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Cloning, Expression, and Characterization of L-Asparaginase from a Newly Isolated *Bacillus subtilis* B11−06

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ABSTRACT: This study focused on the cloning, overexpression, and characterization of the gene encoding L-asparaginase (*ansZ*) from a nonpathogenic strain of *Bacillus subtilis* B11−06. The recombinant enzyme showed high thermostability and low affinity to L-glutamine. The *ansZ* gene, encoding a putative L-asparaginase II, was amplified by PCR and expressed in *B. subtilis* 168 using the shuttle vector pMA5. The activity of the recombinant enzyme was 9.98 U/mL, which was significantly higher than that of *B. subtilis* B11−06. The recombinant enzyme was purified by a two-step procedure including ammonium sulfate fractionation and hydrophobic interaction chromatography. The optimum pH and temperature of the recombinant enzyme were 7.5 and 40 °C, respectively. The enzyme was quite stable at a pH range of 6.0−9.0 and exhibited about 14.7 and 9.0% retention of activity following 2 h incubation at 50 or 60 °C, respectively. The K_m for L-asparagine was 0.43 mM, and the V_{max} was 77.51 μM/min. Results of this study also revealed the potential industrial application of this enzyme in reducing acrylamide formation during the potato frying process.

KEYWORDS: *L-asparaginase II, cloning, expression, Bacillus subtilis, acrylamide*

INTRODUCTION

L-Asparaginase (EC 3.5.1.1; L-asparagine amidohydrolase) catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia.[1] Aspartate can then be transaminated to oxaloacetate, an intermediate in the tricarboxylic acid cycle.[2,3] L-Asparaginase exists in various organisms including animals, plant cells, yeast, fungi, and bacteria.[1,4] In 1961, Broome demonstrated that the antilymphoma activity in guinea pig sera was due to L-asparaginase.[5] In 1963, Mashburn and Wriston reported on the L-asparaginase from an *Escherichia coli* strain.[6] Several further microbial strains including *Aspergillus terreus*, *Erwinia aroideae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Withania somnifera*, and *Staphylococcus sp.* were then shown to produce L-asparaginase.[2,7,8] Campbell confirmed that there were two isoforms of L-asparaginase, L-asparaginase I and L-asparaginase II,[9] and that these two enzymes were genetically differentiated from each other. L-Asparaginases derived from different microorganisms show different properties.[3,10,11]

Because the type II enzyme can effectively deplete cytotoxicity in leukemic cells, it has been widely used for the chemotherapy of acute lymphoblastic leukemia (ALL) and shows great commercial value in the pharmaceutical sector.[12−14] However, clinical application of the enzyme is limited by antibody response and by various side effects, which are attributed to the L-glutaminase activity of this enzyme.[15,16] Thus, enzymes that are best suited for ALL treatment should have high activity, a low K_m, and a strong affinity for L-asparagine (L-Asn) over L-glutamine (L-Gln). L-Asparaginase II from *Erwinia chrysanthemi* and *Escherichia coli* has been acknowledged to best meet these criteria and is widely used in the pharmaceutical industry.[1,17,18] In recent years, L-asparaginase has been shown to reduce acrylamide formation in starchy foods.[19,20] Researchers confirmed that acrylamide formation in food was related to the Maillard reaction[21,22] and that L-Asn is the key determinant of acrylamide formation in cereal products. L-Asparaginase can hydrolyze free L-Asn to aspartic acid, and thus it can decrease acrylamide formation at the source.[22]

Previous studies on L-asparaginase gene expression in *Bacillus subtilis* focused on the mechanism of gene regulation.[11,23] To our knowledge, there is no research on the properties of the enzyme derived from a *B. subtilis* expression system. L-Asparaginase can be mass produced in *B. subtilis* in a form that is safe for use in food production.[18] In the current study, *B. subtilis* B11−06 was isolated from soil and found to produce a putative L-asparaginase. The *ansZ* gene, encoding the putative L-asparaginase II, was then cloned. Subsequently, *ansZ* was inserted into the shuttle expression vector pMA5[24] and expressed in *B. subtilis* 168.[11] The L-asparaginase II from the recombinant *B. subtilis* 168 exhibited higher activity than that of the wild-type *B. subtilis* B11−06. We then purified and characterized the recombinant protein and examined its industrial application in reducing acrylamide formation during the potato frying process.

