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Gene cloning and expression of the L-asparaginase from *Bacillus cereus* BDRD-ST26 in *Bacillus subtilis* WB600

Yue Feng,[1,2] Song Liu,[1,2,*] Yun Jiao,[2] Yunlong Wang,[1,2] Miao Wang,[4] and Guocheng Du[2,3]

National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China,[1] School of Biotechnology, Jiangnan University, Wuxi 214122, China,[2] The Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China,[3] and State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China[4]

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L-Asparaginase (ASN; EC 3.5.1.1) shows great commercial value because of its ability to reduce toxic levels of acrylamide in foods. To achieve high-efficiency production of L-asparaginase, an open reading frame of 978 bp encoding asparaginase (BcA) was amplified from *Bacillus cereus* BDRD-ST26, followed by its expression in *Bacillus subtilis* WB600, with the highest yield of 374.9 U/ml obtained using an amyE-signal peptide. A four-step purification protocol was used to purify BcA, resulting in a 15.1-fold increase in purification yield, with a specific activity of purified BcA at 550.8 U/mg and accompanied by detection of minimal L-glutaminase activity. Maximum BcA activity was detected at 50°C and pH 9.0 in 20 mM Tris–HCl buffer, with a half-life at 50°C of 17.35 min and a K_m and k_{cat} of 9.38 mM and 63.6 s^{-1}, respectively. Compared with untreated potato strips, 72% acrylamide (2.35 mg/kg) was removed from potato strips pretreated with BcA. These results indicated that this novel BcA variant represents a potential candidate for application in the food-processing industry.

[**Key words:** *Bacillus cereus*; *Bacillus subtilis*; Biochemical characterization; Glutaminase free L-asparaginase; Secretory expression]

Acrylamide is a neurotoxin produced from the Maillard reaction, where reducing sugars and free L-asparagine are heated at > 120°C (1). As a result, acrylamide can be detected in heat-treated starched-based foods and especially in coffee, fried potato crisps, and breads (2). L-Asparaginase (ASN; EC 3.5.1.1) has recently been widely applied as an acrylamide-mitigation agent based on its capacity to catalyze L-asparagine conversion to ammonia and L-aspartic acid (3). Although ASNs have been identified in numerous microorganisms, including *Escherichia coli*, *Erwinia chrysanthemi*, *Aspergillus niger*, and *Aspergillus oryzae* (4), their low specific activity requires a large dose for effective application (5). Therefore, discovery and production of ASNs exhibiting high specific activity represent effective approaches to expanding their industrial application.

Recently, ASNs from *Thermococcus kodakaraensis*, *Enterobacter cloacae*, and *Rhizomucor miehei* were identified and purified, with these enzymes representing promising alternatives due to their high specific activity (6–8). However, the yield of ASNs from these wild-type microbes is extremely low, showing difficulties associated with significant improvements in process optimization (4). To achieve high-efficiency expression of ASNs, a variety of ASN-specific genes have been expressed in *E. coli* (3,5). Considering the important application of this enzyme in the medicine and food fields, safety is extremely important, making *Bacillus subtilis*, a generally regarded as safe expression system, and a popular host for recombinant expression. Previously, the highest production (525.98 U/ml) of an ASN from *Pectobacterium carotovorum* was reported in *B. subtilis* WB800N following medium optimization in a 3-L bioreactor (5). These results indicated the potential for industrial production of ASN using *B. subtilis* strains.

ASNs from bacterial resources could be divided into types I (localizing to the cytoplasm based on the lack of a signal-peptide sequence) and II (periplasmic localization) based on their quaternary structure and localization (4). Previous reports showed that the specific activity of type I ASN was higher than that of type II (6,9–13). It was showed that the specific activity of type I ASN (98.7 U/mg) from *B. subtilis* was higher than that of type II (45.4 U/mg) (9,10). Similar result was also observed in ASN from *Saccharomyces cerevisiae* (11,12). According to sequence alignment and signal-peptide prediction, two possible ASN (Type I and II) genes were encoded by the genome of *Bacillus cereus* strains. In this study, to efficiently produce ASN with acceptable specific activity, the type I ASN from *B. cereus* BDRD-ST26 (BcA) was cloned and expressed in *B. subtilis* WB600. The properties and applications of this ASN in the production of acrylamide-free food were also investigated.

MATERIALS AND METHODS

Strains and plasmids *B. cereus* BDRD-ST26 and *B. subtilis* 168 (ATCC 23857) were the donors of the *ASN* sequence and the signal-peptide sequences amyE, lipA, and wapA, respectively. *B. subtilis* WB600 (Bacillus Genetic Stock Center, Columbus, OH, USA) was the host for ASN expression using vectors constructed in *E. coli* JM109 (Novagen, Madison, WI, USA). Plasmid pP43NMK (a gift from Dr. Xiao-Zhou Zhang,

* Corresponding author at: National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China. Tel.: +86 510 85918307; fax: +86 510 85918309.
E-mail address: liusong@jiangnan.edu.cn (S. Liu).

