Journal of Microbiology (2013) Vol. 51, No. 2, pp. 222-228
DOI 10.1007/s12275-013-2669-9

Expression, Purification, and Biochemical Properties of Arginase from *Bacillus subtilis* 168

Jin-Ju Yu[1], Ki-Bum Park[1,2], Su-Gon Kim[1], and Suk-Heung Oh[1]*

[1]*Department of Food and Biotechnology, Woosuk University, Jeonju 565-701, Republic of Korea*
[2]*Research and Development Center, WiniaMando Inc., Asan 336-843, Republic of Korea*

(Received December 3, 2012 / Accepted April 1, 2013)

The arginine-degrading and ornithine-producing enzymes arginase has been used to treat arginine-dependent cancers. This study was carried out to obtain the microbial arginase from *Bacillus subtilis*, one of major microorganisms found in fermented foods such as *Cheonggukjang*. The gene encoding arginase was isolated from *B. subtilis* 168 and cloned into *E. coli* expression plasmid pET32a. The enzyme activity was detected in the supernatant of the transformed and IPTG induced cell-extract. Arginase was purified for homogeneity from the supernatant by affinity chromatography. The specific activity of the purified arginase was 150 U/mg protein. SDS-PAGE analysis revealed the molecular size to be 49 kDa (Trix·Tag, 6×His·Tag added size). The optimum pH and temperature of the purified enzyme with arginine as the substrate were pH 8.4 and 45°C, respectively. The K_m and V_{max} values of arginine for the enzyme were 4.6 mM and 133.0 mM/min/mg protein respectively. These findings can contribute in the development of functional fermented foods such as *Cheonggukjang* with an enhanced level of ornithine and pharmaceutical products by providing the key enzyme in arginine-degradation and ornithine-production.

Keywords: expression, purification, arginase, *B. subtilis*, ornithine

Introduction

Arginase (L-arginine amidinohydrolases, E.C. 3.5.3.1) is an enzyme that catalyzes the hydrolysis of arginine into ornithine and urea (Bewley *et al.*, 1996). Such reactions occur mostly in the urea cycle of mammals and the arginine catabolism of aerobic microbes (Bewley *et al.*, 1996). In mammals, arginase depletes the arginine levels by hydrolyzing arginine into ornithine and many studies claim that this helps to inhibit the growth of various cancer cells (Rijin *et al.*, 2003; Cheng *et al.*, 2007; Tsui *et al.*, 2009; Hsueh *et al.*, 2012). For example, recombinant human arginase inhibits the proliferation of human hepatocellular carcinoma (Cheng *et al.*, 2007) and human prostate cancer cells (Hsueh *et al.*, 2012) through arginine depletion. Moreover, arginase influences the level of arginine and ornithine within the cell and therefore, involved in the synthesis of glutamate and proline, as well as synthesis of nitric acid in macrophages (Hwang *et al.*, 2001). Arginase, however, takes on a different role in plants as its main function is nitrogen fixation, which is a crucial step in fruit ripening (Todd *et al.*, 2001).

Two types of arginine catabolism reactions in microbes have been reported: the synthesis of ornithine through the arginase-urease pathway (Kuensch *et al.*, 1974) and the synthesis of ornithine through arginine deiminase (ADI) pathway (Arena *et al.*, 1999; Spano *et al.*, 2004). The arginase-urease pathway results in the synthesis of ornithine and urea, which are the same products as in the urea cycle of mammals, while the ADI pathway is a metabolic reaction that forms citrulline, ornithine, and ammonia. Arginine metabolism in lactic acid bacteria (LAB), such as those found in wine and dairy fermentation, are reported to form ornithine, NH_3 and CO_2 through the ADI pathway (Arena *et al.*, 1999; Liu *et al.*, 2003). The LABs with ADI pathway are *Lactobacillus plantarum*, *Lactobacillus hilgardii*, *Leuconostoc oeni*, *Pediococcus pentosaceus*, *Lactobacillus buchneri*, *Lactococcus lactis*, and *Weissella koreensis* (Arena *et al.*, 1999; Liu *et al.*, 2003; Spano *et al.*, 2004; Kim *et al.*, 2009; Yu and Oh, 2010). Microbes that are known for using arginase-urease pathway includes *Bacillus subtilis*, *Helicobacter pylori*, *Saccharomyces cerevisiae*, and *Neurospora crassa* (Borkovich and Weiss, 1987; Green *et al.*, 1990; Bewley *et al.*, 1996; Kanda *et al.*, 1997; Konst *et al.*, 2010).

Bacillus subtilis is one of dominant bacteria in *Cheonggukjang* and Natto, the traditional fermented soybean foods very popular in Korea and in Japan, respectively (Wang *et al.*, 2009). Nattokinase (NK) is a microbial fibrinolytic enzyme originally purified from natto and its effectiveness in thrombolysis *in vivo* have been characterized (Peng *et al.*, 2005; Yoshinori *et al.*, 2005; Wang *et al.*, 2009). Thus, microbial fibrinolytic enzymes such as nattokinases have been postulated to be used as functional food additives (Peng *et al.*, 2005). By contrast, arginases from food-grade microorganisms of Natto and *Cheonggukjang*, are believed to have not been used as a dietary supplement. Thus, characterization of arginases from microorganisms found in fermented foods such as *Cheonggukjang* is necessary for the application of the enzyme as functional food additives.