MATERIALS AND METHODS

Strains, Plasmids, and Chemicals. *E.coli* JM109, *B. subtilis* 168, and vector pMA5 were preserved in our laboratory. The restriction enzymes, T4 DNA ligase, and ExTaq DNA polymerase were obtained from TaKaRa Bio Co. (Dalian, China). The Mini Chromosome Rapid Isolation kit, Mini Plasmid Rapid Isolation kit, and Mini DNA Rapid Purification kit were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). AKTA prime plus and the Butyl Sepharose HP column were purchased from GE Healthcare, Inc. (Little Chalfont,

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U.K.). The Shimadzu LC-20AB high-pressure liquid chromatograph was purchased from Shimadzu Co. (Kyoto, Japan). The Aminex HPX-87H Column was purchased from Bio-Rad Laboratories, Inc. (California, USA). All other chemicals were of analytical grade.

Medium and Culture Conditions. *E.coli* JM109 and *B. subtilis* 168 were grown at 37 °C in Luria–Bertani (LB) medium during the construction of strains.[1,11] Ampicillin (100 μg/mL) or kanamycin (50 μg/mL) was added to the growth medium when necessary. ADS agar (asparagine dextrose salts agar, g/L):[25] asparagine, 10; dextrose, 2; K_2HPO_4, 1; $MgSO_4$, 0.5; agar, 15, and 0.009% phenol red was used as a pH indicator. The basal medium for producing L-asparaginase contained (g/L): glucose, 20; soya peptone, 10; corn steep liquor, 15; K_2HPO_4, 2.3; $MgSO_4$, 0.75; NaCl, 5, pH 7.0. The optimized medium contained (g/L): sucrose, 35; tryptone, 15; urea, 0.8; corn steep liquor,12; K_2HPO_4, 2.612; KH_2PO_4, 2.041; $MgSO_4{\cdot}7H_2O$, 1.845; NaCl, 5; L-Asn, 1, pH 7.0. The temperature and agitation rate were 37 °C and 160 rpm, respectively, during the cultivation.

Isolation of the Bacteria. Various soil samples were collected from different places (Wuxi, China) and put in sterile polythene bags. The samples were scattered by glass beads and then serially diluted in sterile distilled water under aseptic conditions. Dilutions were plated on LB agar plates and incubated at 37 °C for 20–24 h. The individual colonies were subcultured on ADS agar. Isolates capable of producing L-asparaginase were selected for further study. The 16S rRNA of the isolate was amplified by PCR using the universal primers 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGTGATCCAGCCGCA-3′.[26]

Cloning and Construction of Recombinant Plasmid pMA5-*ansZ*. Total genomic DNA of the isolated *B. subtilis* B11–06 was extracted using a Mini Chromosome Rapid Isolation kit. The *ansZ* gene was amplified from chromosomal DNA of the *B. subtilis* B11–06 strain by PCR using *ansZ* forward (5′-GACGGATCCATGAAAAAACAACGAATGCT-3′) and reverse (5′-GGCACGCGTTTAATACTCATTGAAATAAG-3′) primers (underlined parts were the restriction sites), which were designed according to the L-asparaginase gene of *B. subtilis* subsp. *subtilis* str. 168 (accession number NC-000964) available from the National Center for Biotechnology Information (NCBI) Web site (http://www.ncbi.nlm.nih.gov/).[27] The primers annealed to the 5′- (including Start codon) and 3′-ends (including stop codon) of the *ansZ* gene, respectively. The forward primer contained a *Bam*HI restriction site, while the reverse primer contained a *Mlu*I restriction site. The resulting PCR amplicon and plasmid pMA5 were digested with *Bam*HI and *Mlu*I. The digested fragments were checked by agarose electrophoresis and purified from the gel using a Mini DNA Rapid Purification kit. The purified fragment was then ligated into linearized pMA5. The nucleotide sequence of the *ansZ* insert was analyzed by Sangon Biotech. Protein and nucleotide sequence comparisons were performed using the BLAST server available from the NCBI Web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The recombinant plasmid was transformed into chemically competent *B. subtilis* 168 cells for L-asparaginase production.

Expression of *ansZ* in *B. subtilis*. The recombinant plasmid pMA5-*ansZ* was transformed into *E. coli* JM109. The plasmid was then extracted from *E. coli* JM109 and used to transform *B. subtilis* 168 using the procedure described by Spizizen.[28] Transformants were obtained following growth overnight on selective LB medium supplemented with 50 μg/mL kanamycin. Recombinant cells were grown in the basal medium (50 mL) for 24 h. The cells were harvested by centrifugation at 10 000*g* for 10 min at 4 °C, and the supernatant was used for the L-asparaginase enzyme activity assay. The pellets were washed twice with 20 mL of 10 mM PBS buffer (pH 7.4), suspended in 50 mM Tris-HCl buffer (pH 7.5), and sonicated 10 times for 2 s with 5 s cooling intervals. Cell extracts were centrifuged for 30 min at 10 000*g* in a SIGAMA 3K-15 centrifuge (Sigma-Aldrich Co., Ltd., Shanghai, China) to remove cell debris. The supernatant was used for an enzyme activity assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (12% acrylamide), or were stored at −20 °C until further analysis.

