to characterize the L-glutaminase from the Antarctic bacterium *Bizionia argentinensis* and analyze its capability as flavor enhancer of protein hydrolysates. The enzyme was heterologously expressed and purified from *Escherichia coli*, obtaining optimum and homogeneous yields. Kinetic parameters K_m and V_{max} were located at the lower and upper range of values reported for L-glutaminases, suggesting high catalytic efficiency. Optimum temperature was 25 °C, and the enzyme conserved around 90% of maximum activity at 0 °C and in presence of 15% (v/v) ethanol and methanol. In saline conditions, the enzyme conserved around 80% of maximum activity in 3 M NaCl. Analysis of structural model suggested cold-adaptation features such as low Arg/(Arg+Lys) ratio and fewer intramolecular interactions than mesophilic and thermo-tolerant L-glutaminases. This work provides a novel cold-adapted L-glutaminase with promising features in the food industry.

Keywords L-glutaminases · Cold-adapted enzymes · Food industry

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Introduction

L-glutamine glutaminases (EC 3.5.1.2) are ubiquitous enzymes that catalyze the deamination of L-glutamine through hydrolysis of the γ-amido bond, producing ammonium and L-glutamate [1]. These enzymes have different applications in pharmaceutical and food industries [1, 2]. In medicine, L-glutaminases can be used in amino acid deprivation therapy for the treatment of auxotrophic tumors to produce selective death of glutamine dependent cancerous cells [3]. Other therapeutic applications involve significant inhibitory activity against HIV replication in vivo and as biosensor of glutamine levels in body fluids [1, 4]. The biosensor application was patented in a device useful to measuring L-glutamine in liquid samples [5].

In food industry, L-glutamine glutaminases have been used in the synthesis of L-theanine, a bioactive compound present in green tea with promising health benefits [6]. Considering that L-glutamine glutaminases produce L-glutamate, which elicits the umami taste, they also have been applied to improve the flavor of protein hydrolysates obtained by enzymatic hydrolysis [7].

The hydrolysis of proteins increases their digestibility, favors the assimilation of amino acids, and produces peptides with different beneficial properties for the human health, adding value to proteins from vegetal sources [8]. Considering their beneficial properties, protein hydrolysates are useful in food formulations to develop functional foods. However, the characteristic bitter taste of protein hydrolysates restricts its potential and has been considered a challenge to be resolved [9]. L-glutamine glutaminases enhance the umami and decrease the bitter taste of protein hydrolysates, turning them acceptable for consumption. These enzymes overcome limitations of physicochemical methods such as undesired side effects and costs [7]. Besides, the deamination of L-glutamine induced by L-glutaminases avoids exogenous applications of L-glutamate as disodium salt, contributing to foods with fewer amounts of sodium.

Both applications in the food industry have been patented in different innovations. Okada et. al., in collaboration with the food company Taiyo Kagaku Co. Ltd. (Yokkaichi, Japan), patented a method that involves the use of L-glutaminases from *Penicillium*, *Rhizopus*, *Mucor*, *Aspergillus*, *Hansenulla*, *Schizosaccharomyces*, *Candida*, and different species of *Bacillus* to produce L-theanine [10]. This method is based on the recombinant production of L-theanine and is more efficient than the conventional extraction from tea leaves, given that it produces higher yields of L-theanine without the necessity of vegetal material [6]. The flavor enhancer property of the L-glutaminase from *Bacillus amyloliquefaciens* was patented in the preparation of a strong-taste peptide, which significantly improved the taste of foods [11]. In a similar patent, a commercial L-glutaminase was used to increase the flavor of protein hydrolysate obtained from plant raw material [12]. E-Glutec, the L-glutamine glutaminase from *Escherichia coli* produced by Megazyme (Bray, Ireland), is an example of commercial enzyme. The deamination mediated by E-Glutec was useful to improve the taste of wheat protein hydrolysates. This treatment exhibited the highest umami intensity and lowest bitterness intensity compared to chemical and protein glutaminase (EC 3.5.1.44) treatments [7].

Considering the potential of L-glutamine glutaminases, numerous works have pursued the production and characterization of these enzymes [1, 2, 4]. Several investigations have focused on thermo- and salt-tolerant L-glutaminases because they would be compatible with specific processes that require heat and salinity in the food industry, such as soy sauce cooking [13–17]. However, studies involving characterization of cold active L-glutamine glutaminases have not been reported up to date. Cold-adapted L-glutaminases could be interesting to the food industry because they present high catalytic activity at mild or room temperatures and could be applied in processes aimed to saving energy, thus reducing not only costs but also impact on CO_2 emissions [18].

The aim of this work was to characterize and evaluate the potential of the L-glutamine glutaminase from the psychrotolerant bacterium *Bizionia argentinensis* to enhance the flavor of soy protein hydrolysates. *B. argentinensis* is a gram-negative, marine bacterium isolated from surface seawater in Antarctica, belonging to the Flavobacteriaceae family and can tolerate low temperatures [19]. Our work evinces that this enzyme is promising in L-glutamine deamination at room and lower temperatures.

Materials and Methods

In Silico Analysis Protein sequence of L-glutaminase from *Bizionia argentinensis* JUB59 (GenBank ID: WP_008639909; UniProt Accession ID: G2E9E1) was aligned against non-redundant protein sequences database of NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) using BLASTP to identify conserved domains. Reference protein sequences for L-glutaminase were collected from published works [17, 20, 21] and compared with L-asparaginase sequences [20]. In addition, BLASTP first 10 matches obtained from alignment of *B. argentinensis* L-glutaminase against NCBI database were also added to the analysis. Sequences were aligned using ClustalX [22]. Phylogenetic tree was constructed using the maximum likelihood method, substitution model LG+G, and the software MEGA 6.0 [23]. Bootstrapping was performed with 100 replications. Suitable template for building high quality models was selected using HHpred web server and homology search in the PDB database using BLAST [24]. The L-glutaminase from *Geobacillus kaustophilus* HTA426 (PDB 2pby) presented the highest sequence identity, coverage, and resolution and was selected as template for structural homology modeling. Five models were calculated using MODELLER version 9.16 [25]. Models were ranked using DOPE Z scores (Discrete Optimized Protein Energy). The top model with the lowest energy was further validated by PROCHECK and used in the structural analysis [26]. Intramolecular H-bonds and salt-bridges were calculated with UCSF Chimera 1.10.2 [27].