Previously, arginase from cells of *B. subtilis* KY3281 was purified in a crystalline form using the conventional methods such as ammonium sulfate precipitation, ion-exchange, and

*For correspondence. E-mail: shoh@woosuk.ac.kr; Tel.: +82-63-290-1433; Fax: +82-63-290-1429

gel-filtration chramatography, etc (Nakamura *et al*., 1973). Recently, Konst *et al*. (2010) reported the stabilization and immobilization of *B. subtilis* arginase to overcome the difficulties in selectively splitting L-arginine into L-ornithine through chemical reactions. The arginase used by Konst *et al*. (2010) was the *B. subtilis* arginase stock solution (Megazyme International Ireland Ltd.), which consisted of 38 kDa subunits according to SDS-PAGE analysis. However, arginase from the microorganism of *B. subtilis* 168 has neither been purified by expressing the gene for cloned arginase nor characterized.

In the present study, we report the cloning and expression of the microbial arginase from *B. subtilis* 168 in *E. coli*. We also describe the purification and characterization of the arginase to facilitate further functional studies and nutraceutical applications of this enzyme.

Materials and Methods

Bacterial strains, growth conditions, and plasmid

E. coli DH5α [*supE*44, Δ*lac*U169(φ80*lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1] was used for T vector cloning and BL21(DE3) [F–*omp*T *hsd*SB(rB–, mB–) *gal dcm* (DE3)] was used for protein expression of arginase from *B. subtilis* 168. The *E. coli* culture was grown in LB medium with or without ampicillin (50 µg/ml) at 37°C. The initial cloning was done through pGEM T-vector (Promega Co., USA), while the expression vector used the pET32a(+) vector (Novogen, USA) which includes S·Tag (Internal tag), Trix·Tag (N-terminal tag), and 6×His·Tag (Internal, C-terminal tag).

B. subtilis arginase gene (BSA) cloning

The cloning of arginase gene (BSA) in *B. subtilis* 168 for PCR amplification was conducted by utilizing PCR primers 5′-GGTACCATGGATAAAACGATTTCGG-3′ (*Kpn*I site underlined, forward) and 5′-AAGCTTTTACAGCAGCTTCT TCCC-3′ (*Hin*dIII site underlined, reverse) (NCBI accession number NP-391912.1). PCR amplification was performed with a Biometra thermocycler (Tampa, USA) for 30 cycles using amplification mixture contained 100 ng of genomic DNA, 0.5 µM of primer DNA, 0.2 mM dNTPs, 10× Ex Taq buffer solution, and Taq polymerase (TaKaRa Bio Inc., Japan) 0.025 U/µl. PCR conditions were set at 30 sec of denaturing at 95°C, 30 sec of annealing at 58°C, and 1.5 min of extension at 72°C. The ligation of final product was conducted through the pGEM T-easy vector (Promega Co.) using T4 ligase. The DH5α strain was transformed by incubating with the pGEM T-easy vector. The *B. subtilis* 168 gene encoding for arginase was obtained from the pGEM T-easy vector by using *Kpn*I (10 U) and *Hin*dIII (50 U) at 37°C for 2.5 h. The resulting arginase gene of *B. subtilis* 168 pronounced as BSA throughout this experiment.

Expression of pET32a-BSA/BL21(DE3)

The *Kpn*I and *Hin*dIII-digested 900 bp BSA gene was inserted into the 6,000 bp pET32a (+) vector using TaKaRa's Mix ligation kit (TaKaRa Bio Inc.) the ratio of insert: vector= 5:1 (mole). After the ligation reaction (4°C, overnight), the BL21(DE3) *E. coli* was incubated with the ligation mixture in the heating block at 42°C for 10 min and then incubated in a LB media (pH 7.4, Difco, USA) at 37°C for 1 h to obtain the transformed BL21(DE3) colony. The resulting pET32a-BSA plasmid was digested by incubating at 37°C for 2.5 h with *Kpn*I (0.5 µl, 10 U) and *Hin*dIII (0.1 µl, 50 U). The *E. coli* containing recombinant plasmid was named as pET32a-BSA/BL21(DE3).

The pET32a-BSA/BL21(DE3) was incubated in LB (pH 7.4) with 50 µg/ml of ampicillin, while the control group BL21 (DE3) was incubated in LB (pH 7.4) (37°C, overnight) to obtain the seed cultures. 1% (v/v) pET32a-BSA/BL21(DE3) culture was introduced to the LB (pH 7.4) added with 50 µg/ml of ampicillin and the control group BL21(DE3) in LB (pH 7.4), and incubated (37°C, 220 rpm) for 2–2.5 h until the optical density reached a value of 0.4–0.5 at 600 nm. At the optical density points, isopropyl-beta-d-thiogalactopyranoside (IPTG, final concentration 1 mM, Sigma, USA) was added and further cultured to induce the BSA protein synthesis (16°C, 100 rpm, 12–16 h).

Crude extract of pET32a-BSA/BL21(DE3)

The transformed [pET32a-BSA/BL21(DE3)] culture was centrifuged (12,000×g, 4°C, 20 min) and pellets were collected. The resulting pellets were dissolved in lysis buffer (20 mM sodium phosphate buffer, 1% Triton X-100, pH 7.0) at a ratio of 1:9 (w/v), after which 200 µg/ml of lysozyme was introduced for a lysis reaction for 30 min at 37°C. Following the lysis, 1 mM of phenylmethylsulfonyl fluoride (PMSF) was added and the solution was left on ice for 5 min, after which it was homogenized (T10 basic, IKA-Werke GmbH & Co., Germany) at 3,400×g for 1 min (repeated 10 times), followed by centrifugation (12,000×g, 4°C, 20 min), the supernatant was collected in a fresh tube and was used as an extract solution (Kanda *et al*., 1997). The quantity of protein was determined by the Bradford method (Bradford, 1976) using the Bio-Rad protein assay reagent (Bio-Rad, USA). The *E. coli* BL21(DE3) carrying no plasmids and cultured in the absence of IPTG was labeled as B sample, while the *E. coli* BL21(DE3) carrying no plasmids cultured in the presence of IPTG was labeled as BI sample, the pET32a-BSA/BL21(DE3) extract solution which was not induced for expression was called the UIC (uninduced transformed crude extract) sample, and the pET32a-BSA/BL21(DE3) extract induced by IPTG for expression was called the TIC (transformed induced crude extract) sample.