Enzyme Activity Assay. Enzyme activities of the intracellular and extracellular parts of the protein were performed using an ammonia-sensitive electrode, which can detect dissolved ammonia accurately in aqueous solutions.[29−31] This apparatus included an ammonia electrode attached to a digital pH meter and a magnetic stirrer. The reaction mixture (25 mL) consisted of 10 mL of 50 mM Tris-HCl buffer (pH 7.5) and 10 mL of 50 mM L-Asn. Supernatant or cell extract (0.5 mL) was added, and the reaction proceeded at 37 °C for 15 min. The reaction was then terminated by addition of 5 mL of a 15% (w/v) trichloroacetic acid solution. The control tube was terminated before 15 min of incubation. The ammonium electrode was calibrated by using ammonium chloride with different concentrations. One unit of the L-asparaginase was defined as the amount of enzyme required to produce 1 μmol of ammonia per minute under assay conditions. To evaluate the accuracy of this method, L-asparaginase activity was also detected by the Nessler assay using Nessler's reagent.[3,4] Protein concentration was determined by the Bradford method with bovine serum albumin as the standard.[32]

Enzyme Purification. The fermentation broth was centrifuged at 10 000*g* for 15 min at 4 °C. The supernatant was collected and precipitated using 80% (w/v) ammonium sulfate. The precipitate was then dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 1.5 M ammonium sulfate. The dissolved enzyme was loaded onto a Butyl Sepharose HP column equilibrated with the same buffer. Nonabsorbed proteins were washed off thoroughly with the buffer, and the enzyme was eluted in a linear gradient of 1.5–0.0 mM ammonium sulfate with a flow rate of 1.0 mL/min. The active fractions were collected and dialyzed for an L-asparaginase enzyme assay or were stored at −20 °C until further analysis.

Enzyme Characterization. The optimum pH was confirmed in several buffers (pH 3.0–6.5, 0.05 M citrate–sodium citrate buffer; pH 6.5–9.0, 0.05 M Tris-HCl buffer; pH 9.0–10.0, 0.05 M borax–sodium hydroxide buffer), while the optimum temperature was examined in 50 mM Tris-HCl buffer (pH 7.5) using the standard reaction mixture. The pH and thermal stability of L-asparaginase was determined by incubation at different pH levels at 4 °C for 24 h and at various temperatures for 10 h. Effects of various compounds on enzyme activity were examined in the standard reaction mixture supplemented with various cations and EDTA at concentrations of 1 mM. The enzyme activity of the recombinant L-asparaginase without the addition of metal ions or EDTA was used as the control. Kinetic parameters were performed in 50 mM Tris-HCl buffer (pH 7.5) at 40 °C by changing the concentration of the substrate L-Asn and L-Gln. Eadie–Hofstee plots were used to calculate kinetic parameters K_m and V_{max} according to the enzyme reactions.[33]

Raw Potato Preparation and Frying Condition. Potatoes (Fovorita, moisture content of 74 g/100 g) were washed, peeled, and then sliced (thickness of 2.0 mm) using a slicing machine. All the potato slices were immersed in distilled water for 2 min to remove the starch on the surface. Three samples were pretreated prior to frying, as follows: (i) without any processing; (ii) immersion in distilled water for 30 min; (iii) immersion in an 8000 U/L L-asparaginase solution for 30 min. All samples were fried at 160 °C for 10 min in an electric fryer. After frying, the potato slices were dried on paper, cooled to ambient temperature, and then the acrylamide was extracted for analysis.[34] The acrylamide content of the fried potato slices purchased from the local supermarket were also analyzed.

Acrylamide Determination. Acrylamide was analyzed by high-performance liquid chromatography (HPLC) with ultraviolet detection at 200 nm, as described previously.[34,35] Briefly, 1 g of a sliced potato sample was crushed by a lab mixer homogenizer and was homogenized with 1 mL of distilled water. Then, 2 mL of distilled water was added, and the homogenates were incubated in a water bath at 70 °C with agitation for 30 min. The homogenates were then centrifuged at 10 000*g* for 30 min and were filtered through a 0.45 μm microporous membrane. The filtrates were transferred to a 10.0 mL volumetric flask and diluted to mark with distilled water. Hexane (3 mL) was added to 1 mL of the sample solution to extract long-chain fatty acids. The mixture was shaken thoroughly, and the upper layer was removed with a pipet. The lower aqueous layer was transferred into plastic vials (Zhejiang Aijiyen Technology Co., Ltd., Quzhou, China) for autosampler analysis. Sulfuric acid solution (0.01 M) was