Cloning Genomic DNA of *B. argentinensis* JUB 59 was purified by applying the CTAB method [28]. The coding sequence for the L-glutaminase from *B.* argentinensis JUB 59 (GenBank ID: NZ_AFXZ01000070) was amplified by PCR from the purified genomic DNA, with the primers BamHI_up (5′-GTTAA*GGATCC*ATTGATTATCAATCCATTTTAAACAATATTTACGCG-3′) and XhoI_lw (5′-GGAAT*CTCGAGT*TAAAAAATAGATTGATTAGTAAGGGTTG-3′), using *Pfu* proof-reading DNA polymerase (Inbio Highway, Tandil BA, Argentina). The PCR fragment was purified with a purification kit (EasyPure PCR purification kit, Transgen, Beijing, China) and digested with 5 units of *Bam*HI and *Xho*I (Promega, Madison, WI, USA). The digested PCR fragment was purified and ligated with T4 DNA ligase (Promega) in the vector pET28a (Novagen, Madison WI, USA) digested with the same restriction enzymes, obtaining the plasmid pGBA. Positive clones containing pGBA were screened by colony-PCR [28]. Plasmid DNA was purified from positive clones by using DNA purification kit (EasyPure Plasmid Miniprep kit, Transgen, Beijing, China) and sequenced (Macrogen, Seoul, Korea). Fidelity of constructions was checked by sequence analysis with DNASTAR Lasergene (DNASTAR Inc., Madison WI, USA).

Enzyme Purification Protein purification was performed by affinity chromatography with Ni-NTA from heterologous expression in *Escherichia coli* [29, 30]. Competent cells of *E. coli*

BL21 were prepared and transformed with the plasmid pGBA by following the Inoue method of chemical transformation [28]. A colony of transformed cells was picked and inoculated in 10 mL of LB medium containing 40 μg/mL kanamycin and grown at 37 °C with shaking 220 rpm during 16 h. This culture was inoculated in a 5.0-L Erlenmeyer flask containing 1.0 L of LB medium with 40 μg/mL kanamycin and grown until OD_{600nm} 0.6–0.8. Induction was carried out by adding 0.25 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) and incubation at 25 °C during 16 h with shaking (220 rpm). The cells were harvested by centrifugation (6000×*g*, 4 °C, 20 min) and rinsed with 200 mL of sterile distilled water. The cells were resuspended in 20 mL of lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 5 mM imidazole; phenylmethylsulfonyl fluoride (PMSF) 2 mM) and lysed by sonication (three pulses of 40 mA and 10 s) (Sonics, Newtown, USA). The cellular extract was centrifuged at 12,000×*g*, 4 °C during 20 min, and the supernatant was extracted and loaded in a column packed with 2 mL of Ni-NTA resin (MCLAB, South San Francisco, CA, USA) previously equilibrated with washing buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 5 mM imidazole). The column was washed with 15 mL of washing buffer and eluted with 10 mL of elution buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 200 mM imidazole). Aliquots of 1 mL were collected and protein content and purification degree were analyzed by SDS-PAGE [28]. Fractions containing high concentrations of pure L-glutaminase were pooled and dialyzed in 1 L of 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl buffer and stored at −20°C.

Enzyme Kinetics L-glutaminase activity was determined by measuring indirectly the product L-glutamic acid, following previously reported procedures [13, 31, 32]. The reaction was carried out in 0.5 mL of buffer 100 mM Tris-HCl (pH 7.5), 150 mM NaCl containing 10 μM L-glutaminase, and variable concentrations of L-glutamine (from 0 to 45 mM). After incubation at 30 °C during 10 min, the reaction was stopped by boiling for 10 min. The tubes were centrifuged at 4 °C, 10,000×*g*, 10 min, and the supernatant (400 μL) containing the product L-glutamate was added to 600 μL of 50 mM Tris-hydrazine buffer (pH 9.0), 1.5 mM NAD^+, 0.5 mM ADP, and 5 units of L-glutamate dehydrogenase (Roche, Basel, Switzerland). The tubes were incubated at 30 °C during 60 min to promote NADH generation. The NADH concentration was determined by measuring the absorbance at 340 nm (ε_{340} = 6220 $M^{-1}cm^{-1}$) using a Shimadzu UV-1800 UV-VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan). L-glutaminase concentration was determined by the Bradford method, using bovine serum albumin as standard [33]. Triplicates of activity assays were performed at each L-glutamine concentration. Kinetics parameters were calculated by adjusting values of activity versus L-glutamine concentration to Michaelis-Menten model, with SigmaPlot 12.3 (Systat Software Inc., Erkrath, Germany).

Effect of Temperature on L-glutaminase Activity and Stability The effect of temperature on reaction course was assayed by incubating 10 μM L-glutaminase and 32 mM L-glutamine in 100 mM Tris-HCl (pH 7.5) at 0, 4, 15, 25, 30, 40, and 60 °C during 10 min in 1.5 mL tubes. The reaction was stopped by incubation of tubes at boiling water for 10 min. L-glutamate product was determined by the coupled reaction of L-glutamate dehydrogenase and further measurement of NADH at 340 nm as described previously. To evaluate enzyme stability at different temperatures, 1.5 mL tubes containing 0.7 mL of 12 μM L-glutaminase in 100 mM Tris-HCl buffer (pH 7.5) were incubated at 4, 15, 20, 25, 30, 50, and 60 °C for 15 min and 1, 2, 3, 4, 5, and 6 h. The samples were centrifuged at 4° C, 11,000×*g* for 10 min, and L-glutaminase

was extracted from the supernatant and incubated with 32 mM L-glutamine in 500 μL of Tris-HCl 100 mM (pH 7.5) at 30 °C for 10 min. Enzyme activity was determined by triplicate as previously described, and relative activity was calculated at each temperature considering 100% the activity observed at 0 min.

Effect of pH on L-glutaminase Activity and Stability A published procedure was followed to estimate the effect of pH on L-glutaminase activity and stability [17]. Briefly, L-glutaminase was dialyzed against 100 mM mixed buffer composed of acetate, phosphate, and glycine adjusted to the following pH values: 4, 5, 6, 7, 7.5, 8, 9, 10, and 11. To determine the effect of pH on enzyme stability, 120 μM L-glutaminase was incubated at the mentioned pH values for 3 h and 25 °C. After incubation, 12 μM L-glutaminase aliquots were incubated with 32 mM L-glutamine in 100 mM Tris-HCl buffer (pH 7.5) for 10 min and 30 °C. The reaction was stopped by boiling during 10 min and enzyme activity was determined as described previously. Relative activity was calculated at each pH considering 100% the activity observed at pH 7.5. The experiment was performed by triplicate.

