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Cloning, Expression, and Characterization of Para-Aminobenzoic Acid (PABA) Synthase from *Agaricus bisporus* 02, a Thermotolerant Mushroom Strain S

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The *pabS* gene of *Agaricus bisporus* 02 encoding a putative PABA synthase was cloned, and then the recombinant protein was expressed in *Escherichia coli* BL21 under the control of the *T7* promoter. The enzyme with an N-terminal GST tag or His tag, designated GST-*Ab*ADCS or His-*Ab*ADCS, was purified with glutathione Sepharose 4B or Ni Sepharose 6 Fast Flow. The enzyme was an aminodeoxychorismate synthase, and it was necessary to add with an aminodeoxychorismate lyase for synthesizing PABA. *Ab*ADCS has maximum activity at a temperature of approximately 25°C and pH 8.0. Magnesium or manganese ions were necessary for the enzymatic activity. The Michaelis-Menten constant for chorismate was 0.12 mM, and 2.55 mM for glutamine. H_2O_2 did distinct damage on the activity of the enzyme, which could be slightly recovered by Hsp20. Sulfydryl reagents could remarkably promote its activity, suggesting that cysteine residues are essential for catalytic function.

Keywords: *Agaricus bisporus*, *para*-aminobenzoic acid, aminodeoxychorismate synthase, aminodeoxychorismate lyase, Michaelis-Menten constant

Introduction

Folate, composed of a pteridine ring, *para*-aminobenzoic acid (PABA), and glutamic acid(s), is an essential cofactor for all living cells and plays critical roles in a diverse range of metabolic pathways, mainly in one-carbon transfer reactions such as amino acid interconversions, and purine and pyrimidine biosynthesis [22]. During the past decade, more and more details were disclosed for *de novo* synthesis of folate from bacteria and plants; however, little was known about it from mushroom [1, 3, 9]. In *Escherichia coli*, there are three enzymes required for the conversion of chorismate to PABA. PabA is an aminase that supplies ammonia from glutamine hydrolysis; PabB is a member of a family of structurally similar chorismate-utilizing enzymes that catalyze the amination of chorismate, yielding 4-amino-4-deoxychorismate (ADC); PabC is a pyridoxal phosphate-dependent enzyme that catalyzes the elimination of pyruvate from ADC, forming PABA [4]. In most bacteria, *pabA* and *pabB* are isolated genes, but *pabA* and *pabB* homologs are found as one fused gene in a number of actinomycetes and all of the eukaryotes analyzed so far [6, 8, 12, 23]. PabA and PabB associate with one another to form the aminodeoxychorismate synthase (ADCS, E.C. 6.3.5.8) (Fig. 2A, Step 1), whereas PabC acts as the aminodeoxychorismate lyase (ADCL, E.C. 4.1.3.38) (Fig. 2A, Step 2).

Agaricus bisporus (Lange) Imbach is the most frequently cultivated species of edible mushrooms [13, 14]. In contrast with the commercially cultivated common strain *A. bisporus* 8213, which requires 16~19°C during the fruiting period, thermotolerant strain *A. bisporus* 02 can survive at 25°C and the maximum temperature of 32°C. Suppression subtractive hybridization (SSH) can be used to compare two mRNA populations and obtain cDNA representing genes that are either overexpressed or exclusively expressed in one population as compared with another [7, 11, 19].

During the application of SSH to compare the difference between thermotolerant strain 02 and thermosensitive strain 8213, an EST sequence of *1F6* (GH159019) enriched in the strain 02 transcriptosome was cloned, and then the full-length of the *1F6* gene named *pabS* (GenBank Accession No. FJ617437) was cloned and reported for enhancing the thermotolerance of mushroom [18]. To analyze the function of the *pabS* gene, it is necessary to further identify and characterize its encoding protein *in vitro*.