used as mobile phase. Samples were then run on an Aminex HPX-87H (300 mm × 7.8 mm) column with a temperature of 30 °C, flow rate of 0.5 mL/min, and a sample size of 20 μL, while ultraviolet detection was maintained at 200 nm. The experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Isolation of the Bacteria. Four L-asparaginase-producing strains were isolated from the soil samples. Their enzyme activities were assayed by an ammonia-sensitive electrode. Dissolved ammonia, which is produced by the catalysis of L-asparaginase, was measured using the ammonia-sensitive electrode, and the maximum enzyme activity of these strains was 0.039 U/mL. The strain with the greatest activity was Gram-positive. Sequencing of the 16S rRNA gene revealed that the isolated strain showed 99% similarity to the same region in *B. subtilis*. The strain was named *B. subtilis* B11−06, and the sequence was submitted to GenBank with accession number KF028775.

Cloning and Construction of Recombinant Plasmid pMA5-*ansZ*. The *ansZ* gene was successfully amplified from the genomic DNA of the isolated *B. subtilis* B11−06 strain by PCR. Electrophoresis revealed that the amplicon consisted of 1128 bp (Figure 1A), and BLAST analysis showed that the gene had a high level of similarity to that of several *ansZ*-containing strains published in the NCBI database, including *B. subtilis* BEST7003 (AP012496.1), *B. subtilis* subsp. *subtilis* str. 168 (AL009126.3), *B. subtilis* QB928 (CP003783.1), and *B. subtilis* BSn5 (CP002468.1), with similarities of 100%, 100%, 100%, and 99%, respectively. The predicted amino acid sequence of the cloned *ansZ* gene shared 100% similarity with *B. subtilis* 168, and the gene sequence was submitted to GenBank with accession number KF444946. The digested PCR amplicon was cloned into the expression vector pMA5 under the control of the *Hpa*II promoter, resulting in recombinant vector pMA5-*ansZ* (Figure 1C). The recombinant plasmid was digested with *Bam*HI and *Mlu*I to produce two major fragments with sizes of 1128 and 7500 bp (Figure 1B). The result revealed successful construction of the recombinant plasmid.

Expression of the Recombinant Vector in *B. subtilis*. The recombinant plasmid pMA5-*ansZ* was subsequently transformed into the expression host *B. subtilis* 168 to construct the recombinant strain *B. subtilis* 168/pMA5-*ansZ*, which expressed *ansZ* under the control of the *Hpa*II promoter. Recombinant *B. subtilis* was selected with kanamycin and verified by DNA sequencing. The positive clones showed intracellular and extracellular enzyme activities of 4.28 and 5.70 U/mL, respectively. The total enzyme activity was substantially higher than that of the native enzyme producer *B. subtilis* B11−06.

The one-factor-at-a-time and orthogonal array design methods were used for medium optimization.[36,37] The one-factor-at-a-time method was used to investigate the effects of carbon sources, nitrogen sources, inorganic salt, and corn steep liquor on L-asparaginase production. Of the carbon sources, sucrose at a concentration of 35 g/L was most suitable for L-asparaginase production. Of the nitrogen sources, tryptone and urea were conducive to enzyme production at concentrations of 15 and 1 g/L, respectively. Corn steep liquor was used as a growth factor to stimulate cell growth and improve enzyme activity. Subsequently, the concentrations of sucrose, tryptone, urea, and corn steep liquor were optimized by the orthogonal array method. The order of factor effects on L-asparaginase production was found to be corn steep liquor > urea > tryptone > sucrose. Thus, the optimal medium composition (g/L) after one-factor-at-a-time and orthogonal array design methods were applied was determined to be sucrose, 35; tryptone, 15; urea, 0.8; corn steep liquor, 12; K_2HPO_4, 2.612; KH_2PO_4, 2.041; $MgSO_4.7H_2O$, 1.845; NaCl, 5; L-Asn, 1. As a result, the highest enzyme activity reached 89.48 U/mL in a 5 L fermenter, which was approximately 8.9-fold higher than the activity in the basal medium. There are many reports on L-asparaginase production by recombinant strains. Hatanaka et al. and Eisele et al. cloned the *Streptomyces* and *Flammulina velutipes* L-asparaginase genes and expressed them in *E. coli*, resulting in enzyme activities of 23.02 and 16 U/mL, respectively.[38,39] Gilbert cloned the *E. chrysanthemi* asparaginase gene and transformed it into *E. coli* and *Erwinia. carotovora*, with enzyme activities of 49 and 20.2 U/mL, respectively.[40] To the best of our knowledge, the L-asparaginase-producing system described in the current study represents the highest level activity of a recombinant strain. Furthermore, the system is safe, economically viable, and easy

