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Identification, Expression and Preliminary Characterization of a Recombinant Bifunctional Enzyme of *Photobacterium damselae* subsp. *piscicida* with Glutamate Decarboxylase/Transaminase Activity

Francesca Andreoni*, Anna Rita Mastrogiacomo, Giordano Serafini, Gionmattia Carancini, and Mauro Magnani

Department of Biomolecular Sciences, University of Urbino "Carlo Bo", via Arco d'Augusto, 2, 61032 Fano (PU), Italy

Glutamate decarboxylase catalyzes the conversion of glutamate to gamma-aminobutyric acid (GABA), contributing to pH homeostasis through proton consumption. The reaction is the first step toward the GABA shunt. To date, the enzymes involved in the glutamate metabolism of *Photobacterium damselae* subsp. *piscicida* have not been elucidated. In this study, an open reading frame of *P. damselae* subsp. *piscicida*, showing homology to the glutamate decarboxylase or putative pyridoxal-dependent aspartate 1-decarboxylase genes, was isolated and cloned into an expression vector to produce the recombinant enzyme. Preliminary gas chromatography-mass spectrometry characterization of the purified recombinant enzyme revealed that it catalyzed not only the decarboxylation of glutamate but also the transamination of GABA. This enzyme of *P. damselae* subsp. *piscicida* could be bifunctional, combining decarboxylase and transaminase activities in a single polypeptide chain.

Keywords: *Photobacterium damselae* subsp. *piscicida*, bifunctional enzyme, glutamate decarboxylases, γ-aminobutiric acid transaminase

Introduction

γ-Aminobutiric acid (GABA) is a non-protein amino acid found in animals, plant and microorganisms. It plays an important role in the mammalian central nervous system as an inhibitory neurotransmitter, with hypotensive, anti-diabetic, diuretic and analgesic effects [1–3]. GABA is therefore commonly used as a functional bioactive component in foods and pharmaceuticals [4]. In organisms, GABA is synthetized by the irreversible α-decarboxylation of L-glutamate catalyzed by the pyridoxal 5'-phosphate (PLP) dependent glutamate decarboxylase (GAD, EC 4.1.1.15). In most microorganisms, GAD enzymes help to prevent intracellular acidification by proton consumption of the glutamate decarboxylation reaction [5]. In *Bacillus megaterium*, it has been reported that GAD is involved in spore germination [6]. In *Saccharomyces cerevisiae*, GAD is required for normal oxidative stress tolerance [7]. In plants, GABA accumulation has been reported in response to stress conditions such as anoxia, decreasing cellular pH, temperature changes, mechanical handling, and pathogen infections, and it has been associated with plant growth response [5, 8]. Furthermore, in plants and microbes, GABA is an important intermediate of the GABA shunt, the metabolic pathway that converts glutamate to succinate,

*Corresponding author
Tel: +390722304978, Fax: +390722304970
E-mail: francesca.andreoni@uniurb.it

directing the carbon atoms into the Krebs cycle [9]. The reversible conversion of GABA to succinic semialdehyde by GABA transaminase is followed by the irreversible oxidation of succinic semialdehyde to succinate by succinic semialdehyde dehydrogenase [10]. GAD is widely distributed in nature among eukaryotes and prokaryotes. Recently, a number of studies have focused on the biotechnological production of GABA for GABA-enriched foods or pharmaceutical preparations, and GAD enzymes from several microorganisms, in particular, lactic acid bacteria, have been characterized [5, 11]. Most bacteria contain only one GAD, but two isoforms have been described in *Escherichia coli, Listeria monocytogenes* and *Shigella flexneri* [12–14]. Among these, *E. coli* GadA and GabB isoforms have been well characterized and their crystal structures have been elucidated [5, 15].

Photobacterium damselae subsp. *piscicida* (PDP) is a gram-negative halophilic bacterium of the *Vibrionaceae* family, and it is the causal agent of fish photobacteriosis [16]. PDP is a facultative intracellular pathogen. Indeed, all PDP isolates come from internal tissues of fish, and to date, this subspecies has not been isolated from seawater or sediment samples, suggesting that PDP is a bacterium that lives in close partnership with its host [17].