Purification of pET32a-BSA/BL21(DE3) arginase

TIC sample was used for the purification of arginase with affinity chromatography. The column used was the polypropylene, which was the poly-prep chromatography column (0.8×4×9 cm, pore sizes 30–35 µm, Bio-Rad) and the resin utilized was the Ni^{2+} resin (BroBond^{TM} resin, Invitrogen, USA) which had an average bead size of 45–165 µm. The purification was conducted in 3 phases, at a constant temperature of 4°C. The solution obtained in the first phase of purification was termed the flow through fraction (FF) and the second phase of purification was termed the washed

fraction (WF), the third phase was the eluted fraction (EF), and the final purified concentrate (TP, transformed purified protein or PC, purified concentrate).

The purification method involved adding 2 ml of resin in the column for 10 min of equilibration. After eliminating ethanol within the resin, a binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) was added and the fraction was removed. About 1 ml of the TIC sample was applied into the column and processed for 1 h to yield the FF (phase 1, binding step), and 4 ml of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) was added to yield the WF (phase 2, washing step). About 4 ml of the elution buffer 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) was added into the final phase and processed for 30 sec to yield the EF (phase 3, elution step) (Viator *et al.*, 2008). The resulting FF, WF, and EF were subjected to protein quantification using the Bradford assay and separation using 15% (w/v) SDS-PAGE. The fractions confirmed with 15% (w/v) SDS-PAGE to have single band were combined and concentrated using Amicon Ultra-0.5 centrifugal filiter devices (30,000 Da, cut-off, Amicon, Milllipore, Ireland) to yield the TP.

Arginase enzyme activity assay

To measure arginase activity in the UIC, TIC, and TP samples, 100 µl of arginine substrate buffer (20 mM Tris-HCl, 1 mM $MnCl_2$, 40 mM arginine, pH 8.4) was mixed with 7.50–7.58 µg protein of the UIC, TIC, and TP samples and incubated for 50 min at 45°C. A quantitative and qualitative analysis by TLC and HPLC were conducted to measure the extent of arginase activity in converting arginine into ornithine (Hwang *et al.*, 2001; McGee *et al.*, 2004; Yu and Oh, 2010). One unit of arginase activity was defined as the amount of enzyme that liberated 1 mM ornithine per min at pH 8.4 and 45°C. TLC was conducted by utilizing silica gel F_{254} (Merck, Germany), standard ornithine (Merck), standard arginine (Sigma), and mobile phase (butanol: acetic acid: dichoromethanol: water = 5:3:3:3) (Yu and Oh, 2010). The HPLC (Waters Co., USA) analysis of ornithine and arginine level were performed by first passing the samples through a 0.45-µm PVDF filter (Millipore, USA), derivatazing with an 6-aminoquioly-N-hydroxysuccinimidyl carbonate (AQC), and separating the derivatives with a 3.9× 150 mm AccQ·Tag™ (Nova-Pak™C18, Waters Co.) column. Ornithine content was calculated by refering to the standard curve from ornithine HPLC analysis (Yu and Oh, 2010).

K_m and V_{max} analysis

The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) of TP based on different substrate concentrations were determined using Eadie-Hofstee plots (Narimatsu *et al.*, 2009), where the TP conversion of 0.83–33.3 mM of arginine into ornithine was measured. The TP sample was combined with Tris-HCl buffer (pH 8.4) and 1 mM $MnCl_2$ to prepare solutions with substrate concentrations ranging from 0.83–33.3 mM and incubated for 50 min at 45°C, and then boiled for 10 min to suspend all enzyme reactions. The K_m and V_{max} values were calculated by measuring the ornithine levels synthesized per min from the substrate through TLC and HPLC analysis.

Effect of pH and temperature on arginase activity

To measure the optimal pH for arginase activity, buffered solutions ranging from pH 3.4–12.4 were prepared. The solutions were 50 mM citrate buffer (pH 3.3–4.4), 50 mM acetate buffer (pH 5.4), 50 mM sodium phosphate buffer (pH 6.4–7.4), 50 mM Tris-HCl buffer (pH 8.4), 50 mM glycine-NaOH buffer (pH 9.4), 50 mM sodium bicarbonate buffer (pH 10.4–11.4), and 50 mM KCl-NaOH buffer (pH 12.4) (Hwang *et al.*, 2001). The buffer solutions were added to 40 mM arginine and 1 mM $MnCl_2$. Each buffer solution (100 µl) and TP 1.5 µg (2.4 µl) were combined and incubated for 50 min at 45°C, after which the solution was placed in boiling water for 5 min to suspend all enzyme activities. The resulting ornithine contents from the reactions were anayzed by TLC and HPLC. To determine the optimal temperature of enzyme activity, the substrate buffer (pH 8.4) and enzyme were incubated for 50 min at various temperatures ranging from 10–90°C and the ornithine synthesized were determined by TLC and HPLC analysis.