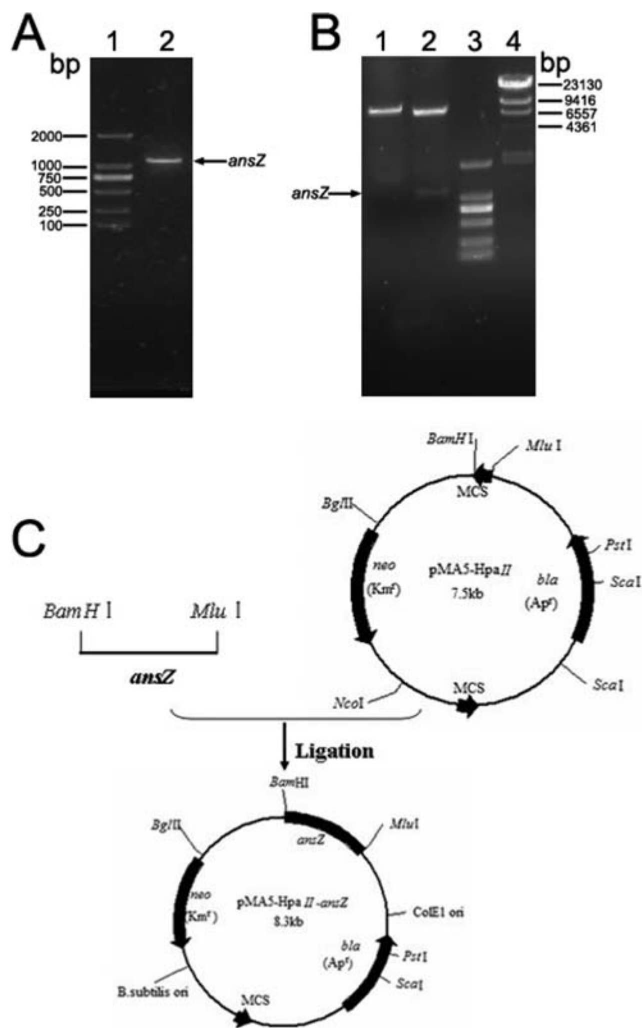

Figure 1. PCR and construction of recombinant vector pMA5-*ansZ*. (A) Lane 1, DL2000 DNA marker; lane 2, *ansZ* PCR product. (B) Lane 1, recombinant vector digested with *Bam*HI; lane 2, recombinant vector digested with *Bam*HI and *Mlu*I; lane 3, DL2000 DNA marker; lane 4, λ Hind III DNA marker. (C) Ligation of the *ansZ* gene with shuttle vector pMA5. The *ansZ* gene predigested with *Bam*HI and *Mlu*I was ligated into the *Bam*HI and *Mlu*I sites of the pMA5 vector.

to scale up. Therefore, the recombinant strain is suitable for large-scale production of L-asparaginase.

Enzyme Assay. The enzyme activity was tested using an ammonia electrode. The precision of this method was investigated by analysis of repeated measurements of L-asparaginase activity, and the mean enzyme activity was 5.70 U/mL in fermentation broth, with a standard deviation of 0.14. The accuracy of the ammonia electrode method was checked by simultaneously determining the L-asparaginase activity with an ammonia electrode and the Nessler method (Table 1). The mean value determined by the two methods was similar, but the Nessler assay was less reproducible than the ammonia electrode. In addition, the Nessler reagent is toxic and has inferior storage stability. Therefore, the ammonia electrode method was safe, accurate, and simple to perform compared to the toxity, complexity, and poor reproducibility of the traditional Nessler assay.

Table 1. Enzyme Assay by Ammonia Electrode and Nessler Assay

	Nessler assay	ammonia electrode
sample	average activity (U/mL)	average activity (U/mL)
one	10.01 ± 0.83	10.30 ± 0.30
two	13.33 ± 1.04	13.49 ± 0.46
three	11.21 ± 0.87	11.89 ± 0.27

Purification of the Enzyme. The recombinant L-asparaginase was purified to homogeneity by a two-step procedure, with a yield of 26.05% (Table 2). As a result, the purified enzyme showed a specific activity of 92.45 U/mg, which was 3.88 times higher than the crude enzyme from supernatant from the fermentation broth. A 12% SDS-PAGE analysis revealed that the subunit of the enzyme had a molecular mass of about 38 kDa when the gel was stained with CBB R-250 (Figure 2).