In this study, a gene from *P. damselae* subsp. *piscicida,* annotated as *gad* based on its homology in sequence to other *gad* genes, was isolated, cloned and overexpressed in *E. coli*. The recombinant enzyme was purified and its activity and properties were assessed.

Materials and Methods

Bacterial strains and culture conditions

P. damselae subsp. *piscicida* NCIMB 2058 was purchased from the National Collections of Industrial and Marine Bacteria (Aberdeen). Bacteria were grown in tryptone soya broth or tryptone soya agar (Oxoid) supplemented with 1.5% NaCl at 25℃. BL21(DE3) carrying pET45b (Novagen) recombinant vector were cultured in Luria-Bertani medium supplemented with ampicillin (50 μg/ml) at 37℃ or 25℃.

Isolation of PDP *gad* gene

The nucleotide sequence of the contig_001_B05 from the genome of *P. damselae* subsp. *piscicida* NCIMB 2058 was obtained by sequencing a genomic cosmid library constructed in pWEB vector (Epicentre Biotechnologies) and deposited in the DDBJ/EMBL/GenBank databases with accession number HQ599848.1 [18]. Open reading frames were predicted using Glimmer software [19] and GeneMark [20] and protein annotation was supported by a Blast homology search against the NCBI non-redundant protein database [21]. Among the open reading frames identified in the contig_001_B05, the sequence for a glutamate decarboxylase was selected and further analyzed against the NCBI CDD database to predict the protein domain [22, 23]. The gene coding for the hypothetical glutamate decarboxylase was isolated from the genomic DNA of *P. damselae* subsp. *piscicida* NCIMB 2058 by PCR using two oligonucleotide degenerate primers (Upper 5'-AACACTGGATCCGAC-TATAGAAGAACGTACT-3', Lower 5'-TGTTTACTC-GAGTTAGAATCTTTCAAGTATT-3'), which introduced *BamHI* and *XhoI* sites (sequences underlined) at the 5' and the 3' of the gene, respectively. The reaction was carried out with KOD XL DNA polymerase (Novagen) according to the instruction manual. The resulting PCR product was digested with *BamHI* and *XhoI* enzymes and ligated into the pET45b expression vector (Novagen), which was handled with the same enzymes to yield plasmid pET45b-GAD.

Expression and purification of recombinant glutamate decarboxylase

The recombinant plasmid pET45b-GAD was transformed into *E. coli* BL21(DE3) competent cells for the expression of the N-terminal His-tag fusion protein. The transformant cells were grown overnight at 37℃ in Luria-Bertani broth containing 50 μg/ml ampicillin. The overnight culture was diluted into the same medium, and the cell growth was continued at the same temperature or alternatively at 25℃ until the optical density (OD) value at 600 nm reached approximately 0.5–0.6. The expression of the recombinant proteins was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3–4 h at 37℃ or 25℃. Cells were harvested by centrifugation at 5000 *g* for 30 min and resuspended in lysis buffer (2 μg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 10 mM imidazole, 20 mM phosphate buffer, 0.3 M NaCl, 3 mM β-mercap-

toethanol, 5% glycerol, pH 7.0). Cells were lysed using an Avestin EmulsiFlex - C50 High Pressure Homogenizer at 14000 psi twice. After centrifugation at 17000 g for 30 min, the recombinant protein was purified from the supernatant (soluble fraction) using a TALON Superflow Metal Affinity Chromatography Column (2.5×10 cm) (Clontech) with an AKTA purifier system (GE Healthcare). The recombinant enzyme was eluted with 500 mM imidazole and 0.5 M ammonium sulfate was added to the elution fractions. The recombinant protein was dialysed into a buffer containing 20 mM phosphate buffer, 0.3 M NaCl, 3 mM β-mercaptoethanol, 5% glycerol and 0.5 M ammonium sulfate, pH 7.0 plus antiproteolytics. The purity of the His$_6$-GAD protein was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by the Bradford method using bovine serum albumin as a standard [24].