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Virginia Polytechnic Institute and State University, Blacksburg, VA, USA) was used for ASN expression.

Cloning of the ASN open reading frame from *B. cereus* BDRD-ST26 The *B. cereus* BDRD-ST26 genome was isolated using the rapid bacterial genomic DNA isolation kit (Sangon Biotech, Shanghai, China). The *ASN* gene (*BcA*) from *B. cereus* BDRD-ST26 (GenBank accession number: MH107029) was cloned from its genome with polymerase chain reaction (PCR) using the primers F1 and R1 (Table S1) designed based on the putative *BcA* sequence of *B. cereus* BDRD-ST26. PCR was performed as follows: 95°C for 5 min for initial denaturation, followed by 34 cycles of 98°C for 10 s, 58°C for 5 s, and 72°C for 1 min and a final extension at 72°C for 10 min. The amplified *BcA* gene was sequenced by Sangon Biotech.

BcA sequence analysis BcA amino acid sequence was deduced based on its nucleotide sequence, and theoretical molecular weight (MW) and isoelectric point were predicted using Vector NTI (Informax, Frederick, MD, USA). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to analyze the potential signal peptide, and a BLAST search was accomplished by submitting the sequence to NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment (MSA) of BcA and other ASNs from *Helicobacter pylori* (GI: 487878605), *Campylobacter jejuni* (GI: 1124155699), *B. subtilis* (GI: 505196916), and *E. coli* (GI: 1255333425) was performed using ClustalX2 (14).

Plasmid construction and BcA expression The amplified *BcA* gene was inserted into the *Kpn*I and *Pst*I restriction sites of pP43NMK and driven by the P43 promoter, resulting in vector P43-BcA. To enhance the secretion efficiency of BcA in *B. subtilis*, three signal peptides (amyE, lipA, and wapA) were cloned from the *B. subtilis* genome using the corresponding primer pairs (Table S1) and fused into the *BcA* sequence encoding the BcA N-terminus with overlap PCR, followed by ligation between the same restriction sites in pP43NMK to ultimately obtain the three recombinant vectors P43-amyE-BcA, P43-lipA-BcA, and P43-wapA-BcA.

To express BcA, these vectors were transformed into competent *B. subtilis* WB600, and the transformants were cultured in Luria–Bertani medium supplemented with kanamycin (50 μg/mL) at 37°C. Overnight seeds (4%, v/v) were transferred into modified terrific broth medium (20 g glycerol, 12 g peptone, 24 g yeast extract, 16.431 g $K_2HPO_4 \cdot 3H_2O$, and 2.43 g KH_2PO_4 in 1 L H_2O) containing the same antibiotic concentration and grown under the same conditions. To analyze enzyme activity, 1 mL cell culture was centrifuged at 9000 *g* for 5 min, and the BcA activity in the supernatant was presented as the activity of the extracellular fraction. The precipitates obtained by centrifugation were resuspended in 1 mL of 20 mM K_2HPO_4–KH_2PO_4 (pH 7.5) and subjected to ultrasonication and further centrifugation, with the BcA activity in the resulting supernatant used to represent the enzyme activity of the intracellular fraction.

Measurement of BcA activity ASN activity was measured by calculating the liberation of ammonia, as described previously (15), using L-asparagine as the substrate. After enzymatic reaction, ammonia concentration was quantified using Nessler's reagent, and the absorbance was obtained at 436 nm. One unit of ASN activity was defined as the amount of enzyme liberating 1 μmol of ammonia per min at 37°C. Specific activity was calculated as the number of units of activity per mg of protein. L-Glutamine served as the substrate under the same reaction conditions to detect the glutaminase activity.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on a 10% gradient gel with NuPAGE MES SDS running buffer (Invitrogen, Carlsbad, CA, USA), and Coomassie Brilliant Blue G250 was used to stain the proteins. Protein concentration was detected using the Bradford method and using bovine serum albumin as a standard (16).

Purification of recombinant BcA BcA purification was performed using a four-step purification protocol. First, 60% saturated ammonium sulfate was added into the supernatant to precipitate the protein, and after centrifugation, precipitates containing BcA were obtained and resolved in buffer A [20 mM K_2HPO_4–KH_2PO_4 and 1.5 M $(NH_4)_2SO_4$ (pH 7.5)]. The BcA solution was then purified by hydrophobic interaction chromatography [HiTrap Phenyl FF (HS); GE Healthcare, Salt Lake City, UT, USA] in a column pre-equilibrated with buffer A. After washing unbound molecules, gradient elution was conducted using buffer B [20 mM K_2HPO_4–KH_2PO_4 (pH 7.5)], and fractions comprising BcA were collected and dialyzed against buffer B overnight. Dialyzed samples were then loaded onto an ion-exchange chromatograph (HiTrap DEAE FF; GE Healthcare) using a column pre-equilibrated with buffer B. Buffer C [20 mM K_2HPO_4–KH_2PO_4 and 1M NaCl (pH 7.5)] was used to elute BcA by gradient elution, with the active fraction collected and subjected to gel-filtration chromatography (HiLoad 16/600 Superdex 200 pg; GE Healthcare) with buffer B. Purified BcAs were stored at 4°C until subsequent analysis.