Results and Discussion

BSA selection and vector cloning

The arginase gene (BSA) was obtained through PCR amplification of genomic DNA of *B. subtilis* 168. The resulting BSA gene (Fig. 1A) was purified using a PCR purification kit, cloned into a pGEM T-easy vector, and the pGEM-BSA plasmid was digested with restriction enzymes *Kpn*I and *Hin*dIII to obtain the sample used in this study. The pET32a digested with restriction enzymes *Kpn*I and *Hin*dIII was ligated with the BSA to obtain recombinant plasmid pET32a-BSA. The recombination of pET32a with the BSA was confirmed by digesting the plasmid and examining the size of pET32a vector as 6,000 kb and the size of BSA PCR product was approx 900 bp (Fig. 1B). The nucleotide sequence analysis revealed that the open reading frame (ORF) of the BSA gene consists of 891 bases and encodes a protein of 296

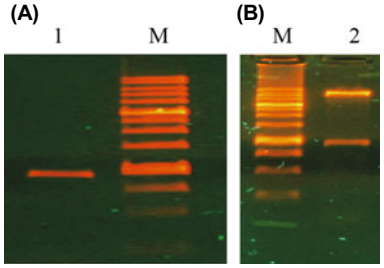

Fig. 1. PCR amplification of BSA from *B. subtilis* 168 (A) and digestion of plasmid pET32a containing the BSA using restriction enzymes (B). Products of PCR amplification and plasmid digestion were analyzed by 1% (w/v) agarose gel electrophoresis and stained using Loading STAR (Dyne Bio, Korea). (A) Lanes: 1, PCR product (BSA) of approx 900 bp amplified; M, DNA molecular weight marker (Intron Biotechnology, Korea) with 10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,500, 1,500, 1,000, 750, 500, 250 bp; (B) Lane 2, digestion products (6,000 bp and 900 bp) of pET32a-*BSA* with *Kpn*I and *Hin*dIII.

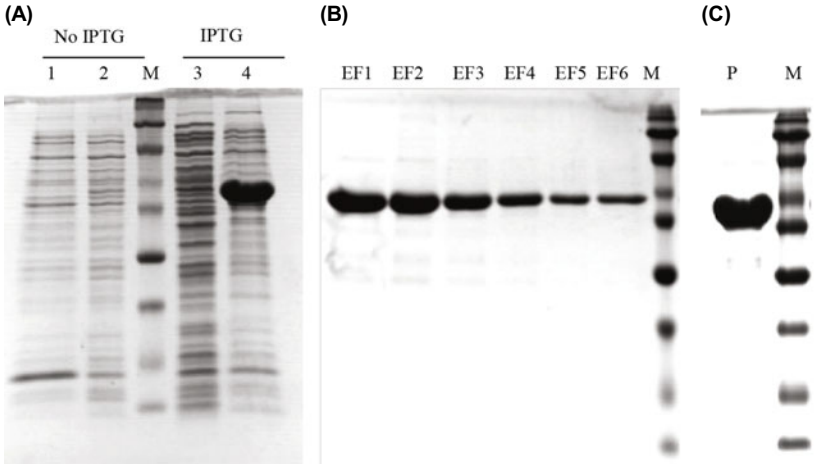

Fig. 2. SDS-PAGE (15%, w/v) analysis of crude extracts and purified 6×his-tagged BSA protein from the untransformed and transformed *E. coli* BL21(DE3). (A) Lanes: 1, BL21(DE3) with no IPTG induced; 2, BL21(DE3) carrying pET32a-BSA with no IPTG induced; 3, BL21 (DE3) with IPTG induced; 4, BL21(DE3) carrying pET32a-BSA with IPTG induced. (B) 6×his-tagged BSA protein during elution step of affinity chromatography. Lanes EF1–EF6, elution fraction. (C) Lanes: P, concentrated BSA protein (6.512 µg/gel line) using Amicon membrane with a 30,000 Da cut-off; M, ProSieve^{R} color Protein markers (Lonza, USA) containing a mixture of nine proteins of molecular weights 178, 120, 78, 51, 40, 25, 18, 12, and 10 kDa.

amino acid residues (data not shown) with a predicted molecular weight of 32.15 kDa. The ORF nucleotide sequence of the BSA is identical to those of arginases from *B. subtilis* RO-NN-1 (Earl *et al*., 2012) and *B. subtilis* (Bewley *et al*., 1996). Comparison of the deduced amino acid sequence of the BSA with those of arginases of *B. subtilis* RO-NN-1 (Earl *et al*., 2012), *B. subtilis* (Bewley *et al*., 1996), *B. brevis* TT02-8 (Shimotohno *et al*., 1997; Todd *et al*., 2001), and *B. caldovelox* (Bewley *et al*., 1996) showed 100, 100, 65, 70% sequence similarities respectively (data not shown).

Expression and purification of BSA enzyme

The synthesis of BSA protein in the transformed pET32a-BSA/BL21(DE3) culture was induced by IPTG. The presence of BSA protein was confirmed by analyzing the cell culture extracts using 15% (w/v) SDS-PAGE. As shown in Fig. 2A, roughly 40–51 kDa of protein was highly induced by IPTG in the pET32a-BSA/BL21(DE3) cell extract. The BSA protein was purified using Ni^{2+} resin (ProBond™ resin, Invitrogen, USA) based affinity chromatography (Fig. 2B). The flow through fraction (FF) obtained during the binding step and the washed fraction (WF) obtained during the washing step did not result in the elution of BSA protein (data not shown). The eluted fraction (EF) obtained at the final elution step showed that the 45 kDa BSA protein was rapidly eluted by the elution buffer (Fig. 2B). Considering the sizes of the ×His·tag (0.66 kDa) and Trix·tag (12 kDa) in the pET32a(+) vector, the size of the arginase enzyme from the *B. subtilis* 168 was determined to be 32.34 kDa.