Enzyme assay

The activity of the purified enzyme was measured as described in Yang *et al.* [25]. The reaction mixture, containing 10 mM L-glutamate (L-Glu), 0.2 mM pyridoxal 5'-phosphate (PLP) and 10–50 µg of the recombinant enzyme in 200 mM acetate buffer, pH 4.5, was incubated for 60 min at different temperatures (20–50℃). 900 µl of ethanol absolute was added at -20℃ to terminate the reaction and the suspension was centrifuged at 8000 g for 15 min at 4℃. 100–200 µl of the supernatant or standard solutions were dried under vacuum using the Savant SpeedVac Concentrator (Thermo Fisher Scientific). Reactions without the GAD enzyme were also set up as a negative control. One unit of GAD activity was defined as the amount of enzyme producing 1 µmol product per min under the experimental conditions.

Derivatization and GC-MS analysis

After drying, the products of the reaction present in the supernatant or standard solutions (GABA and succinic acid) were derivatized to tert-butyldimethylsilylation (TBDMS) derivatives from N-Methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) (Sigma-Aldrich). 25 µl of MTBSTFA followed by 75 µl of acetonitrile were added to the dried residues. The mixture was heated to 80℃ for one hour under stirring with the Hybaid Shake 'n' Stack Hybridization Ovens [26,

27]. 1 µl of the derivatized solution was subjected to GC-MS analysis.

A gas chromatograph (Agilent 6890 N), coupled with double focalization high resolution JMS-GC-Mate II (Jeol) was used for the analysis of the reaction products. The GC-MS was equipped with Jeol Software to confirm reaction products. The mass spectrometer was operated in the electron ionization mode (EI) at 70 eV. The retention time and the characteristic fragments of the EI mass spectra were determined by total ion monitoring (SCAN). Chromatographic separation of products of the reaction was performed using a DB-5msMSD GC column (0.25 mm i.d. × 30 m length, 0.25 µm film thickness). The oven temperature was programmed as follows: 70℃, hold for 3 min, 10℃/min to 310℃, hold for 5 minutes. The carrier gas was helium with a constant flow of 1 ml/min. The injector temperature was 280℃ and the interface temperature was 280℃. Injection volume was 1 µl with 3 minutes of delay time in splitless mode.

Each product of the reaction was identified by comparing its fragmentation pattern with the mass spectrometer database.

Properties of the recombinant enzyme

A pH dependent activity profile of the recombinant enzyme was determined using 0.2 M acetate buffer at different pH values (4.0–6.0) at 25℃, and the remaining activity was measured as described above. The effect of temperature was evaluated at various temperatures (20–50℃) at pH 4.5 for 1 h using acetate buffer. The kinetic parameters of the recombinant enzyme were determined at 25℃ and pH 4.5 (0.2 M acetate buffer) using L-glutamate as a substrate (2–20 mM).

Results

Identification of PDP gad gene

The PDP genomic sequence of the contig_001_B05 (GenBank HQ599848.1) was inspected to identify PDP open reading frames of interest. Among these, one was annotated as coding for a glutamate decarboxylase and considered for further investigation and characterization. The DELTA-Blast search of the predicted protein showed homology with glutamate decarboxylases or putative pyridoxal-dependent aspartate 1-decarboxylase of *Photobacterium* genus, with identity ranging from

100% (*damselae* species) to 78% (*aquae* species), *Vibrio* genus (from 75% of *rimoiensis* species to 69% of *splendidus* species) and *Salinivibrio* species (74–73% identity). Furthermore, the PDP sequence showed 32% identity with L-2,4-diaminobutyrate decarboxylase of *E. coli* ISC56 (query coverage of 66%), 28% identity with glutamate decarboxylase beta of *E. coli* (28% query coverage), 28% identity with a tyrosine decarboxylase of *Lactobacillus brevis* (query coverage of 61%) and 26% identity with a glutamate decarboxylase of *L. brevis* (query coverage of 31%).