Determination of the MW of native BcA Gel-filtration chromatography was performed to determine the MW of native BcA, with cytochrome *c* (12.4 kDa), ovalbumin (44 kDa), serum albumin (65 kDa), and α-globulin (160 kDa) used as standards. All samples were loaded onto a gel-filtration column (HiLoad 16/600 Superdex 200 pg; GE Healthcare) using buffer B, their elution volumes were determined, and a linear fitting of log (MW) versus the elution volumes was established and used to calculate BcA MW.

BcA characterization The optimum temperature for BcA activity was evaluated between 30°C and 70°C and at pH 7.5. BcA half-life ($t_{1/2}$) at 50°C was used to indicate its thermal stability and measured by incubating the enzyme at 50°C for 60 min, followed by determination of its residual activity at 37°C. BcA $t_{1/2}$ at 50°C was calculated by linear fitting of ln (residual activity) versus the incubation time.

The optimal pH for BcA activity was measured at 37°C at different pH values using the following four different buffers: 20 mM NaAc–HAc (pH 3–6), 20 mM K_2HPO_4–KH_2PO_4 (pH 6–8), 20 mM Tris–HCl (pH 8–10), and 20 mM glycine–NaOH (pH 10–12).

To assess the influence of EDTA and metal ions (Li^+, Na^+ K^+ Ca^{2+} Mg^{2+} Zn^{2+} Cu^{2+} Ni^{2+} Ba^{2+} Co^{2+} Mn^{2+} Fe^{3+}, and Al^{3+}) on BcA activity, enzyme solution comprising 20 mM K_2HPO_4–KH_2PO_4 (pH 7.5) with 1 mM of each metal ion and EDTA was incubated for 24 h at 4°C, respectively, followed by determination of relative activity at 37°C.

Apparent kinetic parameters (K_m and k_{cat}) were measured in 20 mM K_2HPO_4–KH_2PO_4 buffer containing between 1.89 mM and 18.9 mM L-asparagine as the substrate at 37°C. Kinetic parameters V_{max} and K_m were calculated from double reciprocal plots. The k_{cat} parameter was determined from the equation $k_{cat} = V_{max}/[E]$, where [E] represents BcA concentration.

Application of BcA to potato chips Potato chips were prepared according to a previous report (17), with some alterations. Briefly, potatoes were washed, peeled, and sliced (thickness: 1.5 mm). Before frying, the potato chips were rinsed with distilled water to remove starch attached to the surface and separated into three identical parts, which were pretreated as follow: (i) no processing, (ii) immersion in distilled water at 45°C for 20 min, or (iii) immersion in 50 U/mL BcA solution under the same conditions. All pretreated potatoes were fried at 170°C for 5 min and then cooled and dried. Acrylamide was extracted from potato chips according to the method described by Zhou et al. (17). Briefly, crushed fried samples (1 g) were defatted with 15 mL petroleum ether three times, followed by removal of the petroleum ether and addition of 15 mL distilled water to dissolve the acrylamide. The samples were centrifuged at 8000 *g* for 20 min to collect the supernatants, and the acrylamide was extracted from the supernatant via ethyl acetate and concentrated by rotatory evaporation.

Acrylamide determination Acrylamide was determined according to a method described previously (17). Briefly, to evaluate the extracted samples, liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed using a C_{18} chromatographic column (2.6 μm, 10 cm × 2.1 mm; Chrom-Matrix Biotechnology Co., Ltd., Wuxi, China). The samples were loaded at a 0.3 mL/min flow rate with a 1% methanol solution used as the mobile phase. Acrylamide precursor and daughter ions were detected at $m/z = 72$ and $m/z = 55$, respectively, in the MS/MS system. All assays were performed in triplicate, and acrylamide concentration was determined as mg/kg potato chips.

RESULTS

Cloning and sequencing of the *BcA* ORF An open reading frame (ORF) of 978 bp encoding BcA was amplified from *B. cereus* BDRD-ST26 with PCR. The theoretical molecular mass and isoelectric point of BcA were predicted as 35.5 kDa and 5.5, respectively, and signal-peptide analysis showed no N-terminal signal peptide in the translated protein sequence. To determine sequence homology, BLAST analysis was performed, revealing that *BcA* displayed the highest similarity to an uncharacterized BcA from *B. cereus* JEM-2 (99%; GenBank accession number: CP018935.1). Furthermore, MSA of the amino acid sequences of ASNs from *B. cereus* and other organisms revealed strict conservation of certain motifs among ASNs (Fig. 1). Specifically, amino acid residues involved in catalytic activity were conserved, with the putative BcA active site (Thr13, Ser56, Thr89, Asp90, and Lys162) structurally equivalent to that in *E. coli* ASN (EcA; Thr14, Ser60, Thr91, Asp92, and Lys163) (4). Further analysis using the GenBank database revealed that BcA was similar to ASNs from *H. pylori* (39.05% identity), *C. jejuni* (37.38% identity), *B. subtilis* (25.7% identity), and *E. coli* (25.4% identity).