In this paper, to understand the gene organization of *pabS* in detail, the corresponding genomic DNA sequence and its 5′ flanking sequence were further cloned. To characterize its encoding protein, *pabS* cDNA was cloned into the pGEX-4t-1 vector and its recombinant protein (*Ab*ADCS) expressed in *E. coli*, and then GST-*Ab*ADCS was purified with glutathione Sepharose 4B. In another protocol, *pabS* cDNA was also cloned into the pET28a vector, and its recombinant protein (*Ab*ADCS) expressed in *E. coli*, and then His-*Ab*ADCS was purified with Ni Sepharose 6 Fast Flow. Coupling with recombinant *E. coli* PabC protein (*Ec*ADCL), the recombinant *Ab*ADCSs (GST-*Ab*ADCS or His-*Ab*ADCS) were characterized *in vitro*.

Materials and Methods

Substrates and Chemicals

Chorismate and *para*-aminobenzoic acid were purchased from Sigma (Shanghai, China). Protein molecular marker and Taq DNA polymerase were purchased from Fermentas (Xiamen, China). Gel Extraction Kit, Plasmid Mini Kit I, and Cycle-pure Kit were purchased from OMEGA Bio-Tek (Xiamen, China). Ni Sepharose 6 Fast Flow and Glutathione Sepharose 4B were purchased from GE Healthcare (Xiamen). pMD19-T vector and M-MLV RTase cDNA Synthesis Kit were purchased from TaKaRa (Dalian, China). Recombinant *A. bisporus* Hsp20 (heat-shock protein 20), PPI (peptidyl-prolyl *cis-trans* isomerase), and BCAT (branched-chain amino acid aminotransferase) were prepared in our laboratory. All chemicals were reagent grade and all solutions were prepared with MilliQ water.

Strains and Culture Conditions

A. bisporus strains 02 and 8213 were provided by the Mushroom Research and Development Station, Fujian Academy of Agricultural Sciences, China. *E. coli* DH5α (TaKaRa, Japan) and BL21 (DE3) RIPL (Stratagene, USA) strains were used in this study for protein expression. The pGEX-4T-1 (GE, Sweden) and pET-28a (+) vectors (Novagen) were used for cloning.

Extraction of *A. bisporus* 02 Genomic DNA and Total RNA

A. bisporus 02 mycelia were inoculated into PDA liquid medium at 24°C. After 2 weeks, Erlenmeyer flasks were transferred to 24°C (non heat stress) or 32°C (heat stress) incubators for 24 h, and then the mycelia were collected with a sterile gauze and washed with sterile water. Genomic DNA was extracted by the modified CTAB method [20]. Total RNA was extracted according to the specifications of the Trizol kit. Then, cDNA of *A. bisporus* 02 was obtained by reverse transcription of RNAs according to the instruction for the M-MLV RTase cDNA Synthesis Kit.

Cloning and Sequencing of *A. bisporus* 02 *pabS* Genomic DNA and cDNA

The *pabS* genomic DNA was amplified by ExTaq from *A. bispous* 02 genomic DNA with primers *pabSF* and *pabSR* (Table S1) designed based on the *pabS* cDNA sequence [17]. The method for amplifying *pabS* cDNA was the same as for *pabS* genomic DNA, except with *A. bispous* 02 total cDNA as the template. The PCR conditions were as follows: 95°C for 2 min; 30 cycles of 94°C for 30 sec, 57°C for 35 sec, and 72°C for 2.5 min; 72°C for 10 min. The PCR products were purified and cloned into the pMD19-T vector, and transformed into *E. coli* DH5α. Finally, positive recombinants were sequenced for cloned PCR fragments.

The sequence of *pabS* cDNA was subjected to BLAST search at the National Centre for Biotechnological Information (NCBI) for similar sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and accordingly representative amino acid sequences were downloaded. Alignments were generated by the ClustalW2 server (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and visualized by ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).