The prediction of conserved protein domains identified the specific hit GadA (COG0076, Glutamate or tyrosine decarboxylase or a related PLP-dependent protein) and revealed that the protein belongs to the aspartate aminotransferase (AAT) superfamily (fold type I) of pyridoxal phosphate (PLP)-dependent enzymes. PLP combines with an alpha-amino acid to form a Schiff base or aldimine intermediate, which, depending on the reaction, is the substrate in four kinds of reactions: transamination, racemization, decarboxylation, and various side-chain reactions depending on the enzyme involved [23]. The analysis of conserved features using the Cn3D tool (4.3.1 version) [28] and alignment of the PDP protein with the most representative members of the family, in particular the 2EO5_A of a 4-aminobutyrate-aminotransferase, showed that the conserved domain contained a PLP binding motif (corresponding to amino acids 173-338), in which a highly conserved residue, lysine 338, is reported to be essential for binding. In the active site, PLP forms an internal aldimine bond (Schiff base linkage) with the catalytic residue lysine, which in most cases, is part of a flexible region [29].

Overexpression and purification of the recombinant enzyme

A pET45b vector was used to clone the *gad* gene of *P.*

damselae subsp. piscicida and produce the recombinant protein in *E. coli* under the control of the T7 promoter as a His-tag N-terminal fusion protein. Increased levels of expression were obtained when the *E. coli* BL21 (DE3) cells with the pET45b-GAD were grown at 25℃ instead of the 37℃ and induced with IPTG at the same temperature. These conditions also enhanced the fraction of recombinant soluble protein that was used for GAD purification. The recombinant protein was eluted from a TALON Superflow Metal Affinity Chromatography Column at the imidazole concentration of 500 mM. To prevent the unstable nature of the enzyme [30], the protein was immediately stabilized with 0.5 M ammonium sul-
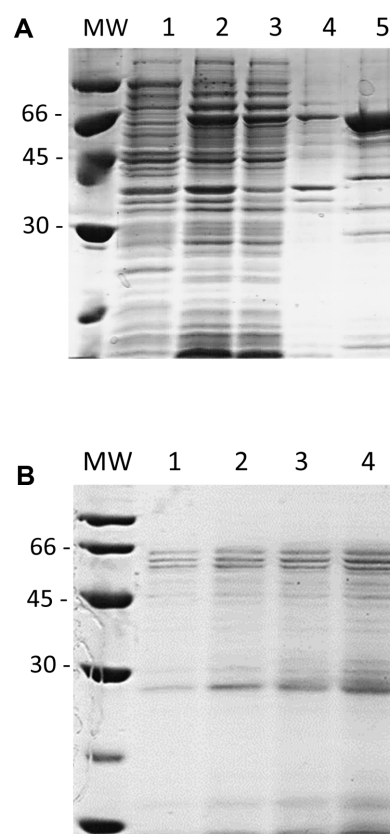

Fig. 1. Expression and purification of the recombinant protein. SDS-PAGE analysis with Comassie brilliant blue staining. (A) Lane 1, total uninduced cell proteins; lane 2, total post-induction cell proteins; lane 3, soluble post-induction cell proteins; lane 4, insoluble post-induction cell proteins, urea treated; lane 5, purified GAD after dialysis stabilized with ammonium sulfate; MW, low molecular weight marker, (B) Lanes 1-4, different quantities (2.5 μg, 5 μg, 7.5 μg, 10 μg, respectively) of purified GAD without ammonium sulfate and its degradation after 1-month storage at -20 ℃.

Table 1. Purification of the recombinant protein from *E. coli*.

Purification steps	Total protein (mg)[a]	Step yield (%)	Overall yield (%)
Cell lysate	320	100	100
Soluble fraction	252	78.7	78.7
Affinity purification	8.1	3.2	2.5
Dialysis	8	98.7	2.5

[a]From 4 L of *E. coli* culture

fate, concentrated and dialyzed in a buffer also containing ammonium sulfate and antiproteolitics. A summary of the purification steps and protein yield is shown in Table 1. The purified fraction showed a major band of 65 kDa molecular mass based on SDS-PAGE analysis, similar to the calculated value of 64.4 kDa of the His_6-GAD (Fig. 1A). In Fig. 1B, it is shown the purified enzyme not stabilized with ammonium sulfate and its degradation after 1-month storage at -20℃.