Effect of NaCl Concentration on L-glutaminase Activity and Stability Twelve micromolars L-glutaminase was incubated in 100 mM Tris-HCl buffer (pH 7.5) and variable NaCl concentrations (0, 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, and 3.0 M) for 3 h at 25 °C. After incubation, 32 mM L-glutamine was added, and the reaction was carried out for 10 min at 30 °C. The reaction was stopped by boiling for 10 min, and enzyme activity was determined as described previously. Relative activity was calculated at each NaCl concentration considering 100% the activity observed at 0.15 M NaCl. The experiment was repeated three times.

Thermal Unfolding Transition BA glutaminase was diluted to a final concentration of 5 μM in 100 mM phosphate buffer (pH 7.4). Thermal unfolding transition was obtained by monitoring the CD signal at 222 nm using a Jasco J-720 spectropolarimeter (Jasco Co., Tokyo, Japan), whilst the temperature of the sample was increased at uniform rate (1 °C per min) from 5 to 90 °C, in a cuvette with a 0.1 cm light path. Temperature was controlled with a thermoelectric circulating bath Peltier PTC-100. The CD data were expressed as mean residue ellipticity (degree cm^2/dmol). Thermal unfolding transition was analyzed assuming a two-state approximation in which only the native and unfolded states are significantly populated. Melting temperature (T_m) was calculated as described previously [34]. The experiments were repeated twice. Far-UV (190–260 nm) circular dichroism (CD) spectra were obtained at 5 °C (native state, before starting thermal transition) and at 90 °C (unfolded state, after finishing thermal transition) using a cuvette with a 0.1 cm light path. Other parameters were bandwidth 2 nm, scanning speed 50 nm/min, and integrations 8.

Effect of $Mg^{2+}$and Chemical Solvents on Enzymatic Activity Ten micromolars purified *B. argentinensis* L-glutaminase was incubated in 100 mM Tris-HCl (pH 7.5) and $MgCl_2$ (25 mM and 125 mM) for 30 min at 25 °C. Similar experiment was performed but in the presence of chemical solvents (ethanol, methanol, isopropanol, acetone, 5, 15, 25 % w/v) instead of $MgCl_2$. After incubation, 30 mM L-glutamate was added and the reaction was carried out by incubation at 30 °C during 10 min. Enzyme activity was measured as described previously, by quantifying NADH concentration at 340 nm. Enzyme activity was reported as relative activity considering 100% enzyme without treatment. The experiments were repeated three times.

Soy Protein Isolate (SPI) Preparation SPI was prepared from defatted soy flour by isoelectric precipitation following published procedures [35, 36]. Briefly, defatted soy flour (America Pampa, Rivadavia, BA, Argentina) was dissolved in sterile distilled water whose pH was adjusted to 8.0 with 2.0 N Na(OH) to produce a 10% (w/v) dispersion. The dispersion was incubated 2 h at room temperature with stirring, and the pH was measured and adjusted to 8.0 every 15 min. The dispersion was filtered through gauze and centrifuged (11,000×*g*) at 4 °C for 30 min. The pH of the supernatant was adjusted to 4.5 with 2.0 N HCl and centrifuged (6000×*g*) at 4 °C for 15 min. The supernatant was discarded, and the pellet was resuspended in sterile distilled water (pH 7.0). Protein concentration was determined by the Bradford method, using bovine serum albumin as standard [33]. Proteins were also identified by SDS-PAGE analysis in 11% (w/v) acrylamide gels [28]. SPI samples were lyophilized and stored at −20 °C.

Enzymatic Hydrolysis of SPI The hydrolysis degree of SPI was estimated by applying the soluble nitrogen-TCA (SN-TCA) method [37]. Briefly, 1.0 g of SPI was dispersed in 10 mL of 50 mM sodium phosphate buffer (pH 7.0) and incubated with proteinase K (0.5 mg/mL) at 37 °C during 15 min, 1h, 2 h, 3 h, 4 h, 5 h, and 6 h. After each digestion time, the enzymatic digestion of SPI was stopped by incubation in boiling water bath during 15 min, to obtain hydrolyzed SPI (hSPI). The non-hydrolyzed proteins were precipitated with 0.2 N trichloroacetic acid (TCA), and the nitrogen content of the supernatant (soluble nitrogen, which is assumed to consist only of amino acids and small peptides) was quantified by using a Kjeldahl auto distillation unit Foss Tecator Kjeltec 2200 KT (Hilleroed, Denmark) with an integrated colorimetric titrator. The hydrolysis percentage was calculated as the percentage of the total nitrogen of suspension without proteinase K treatment, which was measured after complete hydrolysis with 15 mL of sulfuric acid 95–98% (v/v) in the presence of a tablet of copper catalyst (Sigma-Aldrich) at 120 °C for 4 h [37].

Deamination of hSPI by BA Glutaminase The obtained hSPI samples were incubated with 1 mL of BA glutaminase (1 mg/mL) at 25 °C for 6 h to promote the hydrolysis of L-glutamine into ammonium and L-glutamate. Enzyme and substrate blanks (controls without BA glutaminase and without hSPI addition) were also incubated at 25 °C during 6 h. The reaction was stopped by addition of 0.5 mL of 1.5 M TCA. The samples were centrifuged at 12,000×*g* for 10 min to remove precipitates. Aliquots of 0.1 mL were extracted and mixed with 0.2 mL of Nessler's reagent (Sigma-Aldrich, Palo Alto, USA) in 4.0 mL of distilled water. The samples were incubated 15 min at room temperature to favor the development of color, and the absorbance was measured at 450 nm using a Shimadzu UV-1800 UV-VIS spectrophotometer (Kyoto, Japan). The concentration of ammonium was estimated from standard calibration curves made with 0.1 M $(NH_4)_2SO_4$ standard solution [38]. The assays were repeated three times.

Results and Discussion

In Silico Studies

Alignment of L-glutaminase from *B. argentinensis* (BA glutaminase) against non-redundant protein sequences database of NCBI showed the L-glutaminase of *B. myxarmorum*

(WP_148405459) as the homologue with the highest identity (87.5%). However, the sequence showed low homology (around 33–35%) regarding well-characterized and crystallized L-glutaminases such as those from *E. coli*, *B. subtilis*, and *Micrococcus luteus*. Conserved domains corresponding to glutaminase superfamily were identified in the sequence of BA glutaminase, with specific hits for L-glutaminase (PRK00971, pfam04960 EC 3.5.1.2).