Table 2. Purification of Recombinant L-Asparaginase

purification stage	total protein (mg)	total activity (U)	specific activity (U/mg)	recovery (%)	fold purification
fermentation supernatant	34.20	816	23.85	100	1
ammonium sulfate precipitation	10.75	505.90	47.06	61.99	1.97
butyl Sepharose HP	2.30	212.60	92.45	26.05	3.88

Characterization of the Enzyme. The optimum pH of L-asparaginase for hydrolyzing L-Asn was pH 7.5 (Figure 3A). It was different from that of the ErA II, which had an optimum pH of 9.5, but was more similar to that of EcA II (pH 7).[42,43] The enzyme showed maximum activity at 40 °C (Figure 3B), which was similar to other reported bacterial sources.[4,12,39] The recombinant enzyme was stable at pH 6.0−9.0 (Figure 4A). More than 86% of the maximum enzyme activity was retained after incubation at pH 8.0 for 24 h. The enzyme exhibited high stability at −20 and 0 °C with only a small decrease in activity after incubation for 10 h (Figure 4B). It retained about 14.7% of its residual activity after 2 h incubation in 50 °C and showed about 9.0% retention of activity after 2 h incubation in 60 °C. The thermostability was higher than that of YpA[41] and RrA.[42] Differences in thermostability may relate to the hydrogen bonds of the enzyme spatial structures. The effects of ions and EDTA on L-asparaginase were examined (Figure 5), with the results showing that most metal ions slightly inhibited enzyme activity; however, strong inhibition was observed in the presence of Fe^{3+}. The addition of EDTA had no effect on

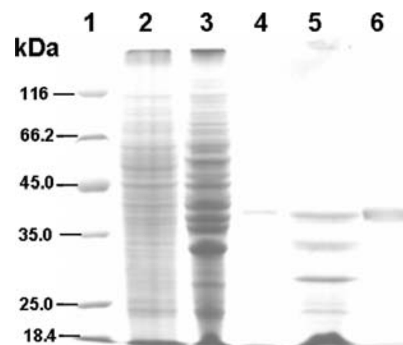

Figure 2. SDS-PAGE analysis of recombinant L-asparaginase. Lane 1, protein marker; lane 2, *B. subtilis* 168 cell extract; lane 3, *B. subtilis* 168/pMA5-*ansZ* cell extract; lane 4, the fermentation supernatant from *B. subtilis* 168/pMA5-*ansZ*; lane 5, ammonium sulfate precipitation; lane 6, butyl Sepharose HP.

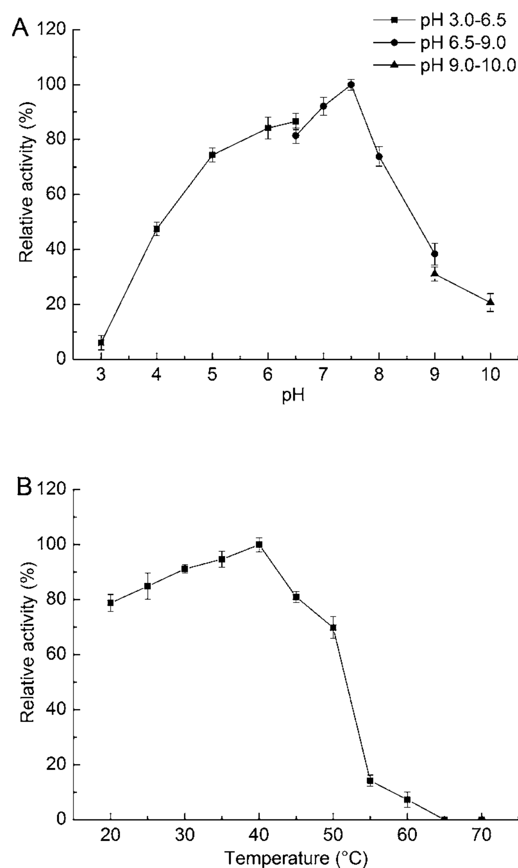

Figure 3. Effects of pH (A) and temperature (B) on recombinant L-asparaginase activity. (A) Optimal pH was determined by assaying the enzyme at various pHs (pH 3.0−6.5, 0.05 M citrate−sodium citrate buffer; pH 6.5−9.0, 0.05 M Tris-HCl buffer; pH 9.0−10.0, 0.05 M borax−sodium hydroxide buffer), at a temperature of 37 °C. (B) Influence of temperature on enzyme activity was investigated by assaying the activity from 20−70 °C in 0.05 M Tris-HCl buffer (pH 7.5).