Cloning and Sequencing of *A. bisporus* 02 *pabS* 5′ Flanking Sequence

An attempt was made to obtain the *pabS* 5′ flanking sequence by self-formed adaptor PCR (SEFA PCR) [25]. PCR amplification of the *pabS* 5′ flanking sequence of the genomic DNA was first performed using three gene-specific primers (*pabS-Sp1*, *pabS-Sp2*, and *pabS-Sp3*) (Table S1) located sequentially on the genomic DNA sequence of the *pabS* gene (KJ609303). The PCR mixture included 15 µl of 2× GC buffer I, 5 µl of 2.5 mM dNTP, 1.5 U of LA-Taq (TaKaRa), and about 1 µg of *A. bisporus* 02 genomic DNA, and deionized water was added to 30 µl. After SEFA PCR, a second round of nested PCR was run with the single primer *pabS-Sp3* (Table S1). To acquire the specific fragment, the third-round PCR was carried out with primers *pabS*-SP4 and *pabS*-SP5 (Table S1). The amplified PCR product was recovered and ligated with the pMD19-T vector, transformed into *E. coli* DH5α, and sequenced.

Expression of *A. bisporus* 02 *pabS* cDNA and Purification of the Recombinant ADCS

The *A. bisporus* 02 *pabS* cDNA ORF was amplified from *pabS*-cDNA-pMD19T recombinant *E. coli* strains with primers *pabSS* and *pabSA* (Table S1) and constructed into *Eco*RI and *Sal*I sites of the pGEX-4t-1 vector; the ligation products were then transformed into *E. coli* BL21 (DE3) cells. The recombinant plasmid was designated *pabS*-pGEX-4t-1 and expressed in the same cells in

Luria–Bertani (LB) medium containing 100 μg/ml ampicillin. In another protocol, the *A. bisporus* 02 *pabS* cDNA ORF was constructed into *Eco*RI and *Sal*I sites of vector pET-28a (+), and the recombinant plasmid was designated *pabS*-pET28a and transformed into *E. coli* BL21 (DE3) cells. The transformed cells of *E. coli* were grown at 37°C in LB medium containing 50 μg/ml kanamycin to an optical density of 0.6 at 600 nm. The expression of the recombinant protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the strain was grown at 16°C for 12 h. The cells were harvested by centrifugation at 8,000 ×*g* for 30 min, resuspended with lysis buffer (0.1 M Tris/HCl (pH 7.5), 1 mM L-glutamine, 0.3 M NaCl, and 10% (v/v) glycerol), and then sonicated with an Ultrasonic processor (750 W, 30% amplitude, 3 sec on and 3 sec off for 20 min) to release intracellular proteins. The cell-free extract was centrifuged at 18,000 ×*g* for 15 min to remove cell debris. Finally, GST-tagged *A. bisporus* ADCS protein (abbreviated GST-*Ab*ADCS) or His-tagged *A. bisporus* ADCS protein (abbreviated His-*Ab*ADCS) was purified on glutathione Sepharose 4B or Ni Sepharose 6 Fast Flow columns according to the manufacturer's instructions. Eluted fractions containing the highest activity were pooled and concentrated by using a 30 kDa Amicon Ultra (Millipore). Proteins were quantified by the Bradford assay using BSA as the standard [5].

Expression of the *pabC* gene of *E. coli* and Purification of the Recombinant ADCL

The *pabC* gene was amplified from the *E. coli* BL21 (DE3) genome with sense primer *pabCS* and *pabCA* (Table S1) designed according to the *E. coli pabC* sequence (GenBank No. ACT42987) and constructed into the *Bam*HI and *Xho*I restriction sites of the pGEX-4t-1 vector. Recombinant vectors were extracted and transformed into BL21 (DE3) cells for protein expression; GST-tagged *E. coli* ADCL protein (abbreviated *Ec*ADCL) was induced by IPTG and purified on a glutathione Sepharose 4B Fast Flow column.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [15] to estimate the protein molecular mass and the purity with a stacking gel (4% polyacrylamide) and a separating gel (10% polyacrylamide).