Phylogenetic tree showed two well-defined clades corresponding to L-glutaminases (EC 3.5.1.2) and L-asparaginases (EC 3.5.1.1) (Fig. 1). BA glutaminase grouped in the L-glutaminases clade, within a well-supported cluster (bootstrap 100) conformed by members of the family Flavobacteriaceae, showing high homology to Bizionia species (Fig. 1).

Three-dimensional structure of BA glutaminase was modeled by homology modeling using the structure of L-glutaminase from *Geobacillus kaustophilus* HTA426 as template (PDB 2pby, homology 35.5%), which showed the highest sequence identity, coverage, and

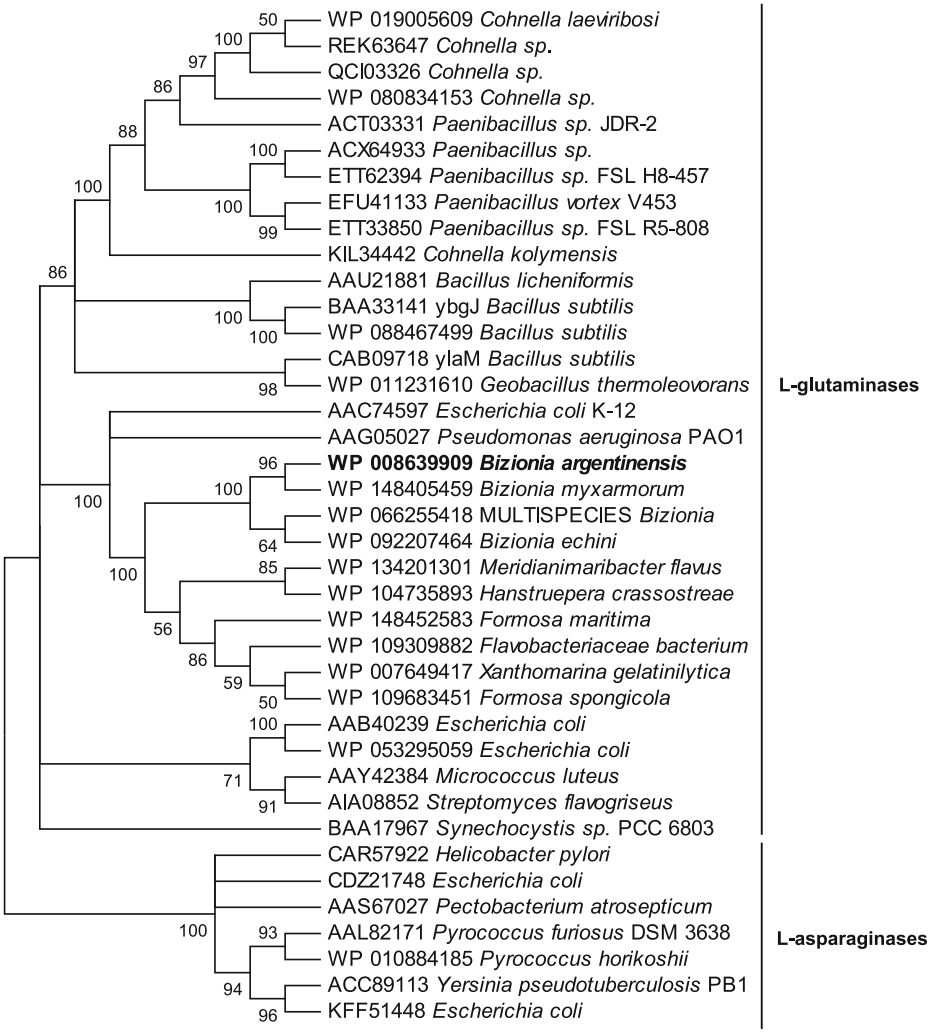


Fig. 1 Maximum likelihood phylogenetic tree showing the relationship between *B. argentinensis* glutaminase (in bold) and reference sequences of L-glutaminases and L-asparaginases

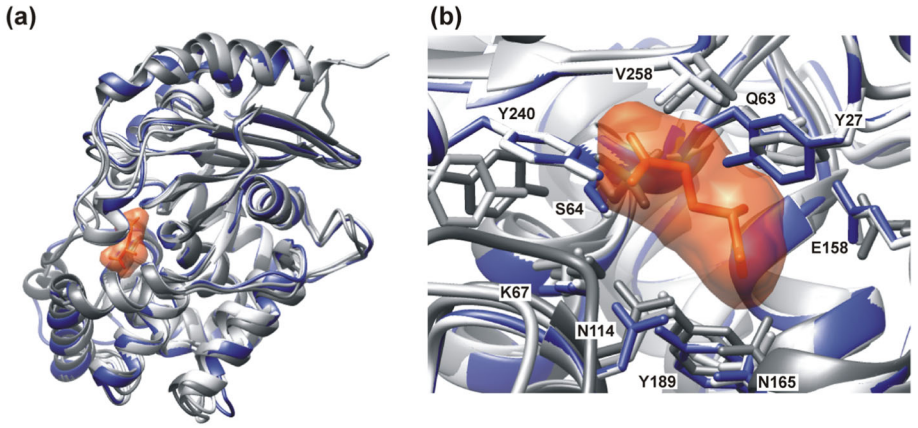
resolution. Major conformational differences were not observed compared with crystallized structures of L-glutaminases (Fig. 2a). In addition, conserved amino acids involved in substrate binding and catalysis were identified in the active site (Fig. 2 b and c). Following the substrate binding model proposed by Brown et al. for the L-glutaminase from *B. subtilis*, the R groups of Y37, Q73, and E170 (Y27, Q63, and E158 in BA glutaminase) form hydrogen bonds with the α-amine of L-glutamine, whereas the α-carboxyl forms hydrogen bonds with the R groups of N126 and N177 (N114 and N165). In addition, the side chain δ-carbonyl oxygen of L-glutamine interacts with the main chain NH groups of S74 and V271 (S64 and V258 in BA glutaminase), whereas the $N^{\varepsilon 2}$ (leaving group) forms a hydrogen bond with the side chain of Y253 (Y240) [39]. In the proposed catalytic mechanism, the hydrolysis of L-glutamine proceeds through four steps. In the first step, the S74 (S64) oxygen performs a nucleophilic attack to the δ-carbon of L-glutamine, forming a glutamyl-enzyme covalent intermediate. The main chain NH groups of S74 and V271 (S64 and V258) increase the polarization of the hydrolysable C-N bond of L-glutamine. K77 (K67) assists in this nucleophilic attack by acting as a general base, accepting the proton from the S74 (S64) Oγ and transferring it to the Y253 (Y240) side chain oxygen. The result of the first step is the tetrahedral intermediate 1. In the second step, the Y253 (Y240) transfers the proton to the substrate Nε2 (the leaving group), favoring the decomposition of the tetrahedral intermediate 1, with the production of ammonium and the acyl-enzyme intermediate. In the third step, a nucleophilic attack of a water molecule accounts on the ester carbonyl carbon of the intermediate, resulting in tetrahedral intermediate 2. In the fourth step, the Y253 (Y240) side chain oxygen accepts the proton from the deacylating water molecule and transfers it to the S74 (S64) Oγ, regenerating the active site and releasing the second product glutamate [39].