Screening signal peptides for BcA expression in *B. subtilis* WB600 To efficiently express BcA in *B. subtilis* WB600, three common signal-peptide sequences, amyE, lipA, and wapA, were selected and fused to the upstream region of the *BcA* sequence (18), followed by insertion of the resulting amyE-BcA, lipA-BcA, wapA-BcA, and wild-type BcA (without a signal peptide) sequences into pP43NMK for expression in *B. subtilis* WB600 under the control of the P43 promoter. We detected different

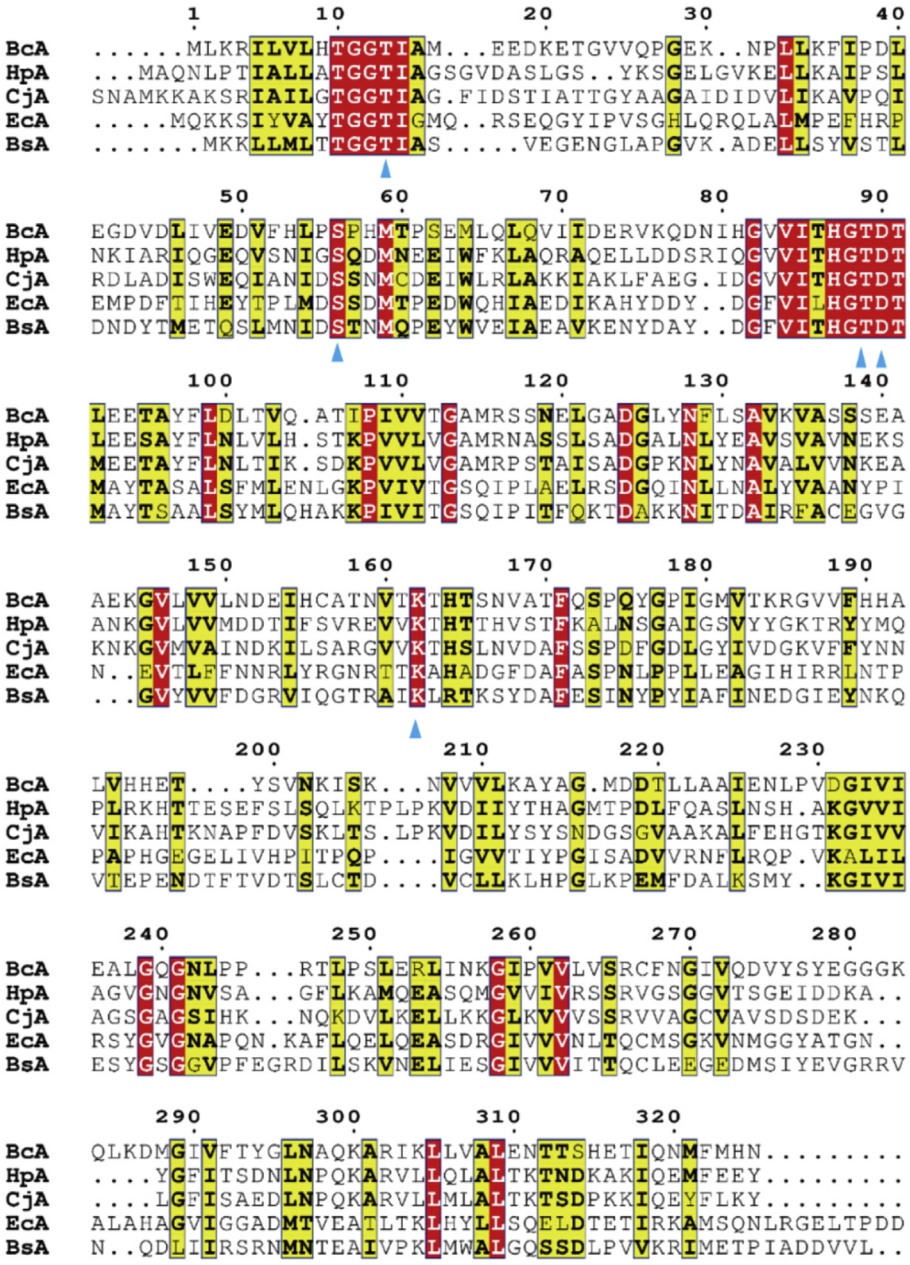

FIG. 1. Sequence alignment with different ASNs. Blue triangles indicate conserved ASN active-site residues (Thr13, Ser56, Thr89, Asp90, and Lys162). Numbering is based on the BcA sequence. Conserved and semi-conserved residues are colored red and yellow, respectively. BcA, *B. cereus* ASN (this study); HpA, *H. pylori* ASN (GI: 487878605); EcA, *E. coli* ASN (GI: 1255333425); CjA, *C. jejuni* ASN (GI: 1124155699); BsA, *B. subtilis* ASN (GI: 505196916). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