Enzymatic Activity of *A. bisporus* ADCS

The enzymatic activity of *A. bisporus* ADCS was assayed according to published procedures [2, 21] with some modifications. The standard reaction system (100 μl) contained 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM L-glutamine, 50 μM chorismate, 10 μg/ml of the recombinant *Ab*ADCS, and 20 μg/ml desalted *Ec*ADCL extract (added as indicated) and was incubated at 25°C for 10~30 min. The PABA peak was monitored by fluorescence (290 nm excitation, 340 nm emission) and quantified on the basis of a standard. One unit of enzyme activity was defined as the amount of *Ab*ADCS protein releasing 1 μmol of PABA per minute. To determine the aminotransferase specificity, different ammonia donors, such as glutamine, asparagine, and NH_4^+, were used as substrate and the product PABA was analyzed by HPLC.

Effects of Environmental Factors on Enzyme Activity

The optimal temperature for enzyme activity was determined at a temperature range of 5–50°C in increments of 5°C for 30 min under standard assay conditions. The thermal stability assay was performed by incubating 5 μl of 20 mg/ml enzyme at different temperatures for 1 h. Aliquots were removed and assayed under standard conditions.

For the determination of optimum pH of the enzyme, activities were measured over a pH range of 5.0–10.0 in increments of 0.5 pH units under standard assay conditions. The buffers used were 0.2 M disodium hydrogen phosphate-0.1 M citric acid (pH 5.0–6.5), 0.05 M Tris-hydrochloride (pH 7.0–8.5), and 0.2 M glycine-sodium hydroxide (pH 9.0–10.5). The pH stability assay was performed by incubating the enzyme at 4°C in different pH buffers for 3 h. Aliquots were removed and assayed under standard conditions.

The effects of various metal ions were assayed at 25°C and pH 8.0, where the enzyme solution was pre-incubated with 5 mM of Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, K^+, Li^+, and Na^+ as chloride salts individually for 30 min. To determine the effect of organic reagents, dimethylsulfoxide, β-mercaptoethanol, ethanol, and isopropanol at a concentration of 1% (v/v); SDS 0.1% (v/v); EDTA (5 mM); dithiothreitol (5 mM); and PMSF (5 mM) were added to the reaction system individually. The production quantity of PABA was determined by HPLC, where the activity was expressed as percent relative activity with respect to maximum activity.

The effect of H_2O_2 was assayed by incubating 3.0 mg/ml His-*Ab*ADCS protein with (A) 5 μmol/l; (B) 50 μmol/l; or (C) 500 μmol/l of H_2O_2 at 4°C for 15 min, and the standard reaction condition was used for determination of PABA, where the activity was expressed as percent relative activity with respect to enzyme activity without H_2O_2 treatment. At 50 μmol/l H_2O_2 as pretreating concentration, His-*Ab*ADCS protein was then incubated with 0.9 mg/ml Hsp20, 0.2 mg/ml PPI, 0.25 mg/ml BCAT protein, or 250 mmol/l DTT at 4°C for 20 min and the recovery of enzymatic activity assayed.

Kinetic Parameters of *A. bisporus* ADCS

Enzyme kinetic parameters of *Ab*ADCS were obtained by measuring the rate of PABA production with one substrate at various concentrations and the other substrate at saturating concentration in the standard reaction condition. For the determination of K_m of chorismate, chorismate concentrations ranged from 0 to 500 μM whereas glutamine concentration did not vary (5 mM). For the determination K_m of glutamine, L-glutamine concentrations ranged from 0 to 5 mM whereas chorismate concentration did not vary (200 μM). The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values were determined from the Lineweaver–Burk plot.

Nucleotide Sequence Accession Number

The nucleotide and 5′ flanking sequence of the *A. bisporus* aminodeoxychorismate synthase gene has been deposited in the GenBank database under the accession numbers KJ609303 and KJ817170.