These results confirmed that the selected sequence of BA glutaminase corresponds to a L-glutaminase enzyme and could present novel functional properties considering that it showed low homology with well-characterized members of this enzyme family.

Purification and Characterization

BA glutaminase was cloned and purified homogeneously in high yield from *E. coli*, obtaining preparations in the range of 1 mg/mL and homogeneous band around 33 kDa, in agreement with the molecular weight predicted from protein sequence (33.32 kDa) (Supplementary Fig. S1). Compared with data deposited in Brenda Enzyme database [40], this molecular weight is similar to the lowest value reported for L-glutaminases, whose molecular weights vary between 32.9 and 140.0 kDa for the enzymes from *E. coli* [39] and *Brevundimonas diminuta* MTCC 8486 [41], respectively (Supplementary Table S1). These results suggested low structural robustness and easiness of production, which are valuable features for industrial applications.

The enzyme was characterized by following published procedures, based on a coupled assay with L-glutamate dehydrogenase and further quantification of NADH at 340 nm [13, 31, 32] (Table 1). The kinetics parameters of BA glutaminase were compared with available data of characterized L-glutaminases calculated upon similar conditions and using information deposited in BRENDA database [40] (Supplementary Table S1). The K_m of BA glutaminase for the substrate L-glutamine (1.1 mM) is located in the lower range of K_m reported for bacterial L-glutaminases up to date, whose values range between 0.104 and 109.5 mM for the enzymes from *G. thermodenitrificans* [42] and *Anabaena* sp. [43], respectively. This result suggested high affinity of BA glutaminase for the substrate L-glutamine. The V_{max} of BA



(c)

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                                    10        20        30        40        50
BA glutaminase                1     ----MIDYQS------ILNNIYARVTRETDKGKIAKYIPELSRVDPNRFG 40
G. kaustophilus (2pby)        1     ---MLVYNQE------ELVRFVEEAKQYARYGKVADYIPALGKANPNELS 41
M. luteus (3iha)              1     ---MRHPIPD------YLASLVTELGAVNP-GETAQYIPVLAEADPDRFG 40
B. subtilis (1mki)            1     MKELIKEHQKDINPALQLHDWVEYYRPFAANGQSANYIPALGKVNDSQLG 50

BA glutaminase               41     IHLKLISGEEFSVGDSDVGFSIQSISKVLTLSMAMS-QVEAKIWNRVDVE 89
G. kaustophilus (2pby)       42     IAIYTPDDEVVSAGDVTVKVTLQSISKIIALALVLIDRGEDEVFHKVGME 91
M. luteus (3iha)             41     IALATPTGRLHCAGDADVEFTIQSASKPFTYAAALVDRGFAAVDRQVGLN 90
B. subtilis (1mki)           51     ICVLEPDGTMIHAGDWNVSFTMQSISKVISFIAACMSRGIPYVLDRVDVE 100
                                                 *   *
BA glutaminase               90     PSGNPFNHLSLLEL-EEGIPRNPLINSGAIVIADILVS---QLKDPKAEF 135
G. kaustophilus (2pby)       92     PTDYPFHSIAKLEE-KPAKPLNPMINAGALVVTSMIQG--GSVSERLERL 138
M. luteus (3iha)             91     PSGEAFNELS-LEA-ESHRPDNAMINAGALAVHQLLVGPEASRKERLDRA 138
B. subtilis (1mki)           101    PTGDAFNSIIRLEINKPGKPFNPMINAGALTIASILPG--ESAYEKLEFL 148

BA glutaminase               136    LKYVQDITGDPSISYNLKVAQSEKETGFNNYAAANLLKSFNNLNNSVDAV 185
G. kaustophilus (2pby)       139    LAFVRRLAGNERISYSDEVARSEFETAFLNRSLCYFLKQHRIIDEDVEEL 188
M. luteus (3iha)             139    VEIMSLLAG-RRLSVDWETYESEMAVSDRNLSLAHMLRSYGVLQDSAEEI 187
B. subtilis (1mki)           149    YSVMETLIG-KRPRIHEEVFRSEWETAHRNRALAYYLKETNFLEAEVEET 197

BA glutaminase               186    LDFYFHQCSLEMSCAQLSKAFFLFANKG-LCLKNVARLTKSQAKRINAIM 234
G. kaustophilus (2pby)       189    MELYTKQCAIEMTCIDLARIGLVLALDGRDPHSSEPLMPLDVARICKTFM 238
M. luteus (3iha)             188    VAGYVAQCAVLVTVKDLAVMGACLATGGIHPMTGERMLPSIVARRVVSVM 237
B. subtilis (1mki)           198    LEVYLKQCAMESTTEDIALIGLILAHDGYHPIRHEQVIPKDVAKLAKALM 247
                                     *
BA glutaminase               235    LTCGFYDEAGEFAFEVGLPGKSGVGGGIAASLP----------NEFSIAV 274
G. kaustophilus (2pby)       239    VTCGMYNSSGEFAIKVGIPAKSGVSGGILAAVP----------GRCGIGV 278
M. luteus (3iha)             238    TSSGMYDAAGQWLADVGIPAKSGVAGGVLGALP----------GRVGIGV 277
B. subtilis (1mki)           248    LTCGMYNASGKYAAFVGVPAKSGVSGGIMALVPPSARREQPFQSGCGIGI 297
                                      *                   *
BA glutaminase               275    WAPGLNPQGNSKLGMQALERFTTLTNQSIF 304
G. kaustophilus (2pby)       279    FGPALDDKGNSLTGVKLLERLSKTYSLSIF 308
M. luteus (3iha)             278    FSPRLDEVGNSARGVLACRRLSEDFRLHL- 306
B. subtilis (1mki)           298    YGPAIDEYGNSLTGGMLLKHMAQEWELSIF 327
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Fig. 2 Modeled structure of BA glutaminase compared with crystallized reference structures. **a** Modeled structure of BA glutaminase (blue) superimposed to crystallized structures of L-glutaminases from *Geobacillus kaustophilus* (2pby, white), *Micrococcus luteus* bound to glutamate product (3iha, light gray), and *Bacillus subtilis* (1mki, dark gray). **b** Active site showing conserved residues involved in substrate binding and catalysis. The product L-glutamate is shown in red. **c** Sequence alignment of the four L-glutaminases. Conserved amino acids are highlighted with gray background, whereas bold letters and asterisks show amino acids involved in substrate binding and catalytic mechanism, respectively