extracellular activities from the five recombinant strains (P43-amyE-BcA, P43-lipA-BcA, P43-wapA-BcA, P43-BcA, and control P43NMK). Recombinant P43-amyE-BcA exhibited the highest extracellular BcA activity (374.9 U/mL), which was ~20-fold higher than that of the other strains, with P43-BcA exhibiting primarily intracellular localization due to the lack of a signal peptide (Fig. 2A). Moreover, SDS-PAGE analysis revealed a band (36 kDa) agreeing with the predicted BcA MW (Fig. 2B), with protein levels of the amyE-BcA fusion representing the highest among the recombinant strains and consistent with results of BcA-activity assays. These results indicated that the amyE signal peptide exhibited the highest efficiency in secreting the active form of BcA in *B. subtilis*.

Purification of recombinant BcA BcA purification, including ammonium sulfate fractionation (60%), hydrophobic interaction chromatography, ion-exchange chromatography, and gel-filtration chromatography, is described in Table 1. The purified enzyme showed a final recovery of 8.25% and a 15.52-fold higher purification rate than that from previous culture supernatant (Fig. 3), with a specific activity of 550.8 U/mg. Additionally, we observed a single band representing the purified enzyme at ~36 kDa observed by 10% SDS-PAGE (Fig. 2C). Gel-filtration chromatography showed that purified BcA displayed a single peak and a corresponding elution volume of 73.69 mL. Plots of the standard proteins (Fig. 2D) suggested that the native molecular mass of BcA was 71.4 kDa, indicating that it might exist as a dimer.

Characterization of recombinant BcA Fig. 3A shows the influence of temperature (30–70°C) on BcA activity. The enzyme exhibited the highest activity at 50°C, which was 1.4-fold higher

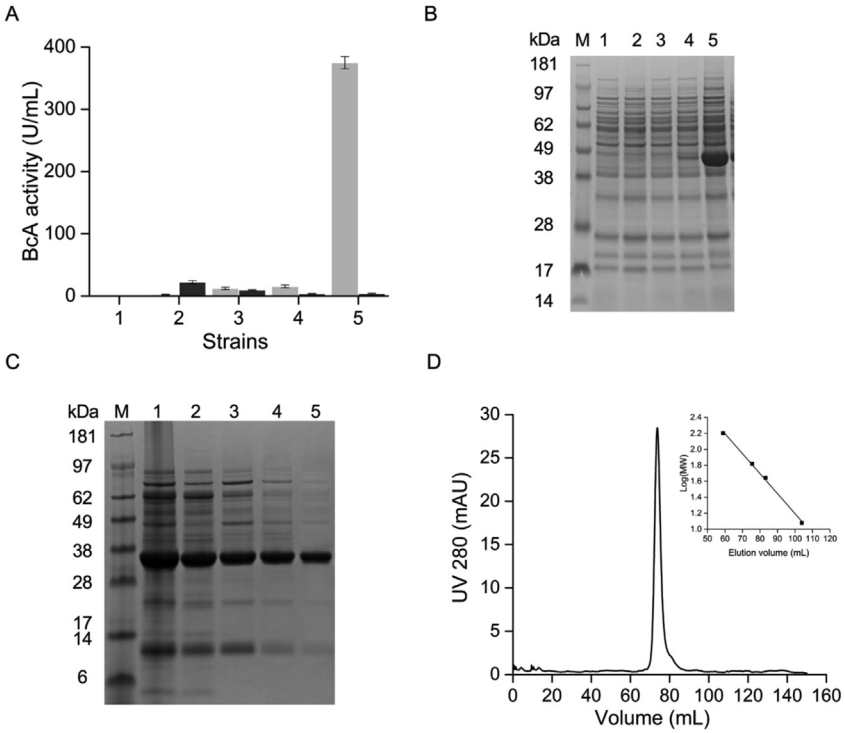

FIG. 2. Comparison of BcA production in recombinant *B. subtilis* WB600 using different signal peptides and purification of recombinant BcA. (A) Extracellular (light grey) and intracellular (dark grey) BcA activities from the recombinant strains. (B) SDS-PAGE analysis of extracellular ASN from the recombinant strains. Lane M, protein marker; lane 1, *B. subtilis* WB600 expressing pP43NMK (without the *BcA* gene); lane 2, P43-BcA (without a signal peptide); lane 3, P43-wapA-BcA (wapA); lane 4, P43-lipA-BcA (lipA); and lane 5, P43-amyE-BcA (amyE). ASN bands are indicated by arrows. (C) SDS-PAGE analysis of purified BcA. Lane M, protein marker; lane 1, culture supernatant; lane 2, ASN partially purified by ammonium sulfate precipitation; lane 3, phenyl hydrophobic chromatography; lane 4, DEAE ion-exchange chromatography; and lane 5, gel-filtration chromatography. (D) Determination of native BcA MW by gel-filtration chromatography. Insets show the fitting curve of the data points of MW analysis.

than that at 37°C. However, BcA activity decreased rapidly at temperatures > 60°C, and no activity was detected at 70°C. To evaluate thermal stability, BcA incubated at 50°C revealed a $t_{1/2}$ of 17.35 min (Fig. 3B).