Results

Cloning of *A. bisporus* 02 *pabS* cDNA, Genomic DNA, and 5′ Flanking Sequence

Cloning genomic DNA, cDNA, and 5′ flanking sequences of the *A. bisporus* 02 *pabS* gene are shown in Fig. S1. Comparing the cDNA and genomic DNA sequences of *pabS* suggests the presence of two introns that obey GT-AT rule (Fig. S2A). The analysis of the whole gene of *pabS* cDNA revealed an open reading frame (ORF) encoding a hypothetical 733 amino acid protein with a molecular mass of 80.6 kDa. Clustal analysis of the *pabS* cDNA corresponding amino acid suggests that a "triad"-family of amidotransferases exists in the N-terminal of *pabS* of *A. bisporus* 02; a conserved glutamate (consensus sequence SPERF) required for the cleavage of the C4 hydroxyl group of chorismate and the conserved sequence PI(M)KGT involved in the nucleophilic attack of C2 of chorismate exist in the C-terminus of *pabS* of *A. bisporus* 02 (Fig. S2B). The motif was recently believed to allow discrimination of PabB enzymes from the closely related enzyme anthranilate synthase, which typically contains a PIAGT active-site motif [4]. Thus, *pabS* cDNA of *A. bisporus* 02 encodes a protein containing putative aminodeoxychorismate synthase. The close canonical recognition sequence for the transcription factors TFIID (TATA box) was found approximately 75 bp upstream of the transcription start point. Promoter analysis with TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) suggested it has at least one heat shock factor (HSF) (AGAAC box) binding site fully matching that of yeast (Fig. S3) [10].

Expression and Purification of *A. bisporus* ADCS

For recombinant protein expression vector construction, the ORF of *pabS* was subcloned (Fig. 1A). To analyze the function of the *pabS* gene of *A. bisporus* 02 encoding protein, we expressed it in *E. coli* under the T7 promoter. Overexpression of the cloned *pabS* ORF induced by IPTG resulted in a high expression of soluble *Ab*ADCS with N-terminal GST tag or His tag. The GST-*Ab*ADCS and His-*Ab*ADCS expressed in *E. coli* BL21 (DE3) were purified on glutathione Sepharose 4B or Ni Sepharose 6 Fast Flow separately; the molecular mass was determined by comparison with a protein marker as approximately 105 kDa and 82 kDa, respectively (Figs. 1C and 1D).