Characterization of the recombinant enzyme

To measure the activity of the purified enzyme, the reactions were set up with L-glutamate as a substrate to confirm the glutamate decarboxylase activity. The GC-MS technique was employed to separate and detect reaction products. The chromatograms showed a peak with the same retention time as the standard GABA, which was absent in the reactions lacking the recombinant enzyme (negative control). The identification of the product of the reaction, made by comparing the mass spectra with those of the NIST database, revealed that this peak corresponded to succinic acid (Fig. 2). The chromatogram and mass spectra of the reaction product were compared both with those of succinic acid (Fig. 3) and GABA standards (Fig. 4), showing that the identified peak in the sample corresponded to succinic acid instead of GABA. Furthermore, the area of the peak, considered
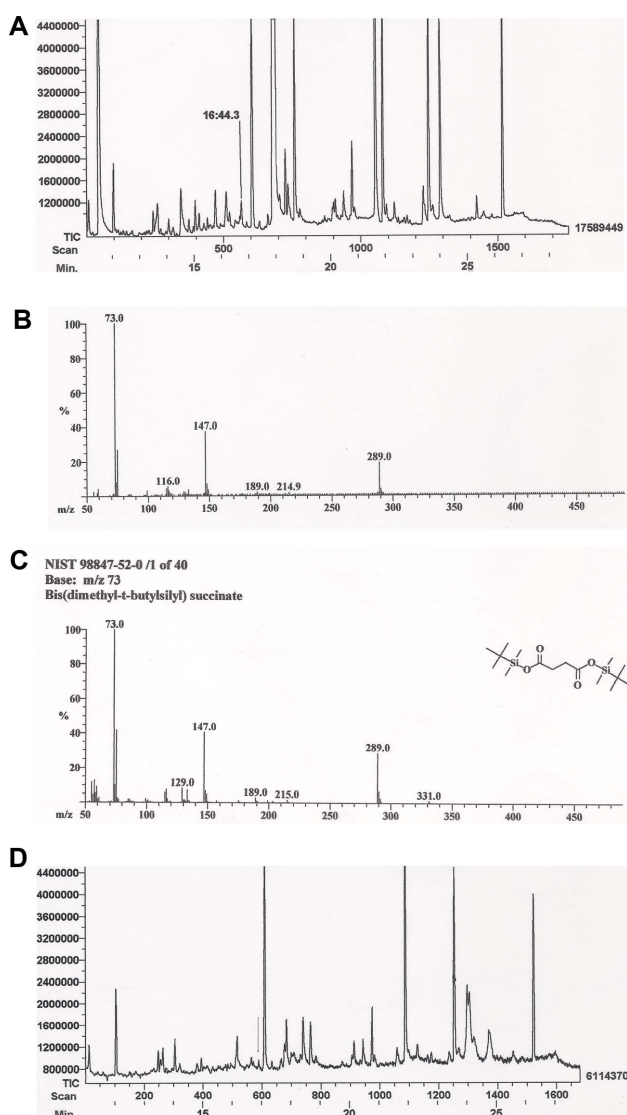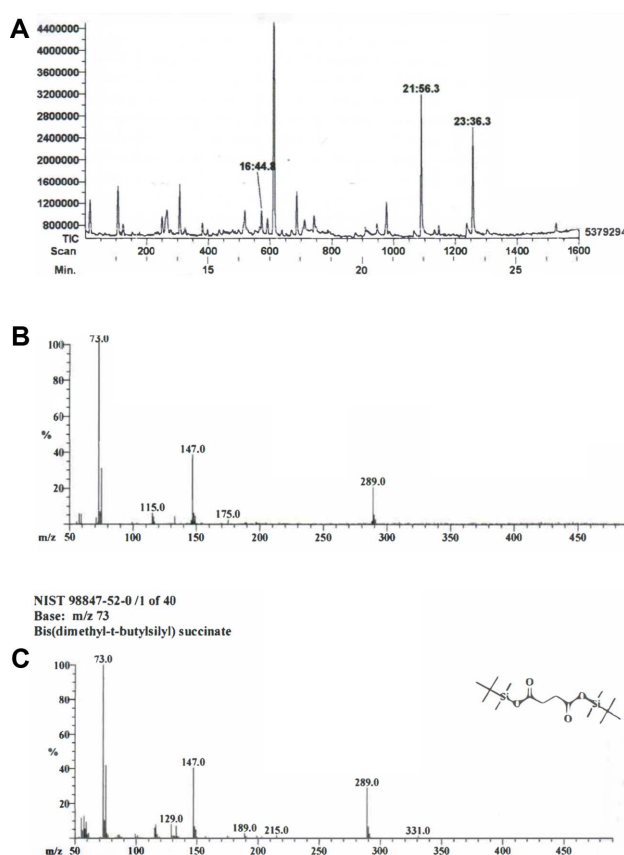