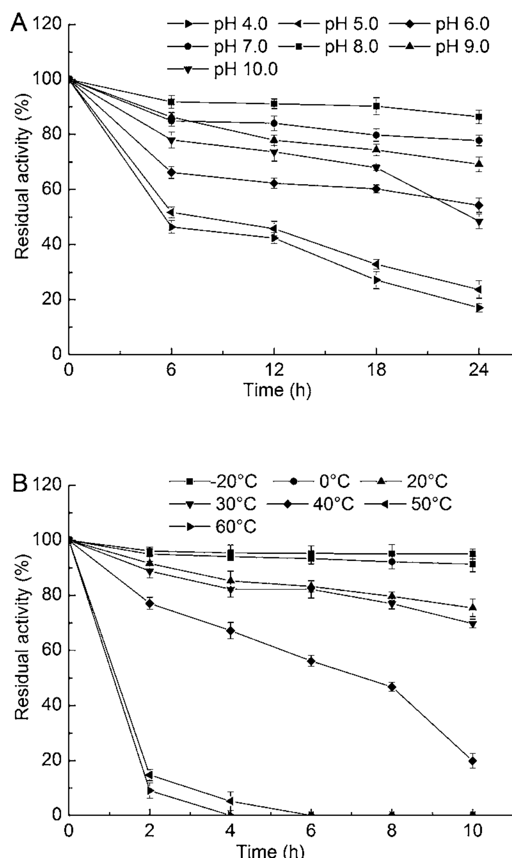

Figure 4. Effects of pH (A) and temperature (B) on recombinant L-asparaginase stability. (A) Influence of pH on stability of the enzyme was determined by incubating at different pH conditions at 4 °C for a certain time and assaying the residual activities. (B) Thermostability of the enzyme was studied by incubating at different temperatures for a certain time in pH 7.5 and assaying the residual activities.

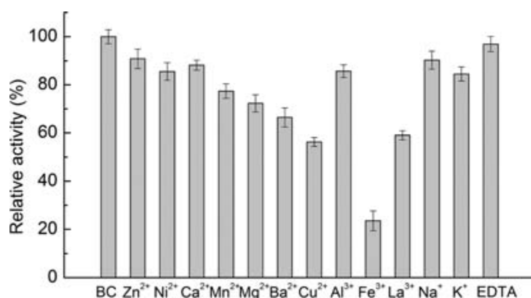

Figure 5. Effects of different metal ions and EDTA at the same concentration (1 mM) on L-asparaginase activity (BC represents blank control: no metal ion or EDTA).

recombinant enzyme activity. This was similar to findings for BsA I, BsA II, and FvNase.[17,18,39]

The K_m and V_{max} values were determined by nonlinear fit analysis based on Eadie–Hofstee plots. The recombinant enzyme hydrolyzed L-Asn with a Michaelis constant K_m value of 0.43 mM (Figure 6A) and a maximum reaction rate V_{max} of 77.51 μM/min. The K_m value showed that the affinity of this recombinant enzyme for L-Asn was slightly lower than that of EcA II and ErA II,[42,43] but it was higher than that of BsA I and BsA II.[17,18] Moreover, the enzyme exhibited very low affinity for L-Gln. The L-glutaminase activity assayed by the ammonia electrode was only 1.3% of that of the L-asparaginase activity. It hydrolyzed L-Gln with a Michaelis constant K_m value of 43.06 mM (Figure 6B). The undesirable side effects of the protein in clinical use have been attributed to the glutaminase activity of the enzyme. Gln is an amino group donor for various biochemical reactions and also the major transport form of amino nitrogen in the blood. L-Asparaginase with high glutaminase activity will decrease the plasma Gln levels, therefore impairing many biochemical functions.[15,16] As a consequence, the recombinant enzyme may be used in the pharmaceutical industry.

Application of L-Asparaginase in Potato Samples. HPLC was used to accurately detect the acrylamide in the samples (Figure 7). After frying, the sample without any

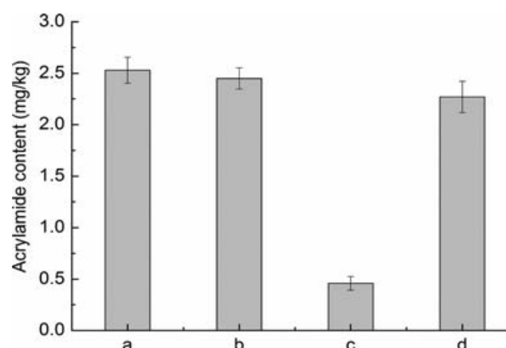

Figure 7. Acrylamide content of potato samples subjected to different treatments prior to frying. All samples, except those obtained from the supermarket, were fried at 160 °C for 10 min in an electric fryer. (a) Raw potato without any processing; (b) Raw potato immersed in distilled water for 30 min; (c) Raw potato immersed in an 8000 U/L L-asparaginase solution for 30 min; (d) Potato slices purchased from the local supermarket.