Arginase enzymes can be found across a wide spectrum of species including animals, plants, and microbes. Prior research indicates varying sizes of arginase in the human liver (120 kDa, Carvajal *et al*., 1995), fungi *Neurospora crassa* (38–41 kDa, Borkovixh and Weiss, 1987) and *Saccharomyces cerevisiae* (37 kDa, Green *et al*., 1990), *Helicobacter pylori* (30–50 kDa, Mendz *et al*., 1998), *Bacillus brevis* Nagano (32 kDa, Kanda *et al*., 1997), *Bacillus caldovelox* (31 kDa, Bewley *et al*., 1996), and *Bacillus brevis* TT02-8 (33 kDa, Todd *et al*., 2001). Among plants, the arginase in ginseng (Panax ginseng C.A. Meyer) was measured as 352 kDa (Hwang *et al*., 2001), while the arginase size in Iris Bulb was reported to reach 191 kDa (Boutin, 1982). The size of the arginase enzyme used in this study (BSA protein) was comparable to the size of arginase enzymes derived from *S. cerevisiae* (Green *et al*., 1990) and *H. pylori* (Mendz *et al*., 1998).

The eluted fractions shown in Fig. 2B were combined and concentrated with an Amicon Ultra-0.5 centrifugal filter device (30,000 Da, cut-off, Amicon, Milllipore, Ireland). SDS-PAGE analysis confirmed that the BSA protein was purified to homogeneity (Fig. 2C). The final product was used as the sample for arginase activity assay. The purification of *B. subtilis* 168-derived BSA protein is listed in Table 1. The total activity of TIC was 35.0 units, while the total activity of TP was measured at 52.6 units. The second phase of purification resulted in a 5.8 fold purification with yield 150%. The higher yield in the second phase of purification are believed to indicate elimination of inhibitors in the TIC solution which increased the activity (Sixt *et al*., 2007). Inhibitors of arginase activities include some purines and pyrimidines and urea (Mendz *et al*., 1998; Hwang *et al*., 2001), and among animal species, amino acids such as lysine and ornithine are also reported to result in inhibition of arginase (Subrahmanyam and Reddy, 1986).

Ornithine producing ability of BSA enzyme

TLC anaysis of the UIC, TIC, and TP reaction mixtures were

Table 1. Purification of BSA enzyme from *E. coli* BL21(DE3) carrying pET32a-BSA

Step	Volume (µl)	Total activity[a] (U)	Total protein[b] (mg)	S.A[c] (U/mg)	Purification (Fold)	Yield (%)
Crude extract (TIC)	1,000	35.0	1.35	26	-	100
Purified (TP)	559	52.6	0.35	150	5.8	150

[a] One unit of arginase activity was defined as the amount of enzyme that liberated 1 mM ornithine per min at pH 8.4 and 45°C.
[b] Protein concentrations were determined by the method of Bradford (1976).
[c] S.A. (Specific activity) was defined as activity units per mg of protein (U/mg).

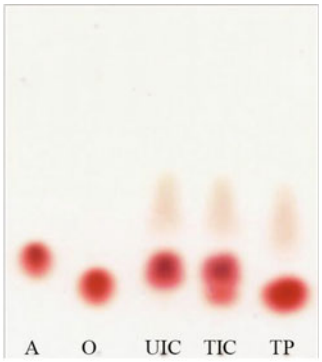

Fig. 3. TLC analysis of the purified BSA enzyme assay product. The assay reaction was conducted at 45°C (pH 8.4) for 50 min as described in 'Materials and Methods'. The protein quantity in the UIC, TIC, TP samples were 7.58, 7.55, and 7.50 μg, respectively. Lanes: A, spot of standard arginine; O, spot of standard ornithine; UIC, spot of uninduced transformed crude extract used reaction product; TIC, spots of transformed and induced crude extract reaction products; TP, spot of transformed and induced and purified BSA reaction product.

used to detect arginase activity by measuring the substrate, arginine and the product, ornithine. As shown in Fig. 3, the UIC reaction mixture contained almost all arginine whereas the TIC mixture had some arginine with roughly half ornithine. The TP reaction successfully converted arginine into ornithine. The arginase activity measurement of UIC, TIC, and TP samples was also conducted by analyzing ornithine contents using HPLC. As shown in Fig. 4, the arginase activity in the UIC, TIC, and TP samples were 1.4 mM/min/mg protein, 21.3 mM/min/mg protein, and 141.0 mM/min/mg protein, respectively. The TP sample exhibited 6.6 times higher ornithine producing ability compared to the TIC sample.

Kinetic properties of BSA enzyme

The K_m and V_{max} values for the purified BSA enzyme with arginine as substrate were determined by using the Eadie-Hofstee plots to measure the level of conversion of arginine to ornithine at pH 8.4 and 45°C. The K_m and V_{max} for the enzyme was estimated to be 4.6 mM and 133.0 mM/min/mg

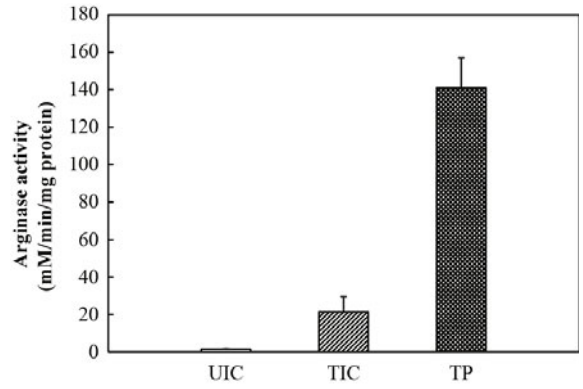

Fig. 4. Comparison of ornithine production abilities between crude extracts and purified BSA enzyme. The levels of ornithine were analyzed by HPLC after reaction at 45°C for 50 min. UIC, uninduced transformed crude extract with substrate buffer (50 mM Tirs-HCl buffer, pH 8.4, 1 mM $MnCl_2$, 40 mM arginine); TIC, transformed and induced crude extract with the substrate buffer; TP, transformed and induced purified protein with the substrate buffer. Values are mean±SD of 3 sets of data.