Fig. 2. Chromatogram and mass spectra of TBDMD derivatives of reaction product by the recombinant enzyme. (A) Sample chromatogram, (B) Peak mass spectrum at 16.44.3 min, (C) Succinic acid spectrum from NIST library, (D) Negative control chromatogram (grey arrow indicates the retention time corresponding to succinic acid).

Fig. 3. Chromatogram and mass spectra of TBDMD derivatives of succinic acid standard. (A) Sample chromatogram, (B) peak mass spectrum at 16.44.8 min and (C) spectrum from NIST library of succinic acid.
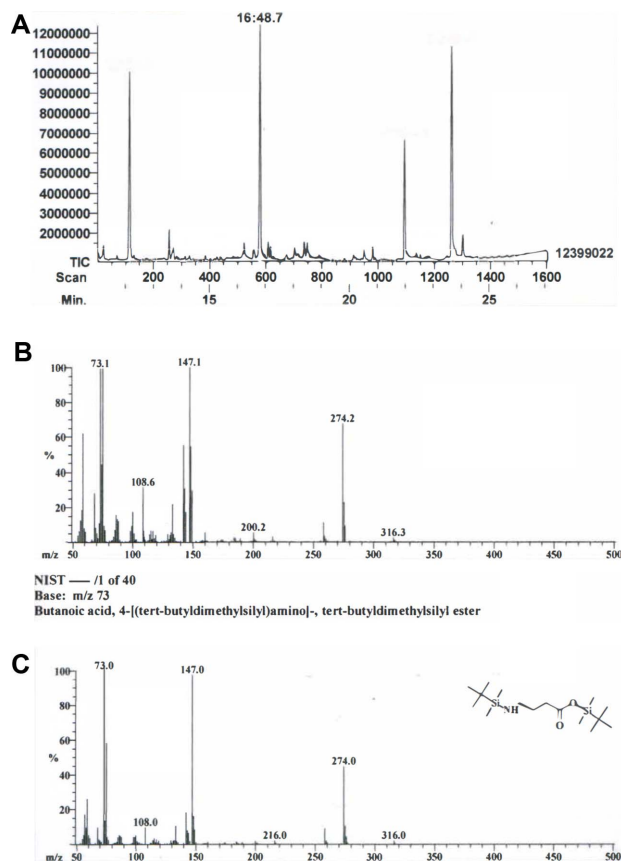
Fig. 4. Chromatogram and mass spectra of TBDMD derivatives of GABA standard. (A) Sample chromatogram, (B) peak mass spectrum at 16.48.7 min and (C) spectrum from NIST library of GABA.
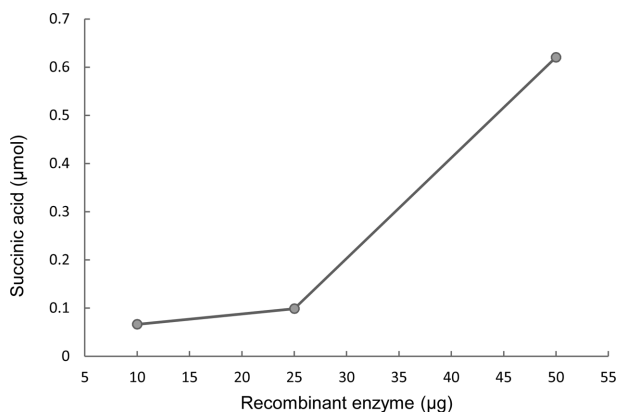

Fig. 5. The amount of succinic acid detected varying the enzyme quantity. The activity of the recombinant protein was measured at pH 4.5 and 25 ℃ temperature for 60 min.