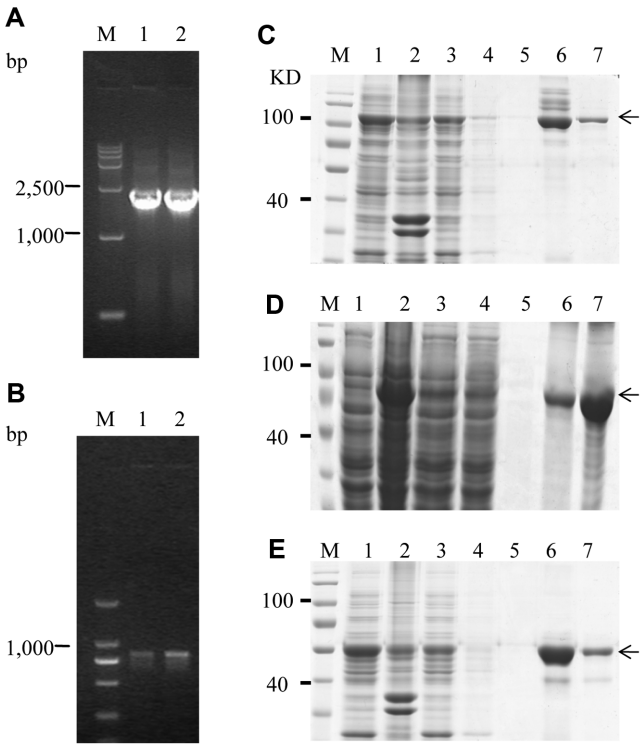

Fig. 1. Cloning of the *AbpabS* and *EcpabC* gene ORFs, and purification of *Ab*ADCS and *Ec*ADCL proteins.
(**A**–**B**) Cloning of *AbpabS* and *EcpabC* gene ORFs: (**A**) Lane M, DNA marker; 1, 2, *A. bisporus pabS* gene ORF; (**B**) M, DNA marker; 1. *pabC* gene ORF cloned from *E. coli* DH5α; 2. *pabC* gene ORF cloned from *E. coli* BL21. (**C**-**E**) Purification of recombinant protein: (**C**) GST-*Ab*ADCS, (**D**) His-*Ab*ADCS, and (**E**) GST-*Ec*ADCL. M, protein marker; 1, supernatant; 2, pellet; 3, flow-through; 4–5, washed protein; 6–7, eluted protein.

Expression and Purification of *E. coli* ADCL

For ADCL protein expression vector construction, the ORF of the *E. coli pabC* gene was cloned (Fig. 1B). After overexpression in *E. coli* BL21 (DE3), an N-terminal GST tag soluble protein (abbreviated *Ec*ADCL) was purified on glutathione Sepharose 4B Fast Flow. The molecular mass of *Ec*ADCL was approximately 55 kDa (Fig. 1E).

Enzymatic Activity of *A. bisporus* ADCS

The sketch map of PABA production catalyzed by *Ab*ADCS and *Ec*ADCL is shown in Fig. 2A. Representative graphs of HPLC analysis for production are shown in Fig. 2B: the PABA standard is shown in graph 1; when added without *Ab*ADCS, and whether with or without *Ec*ADCL, chorismate was not consumed (graph 2 and 4 separately); when added with *Ab*ADCS but without

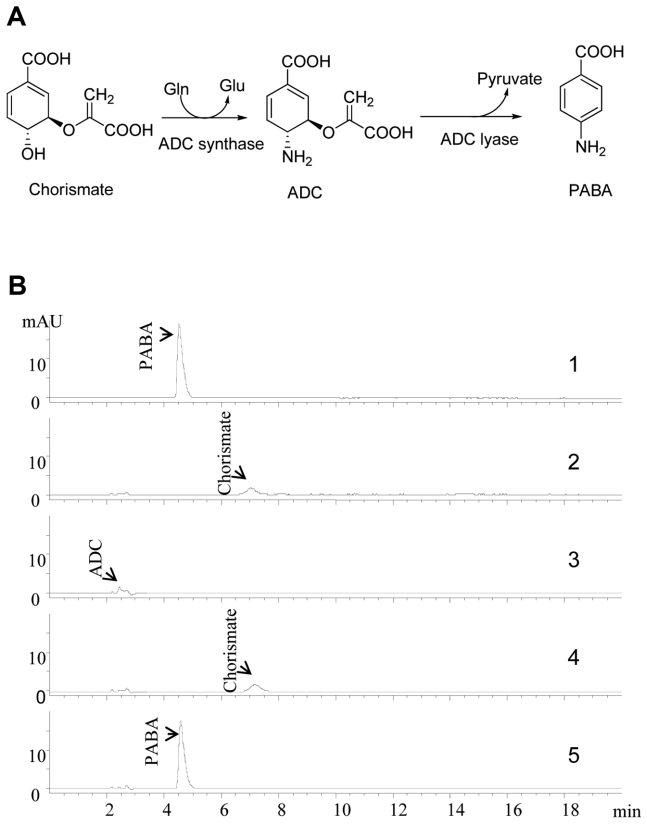

Fig. 2. PABA synthesis procedure and representative graphs of HPLC analysis.

(**A**) Sketch map of PABA production catalyzed by *Ab*ADCS and *Ec*ADCL. (**B**) Representative HPLC analysis graphs for catalyzed PABA production. Graph 1, PABA standard; 2–5, reaction system. 2, without both *Ab*ADCS and *Ec*ADCL; 3, with only *Ab*ADCS; 4, with only *Ec*ADCL; 5, with both *Ab*ADCS and *Ec*ADCL.