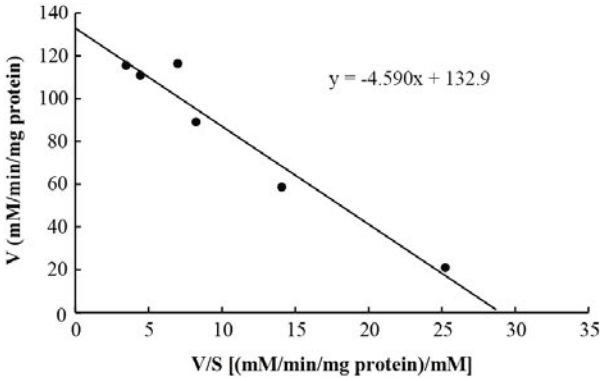

Fig. 5. Eadie-Hofstee plot of velocities of purified BSA enzyme-catalyzed reaction. The reaction mixture contained 50 mM Tris-HCl buffer, pH 8.4, 1 mM $MnCl_2$, a suitable amount of enzyme preparation and different concentrations of arginine ranged from 0.83 to 33.3 mM.

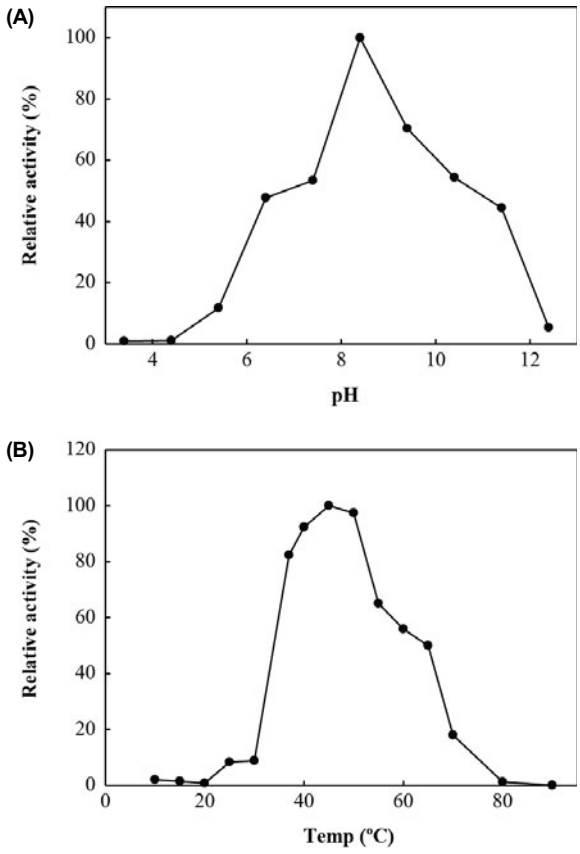

Fig. 6. Optimum pH (A) and temperature (B) of purified BSA enzyme from transformed *E. coli* pET32a-BSA/BL21(DE3). Data were from triplicate assays. The reaction mixture contained in 120 μl: 40 mM arginine, 1 mM $MnCl_2$, a suitable amount of enzyme preparation and 50 mM citrate buffer (pH 3.3–4.4) or acetate buffer (pH 5.4) or sodium phosphate buffer (pH 6.4–7.4) or Tris-HCl buffer (pH 8.4) or glycine-NaOH buffer (pH 9.4) or sodium bicarbonate buffer (pH 10.4–11.4) or KCl-NaOH buffer (pH 12.4).

protein, respectively (Fig. 5). The K_m value (4.6 mM) was relatively smaller compared with K_m values of other arginases such as 12.8 mM for *B. brevis* Nagano (Kanda *et al*., 1997), 13.5 mM for *B. subtilis* KY3281 (Nakamura *et al*., 1973), 15.7 mM for *S. cerevisiae* (Green *et al*., 1990), 22.0 mM for *H. pylori* (Mendz *et al*., 1998), 82.7 mM for ginseng (*Panax ginseng* C.A. Meyer) root (Hwang *et al*., 2001), and 131.0 mM for *N. crassa* (Borkovich and Weiss, 1987). Based on the relatively low apparent K_m value, the BSA enzyme has higher affinity for arginine as substrate than others. The V_{max} values of arginases also vary depending on origins and purified status of enzymes, for examples, purified arginase from *S. cerevisiae* is 887.5 μmol/min/mg protein (Green *et al*., 1990), cell lysates of *H. pylori* strains 108–200 nmol/min/mg protein (Mendz *et al*., 1998), and purified arginase from ginseng (*Panax ginseng* C.A. Meyer) root 29.3 μmol/min/mg protein (Hwang *et al*., 2001).

Optimum pH and temperature of BSA enzyme

The optimal pH of BSA enzyme was determined to be 8.4 (Fig. 6A), suggesting the enzyme is an alkalic arginase. The optimal pH for other arginases such as *B. brevis* Nagano is pH 10.0 (Kanda *et al*., 1997), *B. caldovelox* is pH 9.0 (Pahchett *et al*., 1991), *B. subtilils* pH 9–11 (Viator *et al*., 2008), *B. anthracis* pH 9.0 (Viator *et al*., 2008), ginseng (*Panax ginseng* C.A. Meyer) root pH 9.5 (Hwang *et al*., 2001) and *S. cerevisiae* and *N. crassa* pH 9.5 (Borkovich and Weiss, 1987; Green *et al*., 1990). However, maximal activities of *H. plyori* arginase and mouse liver arginase log pH 6.1 (Mendz *et al*., 1998; McGee *et al*., 2004; Viator *et al*., 2008) and pH 7.4 (Spolarics and Bond, 1998), respectively. The optimum temperature for which BSA enzyme activity was 45°C at pH 8.4 (Fig. 6B). The optimal temperature for other arginases such as *B. caldovelox* (Pahchett *et al*., 1991) and ginseng (*Panax ginseng* C.A. Meyer) root (Hwang *et al*., 2001) is 60°C. However, the optimal temperature for *S. cerevisiae* (Green *et al*., 1990), *B. brevis* Nagano (Kanda *et al*., 1997), *H. plyori* (Mendz *et al*., 1998; McGee *et al*., 2004; Viator *et al*., 2008) arginases is in the range of 30–37°C. As such, the optimal pH and temperature conditions for arginase activity vary across a wide spectrum.