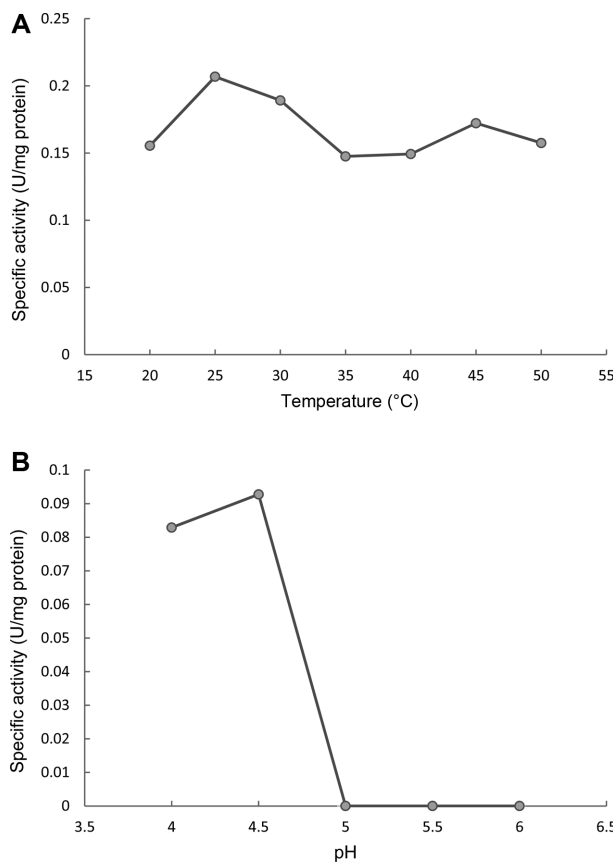as an indication of the reaction rate, varied as a function of the concentration of the enzyme (Fig. 5).

Fig. 6. Effect of temperature and pH on the activity of the recombinant GAD. GAD activity was measured under pH 4.5 at various temperatures for 60 min (A) or under various pHs at 25 ℃ for 60 min (B). One unit of GAD activity was defined as the amount of enzyme producing 1 μmol of succinic acid/min.

Assuming that the recombinant enzyme could produce succinic acid by the transamination of GABA, decreasing concentrations of GABA (from 10 mM to 0.625 mM) were provided to the reaction as a substrate. At lower GABA concentrations, the mass spectra of the detected product was distinctive of succinic acid. Conversely, when the substrate concentration was increased, the mass spectra also showed the mass characteristic of GABA, indicating that conversion of GABA to succinic acid had not been completed.

The optimal pH of the recombinant enzyme was observed at 4.5 and optimal temperature at 25℃ (Fig. 6). The kinetic parameters of the recombinant enzyme were evaluated by Lineweaver-Burk plot using L-glutamate as a substrate. The apparent K_m and V_{max} were 1.76 mM and 119 μM/min, respectively.

Discussion

The sequence of PDP showing homology with glutamate decarboxylases or putative pyridoxal-dependent aspartate 1-decarboxylase was isolated and cloned into an expression vector to produce the recombinant enzyme. For protein characterization, GC-MS was taken into account to separate and detect the reaction product catalyzed by the recombinant enzyme with L-glutamate as substrate. For this study, we evaluated the use of the silylation reagent N-tert-butyldimethylsilyl-N-methyl-trifluoroacetamide for the derivatization of amino acid. MTBSTFA forms tert-butyl dimethilsilyl (TBDMS) derivatives, which are more stable when reacted with polar functional groups containing an active hydrogen. Silylation is a very common derivatization technique, which is useful for detecting a wide variety of compounds, i.e. carboxylic acids, amines, amides, steroids, amino acids [31]. In the silylation reaction, a labile hydrogen from amino acids, acids, alcohols, amines, etc. is replaced by a trimethylsilyl group. The products are generally more volatile and thermally stable. Silylation does not normally require a purification step, and the derivates can be injected directly into GC [32, 33] and identified on the basis of retention time and EI mass spectra.