*Ec*ADCL, a little amount of intermediate postulated to be ADC was generated (graph 3); when added both with *Ab*ADCS and *Ec*ADCL, PABA was produced (graph 5). Therefore, we confirmed the *pabS* gene-encoding protein to be 4-amino-4-deoxychorismate synthase. Comparing asparagines, glutamine, and NH_4^+, we believe that glutamine was a natural ammonia donor for *Ab*ADCS (Table 1).

Effects of Environmental Factors on Enzyme Activity

At pH 8.0, the effect of temperature on *Ab*ADCS activity was determined; the result showed the optimal temperature of *Ab*ADCS was approximately 25°C (Fig. 3A). Incubating the protein at different temperatures for an hour suggests, at 5–30°C, *Ab*ADCS was relatively stable; however, above 35°C, the enzyme activity declined sharply (Fig. 3B).

When assayed at various pH values at 25°C, the recombinant *Ab*ADCS with GST tag or His tag activity showed the optimum pH to be approximately 8.0 (Fig. 3C). The enzyme was active and stable in the pH range 7.0–9.0 (Fig. 3D).

Table 1. Substrate specificity of *Ab*ADCS.

Ammonia donor	Relative activities (%)[a]	
	GST-*Ab*ADCS	His-*Ab*ADCS
L-Glutamine 5 mM	100	100
L-Asparagine 5 mM	11.2	10.7
[NH_4^+-NH_3] 52 mM	29.9	29.5

[a]The highest activities of the enzyme for amino donors (L-glutamine) were taken as 100%.

The effect of metal ions is shown in Fig. 4A, where in the presence of 5 mM Mg^{2+}, *Ab*ADCS activity reached a maximum, whereas in the same concentration of Mn^{2+}, and in Co^{2+} or Ca^{2+}, its activity was lower; on the contrary, in the presence of the same concentration of Cu^{2+} or Zn^{2+}, its activity was inhibited. In the presence of monovalent cations such as K^+, Li^+, Na^+, and negative control, a small amount of PABA product was yielded, probably owning to residual Mg^{2+} in the extract.

The effects of organic solvents are shown in Fig. 4B, where DMSO did not affect the activity of *Ab*ADCS; ethanol, isopropanol, and PMSF slightly inhibited the activity, whereas SDS and EDTA strongly inhibited the activity. Of note reducing agents mercaptoethanol and DTT could activate *Ab*ADCS activity remarkably, which suggests that cysteine residues are essential for its catalytic function.

The damage caused by H_2O_2 and the recovery by other proteins are shown in Fig. 4C. Results suggest that a high concentration of H_2O_2 had serious damage on enzymatic activity; after 50 μmol/l of H_2O_2 for *Ab*ADCS pretreating, and then using proteins for recovery, Hsp20 had mild capability for the recovery of the enzyme, whereas PPI and BCAT further damaged the protein. However, DTT recovered most of the activity of the enzyme.

Kinetic Parameters of *A. bisporus* ADCS

The initial rates were calculated by measuring the production of PABA at 15 min. By plotting the Lineweaver-Burk double-reciprocal graph, we found that the reaction catalyzed by recombinant *Ab*ADCS and *Ec*ADCL proteins obeyed Michealis-Menten kinetics [16]. The resulted plot had a slope equal to K_m/V_{max} and an intercept equal to $1/V_{max}$. For the substrate chorismate, K_m was 116.8~117.0 μM and V_{max} was 12.18~12.64 nmol/(min.mg) of *Ab*ADCS protein (Fig. 5A); for the substrate glutamine, K_m was 2.39~2.53 mM and V_{max} was 7.56~11.42 nmol/(min.mg) of *Ab*ADCS