Bacillus subtilis is one of major bacteria found in *Cheonggukjang* and Natto (Wang *et al*., 2009). Recently, we generated a genetically engineered *Bacillus* strain by introducing the arginase gene derived from food-grade microorganisms such as *B. subtilis* to produce high levels of arginase and ornithine in *Cheonggukjang* (Park *et al*., 2010). Arginine-degrading and ornithine-producing enzyme arginases have been used to treat arginine-requiring cancers (Cheng *et al*., 2007; Hsueh *et al*., 2012). Thus, arginases of microbial origin such as BSA enzyme from *B. subtilis* 168 are expected to have antitumor activity and to be used as functional food additives. Further studies to prove that the BSA enzyme inhibits the growth of various cancer cells are currently under investigation.

Conclusion

This study utilized pET32a-BSA recombinant DNA to transform the BL21(DE3) bacteria and purify the BSA protein to determine its characteristics. The purified BSA protein measured roughly 45 kDa (6×His·tag, 0.66 kDa+Trix·tag, 12 kDa) in size, thus the pure form of BSA protein was estimated to be 32.34 kDa. The purification of TP using affinity chromatography resulted in 5.8 fold purification, and the process eliminated inhibitors present in TIC to drastically increase total activity and yielded 150%. The arginase exhibited optimal activity at pH 8.4 and 45°C. Furthermore, the measurement of arginine to ornithine conversion per min at the optimal conditions indicates high affinity of BSA enzyme for arginine based on the K_m and V_{max} values estimated to be 4.6 mM and 133.0 mM/min/mg protein, respectively. The results provided valuable information for determining the optimal conditions for the production of purified arginases.

Acknowledgements

This study was supported by a research grant from Sunchanggun Fermentation Microorganism Management Center, Jeonbuk, Korea.

References

Arena, M.E., Saguir, F.M., Manca, M.C., and Nadra, M. 1999. Arginine, citrulline and ornithine metabolism by lactic acid bacteria from wine. *Int. J. Food Microbiol.* **52**, 155–161.

Bewley, M.C., Lott, J.S., Baker, E.N., and Patchett, M.L. 1996. The cloning, expression and crystallization of a thermostable arginase. *FEBS Lett.* **386**, 215–218.

Borkovich, K.A. and Weiss, R.L. 1987. Purification and characterization of arginase from *Nerurospora crassa*. *J. Biol. Chem.* **262**, 7081–8086.

Boutin, J.P. 1982. Purification, properties and subunit structure of arginase from iris bulbs. *Eur. J. Biochem.* **127**, 237–243.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Carvajal, N., Torres, C., Uribe, E., and Salas, M. 1995. Interaction of arginase with metal ions: Studies of the enzyme from human liver and comparison with other arginases. *Comp. Biochem. Physiol.* **112B**, 153–159.

Cheng, P.N., Lam, T.L., Lam, W.M., Tsui, S.M., Cheng, A.W., Lo, W.H., and Leung, Y.C. 2007. Pegylated recombinant human arginase (rhArg-peg$_{5,000mw}$) inhibits the *in vitro* and *in vivo* proliferation of human hepatocellular carcinoma through arginine depletion. *Cancer Res.* **67**, 309–317.

Earl, A.M., Eppinger, M., Fricke, W.F., Rosovitz, M.J., Rasko, D.A., Daugherty, S., Losick, R., Kolter, R., and Ravel, J. 2012. Whole-genome sequences of *Bacillus subtilis* and close relatives. *J. Bacteriol.* **194**, 2378–2379.

Green, S.M., Eisenstein, E., McPhie, P., and Hensley, P. 1990. The purification and characterization of arginase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**, 1601–1607.

Hsueh, E.C., Knebel, S.M., Lo, W.H., Leung, Y.C., Cheng, P.N.M., and Hsueh, C.T. 2012. Deprivaion of arginine by recombinant human arginase in prostate cancer cells. *J. Hematol. Oncol.* **5**, 17. Doi:10.1186/1756-8722-5-17.

Hwang, J.H., Kim, H.E., and Cho, D.Y. 2001. Isolation and properties of arginase from a shade plant, ginseng (*Panax ginseng* C.A. Meyer) roots. *Phytochem.* **58**, 1015–1024.

Kanda, M., Ohgishi, K., Hanawa, T., and Saito, Y. 1997. Arginase

of *Bacillus brevis* Nagano: Purification, properties, and implication in gramicidin S biosynthesis. *Arch. Biochem. Biophys.* **344**, 37–42.

Kim, J.E., Kim, S.Y., Lee, K.W., and Lee, H.J. 2009. Arginine deiminase originating from *Lactococcus lactis* spp. *lactis* American Type Culture Collection (ATCC) 7962 induces G_1-phase cell-cycle arrest and apoptosis in SNU-1 stomach adenocarcinoma cells. *Bri. J. Nutri.* **102**, 1469–1476.

Konst, P.M., Turras, P.M.C.C.D., Franssen, M.C.R., Scott, E.L., and Sanders, J.P.M. 2010. Stabilized and immobilized *Bacillus subtilis* arginase for the biobased production of nitrogen-containing chemicals. *Adv. Synyh. Catal.* **352**, 1493–1502.