The effect of pH on BcA activity is shown in Fig. 3C, revealing that the enzyme displayed catalytic activity at a pH range of 6.0–11, with the highest activity achieved in 20 mM Tris–HCl buffer at pH 9.0. Examination of the effects of EDTA and metal ions on BcA activity showed that EDTA and most metal ions, except Zn^{2+} and Cu^{2+}, had no effect on enzyme activity (Fig. 3D). These results indicated that BcA activity was not dependent upon the presence of metal ions.

Kinetic properties were determined using L-asparagine as the substrate, with results indicating K_m and k_{cat} values of 9.38 mM and 63.6 s^{-1}, respectively (Fig. 3E). L-Asparagine represented a more favorable BcA substrate than L-glutamine, with BcA showing extremely low activity in the presence of L-glutamine (Fig. 3F). These results suggested that BcA might represent an attractive anticancer agent. To expand its application in medicine, molecular modification will be carried out in subsequent studies.

TABLE 1. Purification steps for recombinant BcA.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	352.35	12540.5	35.49	1	100
60% $(NH_4)_2SO_4$	38.07	8715.6	228.91	6.45	69.5
Phenyl hydrophobic chromatography	15.20	5040.02	331.47	9.34	40.19
DEAE ion-exchange chromatography	4.4	2103.04	477.34	13.45	16.77
Gel-filtration chromatography	1.87	1034.5	550.8	15.52	8.25

BcA application To evaluate the potential of BcA as an additive for the production of acrylamide-free food, trials were performed on fried potato chips. Fried potato chips without preprocessing contained 3.27 mg/kg of acrylamide (Fig. 4). An obvious mitigation in acrylamide levels was observed in samples pretreated with distilled water and BcA, as >30% of acrylamide was diminished following immersion of the potatoes in distilled water. Furthermore, potatoes pretreated with 5000 U BcA showed a >72% decrease in final acrylamide content (Fig. 4).

DISCUSSION

ASNs have recently received increased attention due to their ability to eliminate L-asparagine, which plays a critical role in the production of acrylamide-free food (3,4). Bacterial ASNs are generally divided into groups I and II based on their quaternary structure and localization (4). Type I ASNs localize to the cytoplasm based on the lack of a signal-peptide sequence, whereas type II ASNs display periplasmic localization. In this study, an ASN gene (*BcA*) was cloned from *B. cereus* BDRD-ST26 (GenBank accession number: MH107029). No endogenic signal peptide was detected at the N-terminus of BcA, and the MW of native BcA was similar to that of type I ASN homodimers from thermophiles (~70 kDa) (3,4). Therefore, we speculated that *B. cereus* BDRD-ST26 BcA represented a type I ASN.

To date, a series of *ASN* genes have been cloned and expressed, including those from eukaryotes (*S. cerevisiae*), bacteria (e.g., *E. coli*, *E. chrysanthemi*, *P. carotovorum*, *B. subtilis*, *Bacillus megaterium*, *Erwina carotovora*), and archaea (*T. kodakaraensis*) (Table 2), with *E. coli* and *B. subtilis* frequently used as the host strains (Table 2). In this study, BcA was expressed in *B. subtilis* using the amyE signal peptide, and the ASN yield (374.9 U/ml) in flasks exceeded that