glutaminase was 657.1 μmol/min.mg and k_{cat} 320 s^{-1}, higher than k_{cat} of typical bacterial L-glutaminases such as those from *B. cereus* (0.222 s^{-1}) [44], *B. subtilis* (67.7 s^{-1}) [39], and *E. coli* (101 s^{-1}) [39]. The k_{cat} of BA glutaminase was lower than the k_{cat} of L-glutaminases from *M. luteus* (1400 s^{-1}) and *Aspergillus sojae* (6249 s^{-1}), which showed the two highest k_{cat}

Table 1 Kinetic parameters of BA glutaminase. Substrate: L-glutamine

	K_m (mM)	V_{max} (μmol/min.mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
BA glutaminase	1.1 ± 0.12	657.1 ± 23.5	320.0 ± 11.8	290.9 ± 15.4

values reported up to date [45, 46]. However, BA glutaminase showed the highest catalytic efficiency (k_{cat}/K_m) between the L-glutaminases reported up to date (Supplementary Table S1). These results suggested that BA glutaminase presents a proficient catalytic efficiency.

Effect of Temperature on Enzyme Activity and Stability

The influence of temperature on enzyme activity was investigated through two different approaches. In the first approach, the deamination reaction was carried out at different temperatures by incubating the enzyme and L-glutamine substrate at different temperatures in the range 0–60 °C (Fig. 3a). The objective was to determine how the temperature affects the progress of deamination reaction and calculate the optimum temperature.

BA glutaminase exhibited the highest specific activity at 25 °C (615.7 μmol/min.mg), and major variations were not observed at lower temperatures, conserving 93–96% of maximum activity between 0 (577.1 μmol/min.mg) and 15 °C (591.2 μmol/min.mg) (Fig. 3a). The optimum temperature was in accordance to the previously reported optimal growth temperature of *B. argentinensis* (22–25 °C) [19]. Most of L-glutaminases have optima temperature equal or higher than 30 °C and vary between 20 and 70 °C for the enzymes from *Anabaena* sp. PCC 7120 and *Bacillus* sp./*G. thermodenitrificans*, respectively [15, 42, 43] (Supplementary Table S1). Compared to mesophilic counterparts, the L-glutamine glutaminase from *Aspergillus oryzae*, whose optimum temperature is 30 °C, retained only 40% of maximum activity at 25 °C [47], whereas the L-glutamine glutaminase from *E. coli* showed low level of activity at 4 °C [48].

In the second approach, the objective was to evaluate the stability of BA glutaminase at different temperatures. The enzyme was incubated at different temperatures varying from 4 to 60 °C during 6 h, enzyme aliquots were extracted after different incubation times, and the

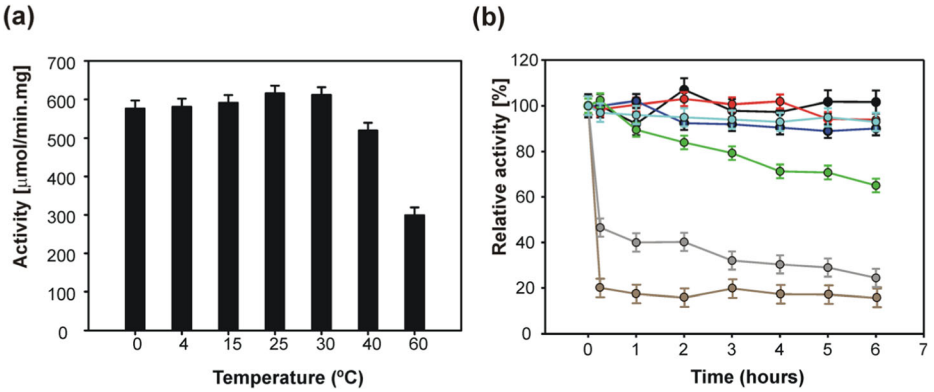

Fig. 3 Influence of temperature on enzyme activity. **a** Activity of BA glutaminase at 0 °C, 4° C, 15 °C, 25 °C, 30 °C, 40 °C, and 60 °C. **b** Relative activity of BA glutaminase measured after incubation at 4 °C (black), 15 °C (red), 20 °C (cyan), 25 °C (blue), 30 °C (green), 50 °C (gray), and 60 °C (brown) in growing times. All activity measurements were performed at saturating concentration of L-glutamine (32 mM)

catalytic activity was determined (Fig. 3b). The enzyme conserved 90–100% of maximum activity between 4 and 25 °C, showing a lineal trend during 6 h. At 30 °C, BA glutaminase decreased progressively the activity, reaching 65% of maximum activity after 6 h. The enzyme diminished more abruptly the activity at 50 °C and 60 °C, conserving 15% of maximum activity after 6 h of incubation at 60 °C (Fig. 3b).

These results suggested that BA glutaminase could catalyze deamination reactions at room temperature and even lower values with high specific activity. Similar to cold-adapted enzymes, the profitable feature of BA glutaminase is the ability to retain a high percentage of maximum activity at low temperature rather than the optimal temperature [18]. This feature could be based on high structural flexibility, which reduces the activation energy of the catalyzed reaction [18, 49]. The structural flexibility has a significant influence on the catalytic efficiency of enzyme activity, given that is required to accommodate substrates and release products during enzyme catalysis [17]. The structural flexibility is restricted in thermo-tolerant enzymes, which base their unusual stability to high temperatures on enhanced structural rigidity [50]. Specifically in L-glutaminases, an intramolecular disulfide bond was identified as a factor involved in the enhanced structural rigidity of the thermo-tolerant L-glutaminase from *Cohnella* sp. A01, but when this disulfide bridge was reduced by addition of β-mercaptoethanol, the enzyme activity increased three times [17].