Kuensch, U., Temperli, A., and Mayer, K. 1974. Conversion of arginine to ornithine during malolactic fermentation in red Swiss wine. *Am. J. Enol. Vitic.* **25**, 191–193.

Liu, S.Q., Holland, R., and Crow, V.L. 2003. The potential of dairy lactic acid bacteria to metabolise amino acids via non-transaminating reactions and endogenous transamination. *Int. J. Food Microbiol.* **86**, 257–269.

McGee, D.J., Zabaleta, J., Viator, R.J., Testerman, T.L., Ochoa, A.C., and Mendz, G.L. 2004. Purification and characterization of *Helicobacter pylori* arginase, RocF: unique features among the arginase superfamily. *Eur. J. Biochem.* **271**, 1952–1962.

Mendz, G.L., Holmes, E.M., and Ferrero, R.L. 1998. *In situ* characterization of *Helicobacter pylori* arginase. *Biochim. Biophys. Acta.* **1388**, 465–477.

Nakamura, N., Fujita, M., and Kimura, K. 1973. Purification and properties of L-arginase from *Bacillus subtilis*. *Agr. Biol. Chem.* **37**, 2827–2833.

Narimatsu S., Kazamori, D., Masuda, K., Katsu, T., Funae, Y., Naito, S., Nakura, H., Yamano, S., and Hanioka, N. 2009. The mechanism causing the difference in kinetic properties between rat CYP2D4 and human CYP2D6 in the oxidation of dextromethorphan and bufuralol. *Biochem. Pharmacol.* **77**, 920–931.

Pahchett, M.L., Daniel, R.M., and Moran, H.W. 1991. Characterisation of arginase from the extreme thermophile *Bacillus caldovelox*. *Biochim. Biophys. Acta* **1077**, 291–298.

Park, K.B., Yu, J.J., and Oh, S.H. 2010. Expression of arginase gene in *Bacillus* sp. enhances ornithine production. Program Abstr. 2010 Int'l Sympo. Annual Meeting., Abstr. P.5-05.

Peng, Y., Yang, X., and Zhang, Y. 2005. Microbial fibrinolytic enzymes: an overview of source, production, properties, and thrombolytic activity *in vivo*. *Appl. Microbiol. Biotechnol.* **69**, 126–132.

Rijin, J.V., Berg, J.V.D., Teerlink, T., Kruyt, F.A.E., Schor, D.S.M., Lavalette, A.C.R.D., Berg, T.K.V.D., Jakobs, C., and Slotman, B.J. 2003. Changes in the ornithine cycle following ionising radiation cause a cytotoxic conditioning of the culture medium of H35 hepatoma cells. *British J. Cancer* **88**, 447–454.

Shimotohno, K.W., Miwa, I., and Endo, T. 1997. Molecular cloning and nucleotide sequence of the arginase gene of *Bacillus brevis* TT02-8 and its expression in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **61**, 1459–1464.

Sixt, S.U., Beiderlinden, M., Jennissen, H.P., and Peters, J. 2007. Extracellular proteasome in the human alveolar space: A new housekeeping enzyme? *Am. J. Physiol. Lung Cell. Mol. Physiol.* **292**, L1280–L1288.

Spano, G., Chieppa, G., Benedue, L., and Massa, S. 2004. Expression analysis of putative *arcA*, *arcB*, *arcC* genes partially cloned from *Lactobacillus plantarum* isolated from wine. *J. Appl. Microbiol.* **96**, 185–193.

Spolarics, Z. and Bond, J.S. 1989. Comparison of biochemical properties of liver arginase from streptozocin-induced diabetic and control mice. *Arch. Biochem. Biophys.* **274**, 426–433.

Subrahmanyam, T.S. and Reddy, S.R. 1986. L-Ornithine and L-lysine need their α-carboxyl groups for effective inhibition of bovine liver arginase. *Indian J. Biochem. Biophys.* **23**, 359–361.

Todd, C.D., Cooke, J.E.K., and Gifford, D.J. 2001. Purification and properties of *Pinus taeda* arginase from germinated seedlings. *Plant Physiol. Biochem.* **39**, 1037–1045.

Tsui, S.M., Lam, W.M., Lam, T.L., Chong, H.C., So, P.K., Kwok, S.Y., Arnold, S., Cheng, P.N.M., Wheatley, D.N., Lo, W.H., and Leung, Y.C. 2009. Pegylated derivatives of recombinant human arginase (rhArgI) for sustained *in vivo* activity in cancer therapy: preparation, characterization and analysis of their pharmacodynamics *in vivo* and *in vitro* and action upon hepatocellular carcinoma cell (HCC). *Cancer Cell International* **9**, 9. Doi:10.1186/1475-2867-9-9.

Viator, R.J., Rest, R.F., Hildebrandt, E., and McGee, D.J. 2008. Characterization of *Bacillus anthracis* arginase: Effects of pH, temperature, and cell viability on metal preference. *BMC Biochem.* **9**, 15. Doi:10.1186/1471-2091-9-15.

Wang, C., Du, M., Zheng, D., Kong, F., Zu, G., and Feng, Y. 2009. Purification and characterization of nattokinase from *Bacillus subtilis* natto B-12. *J. Agric. Food Chem.* **57**, 9722–9729.

Yoshinori, M., Wong, A.H.K., and Jiang, B. 2005. Fibrinolytic enzymes in Asian traditional fermented foods. *Food Res. Int.* **38**, 243–250.

Yu, J.J. and Oh, S.H. 2010. Isolation and characterization of lactic acid bacteria strains with ornithine producing capacity from natural sea salt. *J. Microbiol.* **48**, 467–472.