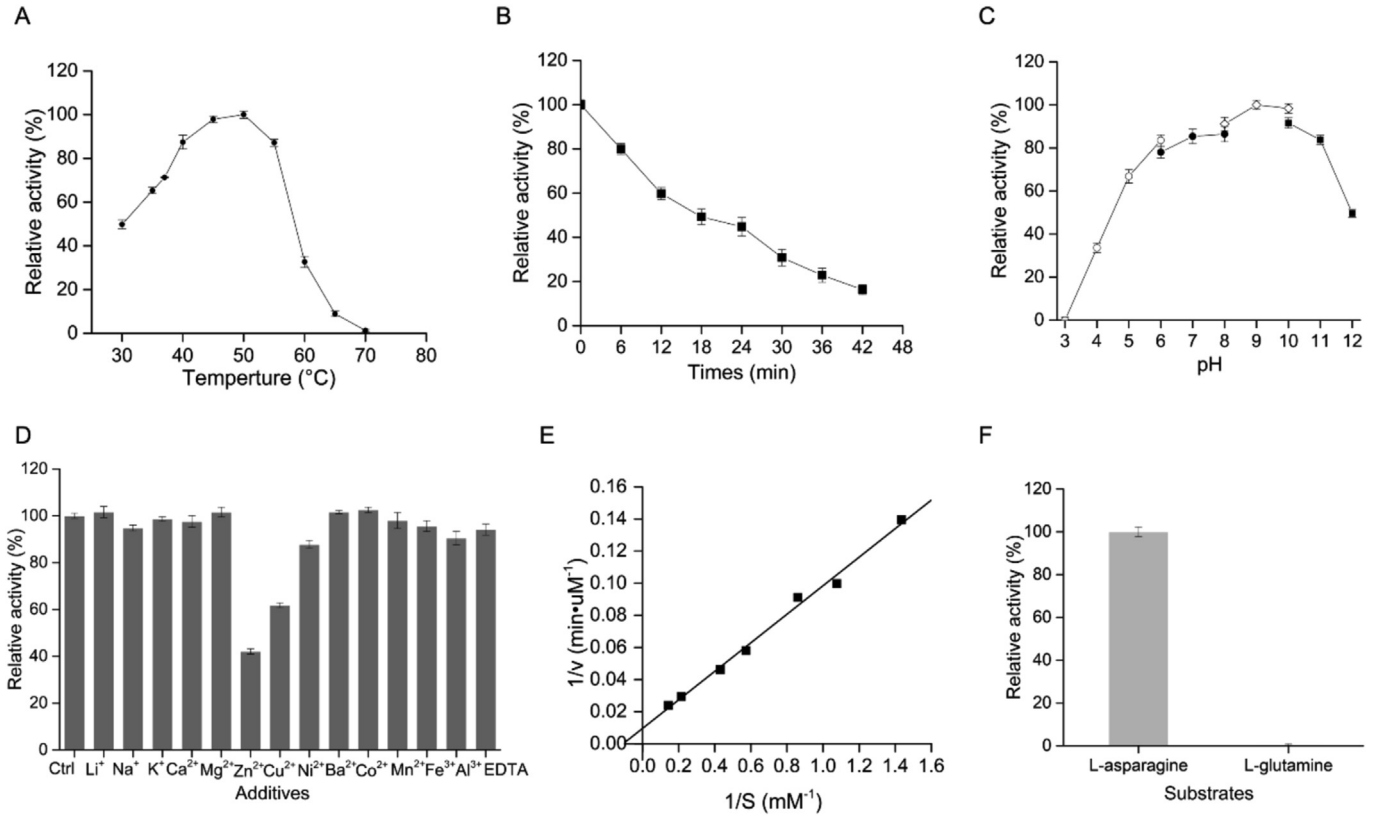

FIG. 3. Enzyme properties of BcA. (A) Effect of temperature on recombinant BcA activity. (B) The thermal stability of recombinant BcA at 50°C. (C) Effect of pH on recombinant BcA activity (pH 3.0–6.0, 20 mM NaAc–HAc, open circles; pH 6.0–8.0, 20 mM, K_2HPO_4–KH_2PO_4, closed circles; pH 8.0–10.0, 20 mM Tris–HCl, open rhombus; pH 10.0–12.0, 20 mM glycine–NaOH, closed squares). (D) Effect of EDTA and metal ions on recombinant BcA activity. (E) Enzyme kinetics of BcA calculated from double reciprocal plots. (F) Substrate specificity of recombinant BcA toward L-asparagine and L-glutamine. Error bars represent the average value of three parallel experiments.

obtained in the previous reports (Table 2). As optimization in a jar fermentor might further improve enzyme production (5), our results potentially contribute to industrial production of ASN.

The catalytic properties of ASNs from different sources are summarized in Table 2. Notably, a significant deviation in enzyme properties was not observed between secreted BcA (P43-amyE-BcA) and intercellular BcA (P43-BcA), suggesting that secretory expression exhibits little effect on the functional properties of BcA. We found that the specific activity of BcA reached 550.8 U/mg, which exceeded that of commercial ASNs from *E. coli* K-12 and *E. chrysanthemi* 3937. Although lower than that of ASNs from *P. carotovorum* and *T. kodakaraensis*, the specific activity of BcA was higher than that of ASNs from *B. subtilis* 168 and *B. megaterium* H-1. Additionally, the optimal temperature for enzyme activity was 50°C, similar to other bacterial ASNs, which exhibit optimal activities at between 40°C and 60°C. BcA thermal stability exceeded that of ASNs from *S. cerevisiae* BY4741, but was substantially lower than that of *T. kodakaraensis* and *B. subtilis* 168. Gaps in thermal stability might be associated with the quaternary structure of the enzymes (3). The optimal pH for BcA activity was similar to that for *T. kodakaraensis* 1656 and *E. carotovora* NCYC 1526, displaying >80% activity at a pH range of 6–11 (Fig. 3C). The K_m of BcA suggested a similar affinity for L-asparagine relative to that observed for *T. kodakaraensis* 1656 and *B. subtilis* 168; however, this was lower than that of commercially used ASNs from *E. coli* and *E. chrysanthemi*. Moreover, BcA displayed lower activity against glutamine relative to that observed in commercial ASNs from *E. coli* K-12 and *E. chrysanthemi* 3937. The findings of high specific activity and low glutamine activity of this novel BcA might reduce enzyme-dosing requirements and glutamine loss during its application in food processing. To be noted, the BcA gene was amplified from a pathogenic bacterium (*B. cereus*), which may restrict its practical application in food industry. Thus, animal experiments will be performed to eliminate its potential harm in future studies.