Cold-adapted enzymes have developed different strategies to deploy high flexibility and catalytic activity at low temperatures. Compared with mesophilic or thermo-tolerant enzymes, these strategies include more glycine residues, lesser proline residues, a lower Arg/(Arg + Lys) ratio, and a lower number of intramolecular interactions such as disulfide bridges, salt bridges, and hydrogen bonds [51]. Structural analysis suggested that BA glutaminase has several of these characteristics commonly found in cold-adapted enzymes (Table 2). This enzyme showed the lowest Arg/(Arg + Lys) ratio and proline residues compared with mesophilic and thermo-tolerant L-glutaminases (Table 2). Besides, BA glutaminase showed the lowest numbers of salt bridges and hydrogen bonds (Table 2). These factors might lead to an enhanced flexibility of the structure of BA glutaminase and consequently to the high catalytic activity observed at low temperature [51].

The effect of temperature on enzyme structure was assayed by monitoring the circular dichroism signal at 222 nm, whose intensity depends on folding state, whilst temperature was

Table 2 Structural features of BA glutaminase compared with mesophilic and thermo-tolerant L-glutaminase

	BA glutaminase model	*M. luteus*[a]	*B. subtilis*	*E. coli*	*Cohnella* sp.
PDB code	ND	3if5	1mki	1u60	ND
Opt. Temp. (°C)	25	50	ND	ND	50
Total amino acids	304	307	328	310	315
Gly %	7.2	9.4	7.9	10	9.8
Pro %	3.9	4.9	5.5	4.2	5.1
Arg/(Arg+Lys)	0.3	0.8	0.4	0.5	0.5
Salt bridges	6	9	9	6	10[b]
H bonds	260	427	694	314	286[b]
Disulfide bonds	0	0	0	0	1
Reference		[13, 55]	[39]	[39]	[17]

[a] Only N-terminal fraction was considered in the analysis

[b] Information obtained from modeled structure as published [17]

ND: Not Determined

increased at uniform rate (Supplementary Fig. S2) [33]. From these data, the melting temperature (T_m) for the thermal unfolding transition of BA glutaminase was calculated, and the obtained value was 60.2 °C, in the range of mesophilic enzymes. At T_m, the half of the enzyme population is unfolded, and the other half is folded [33]. However, BA glutaminase lost 80–85% of activity at 60 °C (Fig. 3b). These results suggest that the catalytic site could be more sensitive to heat than core structural domains, which would be more stable and resistant to temperature. This lower thermal stability of the active site could be attributed to its higher flexibility to catalyze the reaction at low temperatures. In psychrophilic organisms, melting temperatures vary depending on the enzyme type. For example, the reported T_m of the enzymes α-amylase, DNA ligase, and glycosyl hydrolase from the psychrophilic marine bacteria *Pseudoalteromonas haloplanktis* were 44 °C, 33 °C, and 54 °C, respectively [52]. This variation occurs because different domains of the enzyme are involved in structural stability and activity, being the catalytic domain more flexible and therefore more sensible to heat than the structural domains [52].

Effect of Salinity and pH on the BA Glutaminase Activity and Stability

The influence of salinity on enzyme activity was analyzed in the range 0–3 M NaCl (Fig. 4a). The maximum enzyme activity was observed at 0.3 M NaCl, conserving around 80% of maximum activity after 3 h of incubation in 3 M NaCl (Fig. 4a). These results suggested that BA glutaminase could be a halotolerant enzyme. Similar to halophilic enzymes, BA glutaminase has a higher percentage of acidic amino acids (Asp + Glu; 18.0 %) than basic amino acids (Arg + Lys; 15.8 %). Besides, most of acidic amino acids were distributed on the protein surface, leading to a negative surface electrostatic potential, which is key to maintain the stability under high-salinity conditions (Supplementary Fig. S3).

The effect of pH on enzyme stability was analyzed in the range from pH 4.0 up to 11.0 (Fig. 4b). The maximum activity of BA glutaminase was observed at pH 7.5, and high activity was conserved in alkaline pH, showing around 90% of maximum activity at pH 11. BA glutaminase was labile in acidic pH, conserving around 60% of maximum activity at pH 4.0. Similar results were reported for L-glutaminases from *Cohnella* sp. A01 and *Lactobacillus reuteri* [17, 53]. These results follow the common behavior observed for L-glutaminases regarding pH, which are more resistant to alkaline than acidic media.

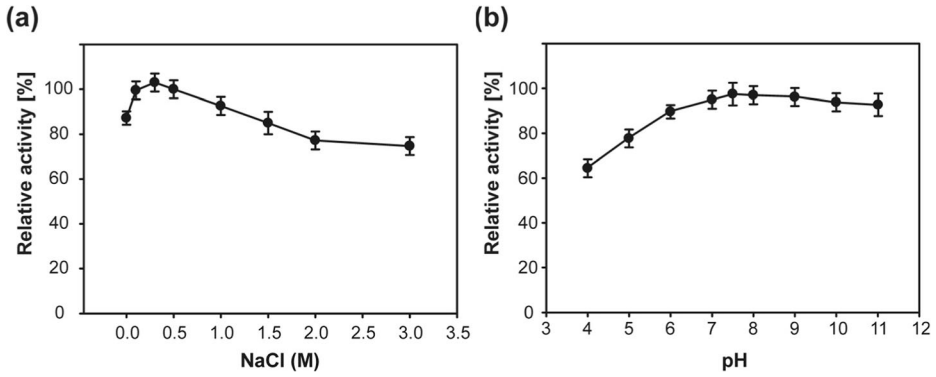

Fig. 4 Influence of salinity and pH on enzyme activity. **a** Relative activity measured at 30 °C after incubation of BA glutaminase at different NaCl concentrations during 3 h and 25 °C. **b** Relative activity at 30 °C after incubation of BA glutaminase at different pHs during 3 h and 25 °C