processing contained 2.53 mg/kg of acrylamide (Figure 7a). Because of the high percentage of reducing sugars and L-Asn in the raw potato, a high level of acrylamide was produced during the frying process. The sample treated with distilled water

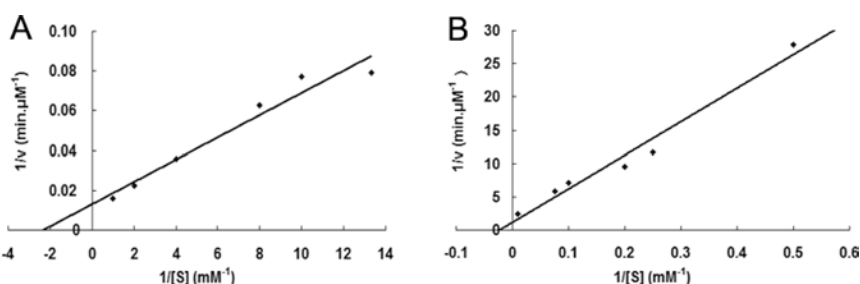

Figure 6. Lineweaver–Burk plots for L-Asn (A) and L-Gln (B).

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showed almost the same acrylamide content, with a level of 2.45 mg/kg. However, the sample immersed in 8000 U/L L-asparaginase solution contained only 0.46 mg/kg acrylamide, corresponding to a decrease in the acrylamide level of about 82% (Figure 7c). This reduction in acrylamide was due to the decrease of L-Asn, which is catalyzed by L-asparaginase. The sample obtained from the supermarket had an acrylamide content of 2.27 mg/kg (Figure 7d), showing that acrylamide exists in fried potato slices. Paleologos et al. detected acrylamide in some food samples by normal-phase HPLC.[35] Pedreschi reported that commercial L-asparaginase could diminish the acrylamide content from 2047 to 158 μg/kg.[20] Kukurova et al. found that the acrylamide level in wheat-based dough was reduced up to 90% by addition of L-asparaginase to the dough.[19] Results of the current study revealed that acrylamide was formed in potatoes during frying and that L-asparaginase could reduce the formation of acrylamide.

In this study, *ansZ*, encoding a type II L-asparaginase, from the *B. subtilis* B11−06 strain was successfully cloned and expressed in *B. subtilis* 168. The enzyme activity was notably higher than the wild-type strain. The recombinant enzyme was then purified, and the enzymatic properties of L-asparaginase from *B. subtilis* B11−06 were investigated. The recombinant enzyme revealed sufficiently high thermal stability for use in the food industry. The application of this enzyme in potato processing proved that the recombinant L-asparaginase could reduce the amount of acrylamide formed during the frying process. This is the first report of the construction of an *ansZ* expression system in *B. subtilis* coupled with investigation of its enzymatic properties, as previous studies have only focused on gene regulation.[11,23] *B. subtilis* is regarded as safe and is already widely used in the food industry. L-Asparaginase II also has a wide range of applications in the pharmaceutical industry. Further research will focus on enhancing the thermostability of the recombinant L-asparaginase by genetic modification. In conclusion, this paper provided a basis for the industry application of recombinant L-asparaginase II.

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Notes

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ABBREVIATIONS USED

ADS, asparagine dextrose salts agar; ALL, acute lymphoblastic leukemia; *ansZ*, L-Asparaginase gene from *B. subtilis*; *Bam*HI, type II Endonuclease from *Bacillus amyloliquefaciens* H; BsA I, *Bacillus subtilis* L-asparaginase I; BsA II, *Bacillus subtilis* L-asparaginase II; CBB R-250, Coomassie Brilliant Blue R-250; EcA II, *Escherichia coli* L-asparaginase II; EDTA, ethylenediaminetetraacetic acid; ErA II, *Erwinia chrysanthemi* L-asparaginase II; Fvnase, *Flammulina velutipes* asparaginase; HPLC, high performance liquid chromatography; L-Asn, L-asparagine; LB, Luria−Bertani broth; L-Gln, L-glutamine; M, mol/L; mM, mmol/L; *Mlu*I, Endonuclease from *Micrococcus luteus*; NCBI, National Center for Biotechnology Information; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RrA, *Rhodospirillum rubrum* L-asparaginase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamid gel electrophoresis; YpA, *Yersinia pseudotuberculosis* L-asparaginase

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