Results of application trials were similar to those of a previous report showing a ~70%–~97% decrease in acrylamide content in potatoes pretreated with ASNs (19,20). The results of the present study suggested that BcA represents a promising candidate for ensuring acrylamide-free food through hydrolysis of the acrylamide precursor asparagine. According to previous studies, high

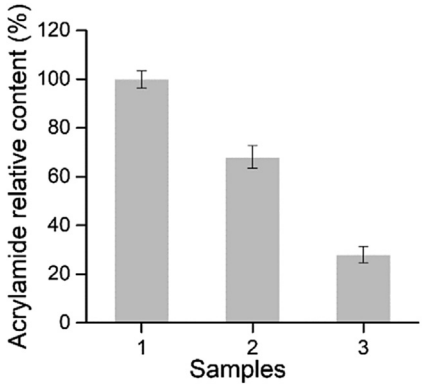

FIG. 4. Application of BcA to fried potato chips. Potato chips were pretreated as follows: 1, untreated; 2, immersion in distilled water; 3, immersion in 5000 U BcA solution. Acrylamide content was detected by LC-MS/MS. Error bars represent the average value of three parallel experiments.

TABLE 2. Comparison of the biochemical properties of selected bacterial ASNs.

Gene source	Host	Specific activity (U/mg)	Yield in flasks (U/mL)	Stability	Optimal temperature (°C)	Optimal pH	GLN/ASN (%)	K_m (mM)	ASN types	References
B. cereus BDRD -ST26 (P43-amyE-BcA)	*B. subtilis* WB600	550.8	374.9	$t_{1/2}$ (50°C): 17.35 min	50	9.0	0.4	9.38	I	This study
B. cereus BDRD -ST26 (P43-BcA)	*B. subtilis* WB600	545.3	22.4	$t_{1/2}$ (50°C): 17.57 min	50	9.0	0.4	9.41	I	This study
P. carotovorum MTCC 1428	*B. subtilis* WB800N	2020.91	105	–	40	8.5	N.D.	0.657	II	21,22
B. subtilis 168	*B. subtilis* WB600	162.9	122.4	$t_{1/2}$ (65°C): 61 min	65	–	1.58	5.3	II	15
E. coli K-12	*E. coli* BLR(DE3)	270	20.95	–	35	8.0	2	0.13	II	23
E. chrysanthemi 3937	*E. coli* BL21(DE3) pLysS	117.8	–	–	35	–	10	0.058	II	24
B. megaterium H-1	*E. coli* BL21(DE3)	146.48	4.25	–	40	7.0	0.06	0.8	II	25
S. cerevisiae BY4741	*E. coli* BL21(DE3)	196.2	–	–	40	8.6	0.38	0.075	I	11
T. kodakaraensis 1656	*E. coli* CodonPlus(DE 3)-RIL	2350	–	$t_{1/2}$ (85°C): 130 min	85	9.5	N.D.	5.5	I	6
E. carotovora NCYC 1526	*E. coli* BL21(DE3) pLysS	430	108.06	–	37	9.5	1.5	0.085	II	26

N.D., not detected; GLN/ASN, the ratio of glutamine activity to asparagine activity.

temperatures contribute to the diffusion and degradation of asparagine from potato chips, resulting in decreased acrylamide content and effective elimination of 90% acrylamide through a combination of traditional blanching and ASN immersion during potato pretreatment (19). Therefore, to expand the potential application of BcA, its thermal stability should be optimized in future work in order to promote its development for use in food industries. Additionally, after one round of application, the residual ASN in the BcA solution decreased by 44%, which might be due to the absorption of the potato spices and the low thermal stability of the enzyme. To improve the reusability of the BcA solution, further investigations will be performed on enzyme immobilization.

In conclusion, in this study, the gene of a novel ASN, *BcA*, was cloned from *B. cereus* BDRD-ST26. Based on its homodimeric structure and cytoplasmic localization, we concluded that this BcA variant represents a type I ASN. After expression in *B. subtilis* using the amyE signal peptide, the extracellular yield of BcA reached 374.9 U/mL in shake-flask cultivation, with a specific activity toward L-asparagine of 550.8 U/mg along with minimal L-glutaminase activity. Notably, the specific activity and yield of BcA were higher than that of commercial ASNs from *E. coli*, *E. chrysanthemi*, and other previously reported ASNs. Application for the elimination of acrylamide in potato chips showed that BcA pretreatment mitigated acrylamide accumulation, suggesting its potential as an additive for the production of acrylamide-free food.

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