Influence of $Mg^{2+}$and Chemical Solvents on Enzyme Activity

It has been reported that the divalent cation Mg^{2+} influences the activity of L-glutaminases, playing an activator or inhibitor role, depending on the enzyme. The presence of 25 mM Mg^{2+} duplicated the maximum activity of L-glutaminase from *Cohnella* sp. [17]. However, the opposite effect was observed in the two L-glutaminases from *E. coli* (YbaS and YneH) and *B. subtilis* (YlaM and YbgJ), whose activities were inhibited by lower concentrations of Mg^{2+} (IC_{50} 0.2 mM and 1.7–5.5 mM) [39]. Therefore, the effect of the divalent cation Mg^{2+} on BA glutaminase activity was assayed (Table 3). An increment around 10% of BA glutaminase maximum activity was observed in the presence of Mg^{2+} 125 mM (relative activity 109.7%, Table 3). These results suggested that there is a slight cofactor effect of Mg^{2+} on BA glutaminase activity and the enzyme does not require this cation for a considerable increment of the catalytic activity. The independence of cofactors could be a valuable feature for industrial purposes.

The effect of chemical solvents on activity of BA glutaminase was also analyzed (Table 3). High relative activity was conserved in presence of ethanol, methanol, and isopropanol at 5% (v/v). Similar behavior was observed in ethanol and methanol 15% (v/v) but not in presence of isopropanol. The enzyme activity diminished considerably in the presence of the chemical solvents at 25% (v/v), and low relative activity was observed with all concentrations of acetone (Table 3). These results suggested that BA glutaminase could function optimally up to 15% (v/v) methanol and ethanol but could not tolerate isopropanol and acetone at concentrations higher than 5%.

Deamination of Hydrolyzed Soy Protein Isolate (hSPI) by BA Glutaminase at Room Temperature

Soy protein isolate (SPI) was hydrolyzed with proteinase K to release small peptides and amino acids and thus favors the accession of L-glutamine to the active site of BA glutaminase. The hydrolysis percentage of the hydrolyzed SPI (hSPI) was calculated at different times, showing higher hydrolysis percentages at longer incubation times (Supplementary Fig. S4).

The obtained hSPI were incubated with BA glutaminase at 25 °C to catalyze the deamination of L-glutamine and thus obtain the flavor enhancer L-glutamate. The presence of L-glutamate was estimated indirectly by measuring ammonium, which is the other product released during the reaction catalyzed by BA glutaminase. The produced ammonium increased with the hydrolysis percentage, showing that BA glutaminase deaminates more efficiently

Table 3 Effect of Mg^{2+} and organic solvents on BA glutaminase activity

	Relative activity (%)		
Control	100 ± 0.8		
Concentration	25 mM	125 mM	
$MgCl_2$	104.6 ± 0.9	109.7 ± 1.1	
Concentration	5% (v/v)	15% (v/v)	25% (v/v)
Ethanol	100.3 ± 1.1	102.6 ± 2.6	19.2 ± 6.8
Methanol	99.2 ± 0.4	94.3 ± 5.7	61.4 ± 8.2
Isopropanol	92.5 ± 5.7	35.4 ± 11.4	12.6 ± 5.8
Acetone	42.7 ± 2.9	32.3 ± 10.2	17.2 ± 2.4

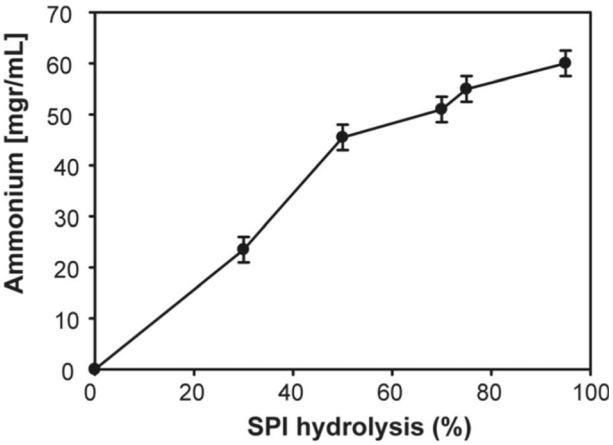

Fig. 5 Production of ammonium from hSPI mediated by BA glutaminase at different protein hydrolysis percentages of hSPI

hydrolyzed proteins (Fig. 5). Besides BA glutaminase was not inhibited by other amino acids and molecules present in hSPI. Similar results were obtained when L-glutamine glutaminase from *E. coli* was applied to deaminate whey protein hydrolysates [7]. This treatment showed the highest deamination percentage and was the most efficient procedure to increase the umami taste compared with chemical deamination and protein glutaminase treatments [7]. These results suggested that BA glutaminase catalyzed efficiently the hydrolysis of L-glutamine residues present in hSPI and the enzyme is more efficient when SPI is more hydrolyzed.

Toxicological studies have demonstrated that the use of the L-glutaminase from *A. niger* in food production is safe for human consumption [54]. In addition, the L-glutaminase from *B. amyloliquefaciens* has been used to improve the taste of foods [11]. The safety of *B. argentinensis* in the food industry has not been confirmed; however, the use of the purified BA glutaminase but not the microorganism could overcome this limitation. Further studies such as toxicity in rats, in vitro bacterial reverse mutation, and in vitro mammalian chromosome aberration tests will support the use of BA glutamine in food processing.

Conclusions

This work involved the characterization of the L-glutaminase from *B. argentinensis*, which presented high catalytic activity at room and lower temperatures. This enzyme also showed proficient features for industrial applications such as high yield of production, high catalytic efficiency, independence of cofactors, stability in high salinity, and the capability to function optimally in the presence of 15% (v/v) ethanol and methanol. In addition, the enzyme could deaminate hSPI optimally at room temperature, suggesting high production of the flavor enhancer L-glutamate and no inhibition of enzyme activity by the hydrolysis products. Besides, this feature dispenses with temperature factor, and therefore BA glutaminase does not require energy inputs to carry out deamination processes. BA glutaminase could be useful to increase the flavor of protein hydrolysates obtained by digestion with cold-adapted proteases, in a process aimed to reduce both energy consumption and costs, producing a positive

impact at industrial scale. In addition, they could be applied to increase the flavor of fermented foods obtained by fermentation at room temperature. This work provides a novel cold-adapted L-glutaminase useful to improve the taste of protein hydrolysates, which is a relevant capability for the development of functional foods in the food industry.

ND not determined

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Declarations

Conflict of Interest The authors declare no competing interests.

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Affiliations

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