Analysis of the chromatograms showed a peak with a retention time similar to that of the expected product from glutamate decarboxylation. Nevertheless, the characteristic fragments of the EI mass spectra of the reaction product peak were different from those of the standard GABA, showing a characteristic mass of 289 compared to 274 of GABA. This peak was identified as succinic acid. GABA and succinic acid have been reported to show the same retention time but different EI mass spectra and their identification is possible based on the mass spectra [34].

These findings led to the hypothesis that the isolated enzyme of PDP could act as a bi-functional enzyme of PDP, combining glutamate decarboxylase and GABA transaminase activities in a single polypeptide chain. The enzyme may catalyze both the decarboxylation of L-glutamate to GABA and the subsequent transamination reaction of GABA. Therefore, it is likely that GABA is an intermediate of the reaction. Indeed, when GABA was provided as a substrate for the reaction, succinic acid was also detected.

The combination of two activities in one protein has been observed in many other cases. Another PLP-dependent enzyme, the L-aspartate 4-decarboxylase has been shown to be a bifunctional enzyme that mainly catalyzes the decarboxylation on carbon-4 of L-aspartate and a very minor part of the transamination reaction. Once the quinonoid intermediate forms, the reaction pathway may diverge to perform transamination or β-elimination [35].

In the case of the PDP enzyme, combining the glutamate decarboxylase and GABA transaminase activities in a single polypeptide chain might direct the glutamate towards the GABA shunt more efficiently. The role of the GABA shunt pathway in bacteria is not fully understood; however, it is thought to play a role in glutamate metabolism, anaplerosis, and antioxidant defense [36]. For *Listeria monocytogenes* [37] and some cyanobacteria [38], the GABA shunt may represent a potential route to compensate for the incomplete TCA cycle. Glutamate metabolism plays an important role in the assimilation of ammonia to amino acids, but also in resistance, mainly against acidic conditions and other stresses. In micro-organisms, acid resistance is also a virulence factor, as it allows pathogens to pass through the extremely acidic conditions of the stomach barrier [39]. The structure of the GAD system seems to be highly variable among species and has not been elucidated in PDP species.

The conversion of glutamate to GABA by glutamate decarboxylase is the first step towards the GABA shunt pathway. It helps to increase the pH of the cell leading to the accumulation of intracellular GABA. The action of GABA aminotransferase plays a part in GABA removal. This involves the reversible conversion of GABA to succinic semialdehyde, where the amino group of GABA is donated to an amino group acceptor, usually an α-ketoglutarate molecule by two coupled half reactions [40]. In our conditions with exceeding PLP, GABA may be catabolized to succinic semialdehyde with the concomitant conversion of the PLP coenzyme to PMP. Without the α-ketoglutarate as amino group acceptor, PLP cannot be regenerated.

The oxidation of succinic semialdehyde causes the formation of the succinate. We hypothesize the purified protein is a bi-functional enzyme, nevertheless the suc-

cinic semialdehyde has not been found, instead succinic acid has been revealed. We suppose that the autoxidation of succinic semialdehyde to succinic acid may occur spontaneously at the work conditions as shown by some authors [41, 42]. In fact, the oxidation of aldehydes by oxygen is a well-known reaction and the uncatalyzed oxidation of aldehydes using oxygen from air at room temperature is an effective method and a safe process to produce carboxylic acids [43].

The recently sequenced PDP genome (Gene Bank PRJDB5709) shows another glutamate decarboxylase gene, a PLP dependent amino transferase and a NAD-dependent succinate-semialdehyde dehydrogenase, which have been annotated by automated computational analysis using protein homology as a gene prediction method; however, these proteins have not been characterized. Since bacteria can possess more than one gene encoding GABA aminotransferase and succinate-semialdehyde dehydrogenase [44], we cannot rule out the presence of secondary GABA aminotransferase and succinate semialdehyde dehydrogenase activities in PDP. Our preliminary characterization suggests that the isolated enzyme of PDP may include these activities in a single polypeptide chain. Additional experiments will be needed to elucidate the mechanism of the reactions and the role of other cofactors.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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