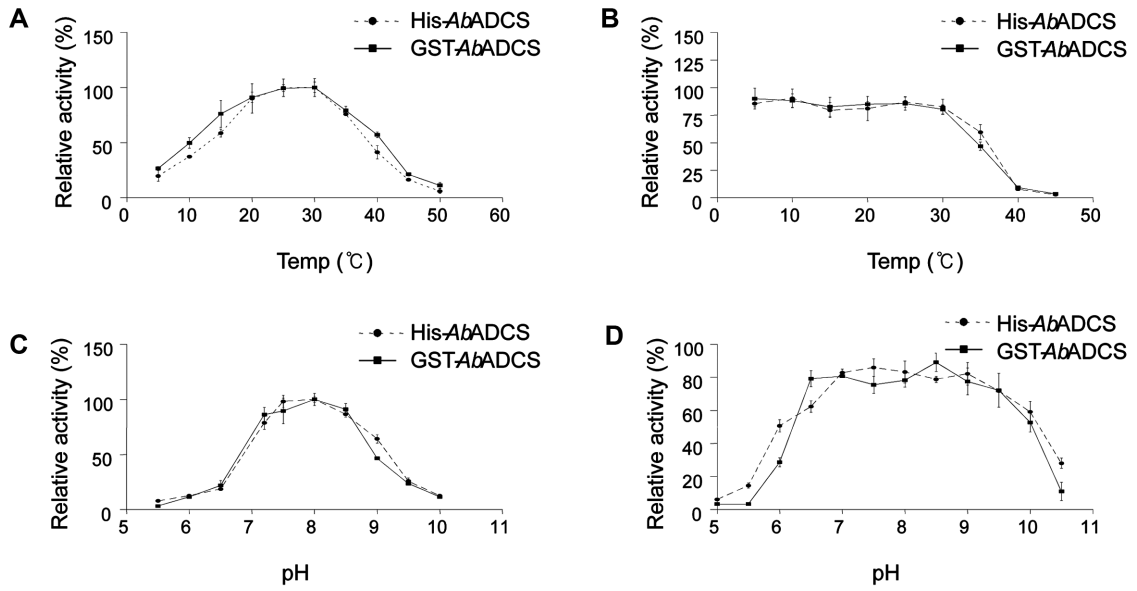

Fig. 3. Effects of temperature and pH on *Ab*ADCS enzyme activity.
(**A**) Effects of temperature on enzyme activity. (**B**) Stability of enzymes after incubating at 5–45°C for 30 min. (**C**) Effects of pH on enzyme activity. (**D**) Stability of enzymes after incubating at pH 5–10.5 for 3 h.

protein (Fig. 5B). As the His tag had a far smaller molecular size than the GST tag, we postulate the character of recombinant His-*Ab*ADCS be more approaching to natural *Ab*ADCS than GST-*Ab*ADCS. Detailed kinetic parameters of recombinant His-*Ab*ADCS are listed in Table 2.

Discussion

PABA is a precursor for the synthesis of folic acid. As an enzyme cofactor, folic acid is involved in numerous basic biological reactions, including nucleotide biosynthesis, DNA repair, and DNA methylation [26]. It was supposed the *pabS*-encoding protein in *A. bisporus* could scavenge the reactive oxygen species (ROS) during heat stress [18].

Herein, we cloned its genomic DNA and 5′ flanking sequence, expressed its encoding protein in *E. coli*, and analyzed its enzymatic character *in vitro*. The genomic DNA of *pabS* has only two introns, which is distinctly different from other similar number of bases of heat shock proteins, such as *A. bisporus Hsp90* (GenBank Accession No. KJ609304), which has six introns, or *Hsp70* (GenBank Accession No. KJ609305), which has seven introns; this is probably due to its housekeeping function requiring a more rapid mechanism for RNA splicing.

Preliminarily, we considered the GST tag could promote the solubility of the expressing protein and constructed the *pabS*-pGEX-4t-1 vector and expressed GST-*Ab*ADCS. As we were afraid that the GST tag was too big and would affect the *Ab*ADCS configuration, we used thrombin to cut the tag off; however, the product had so many bands on SDS-PAGE that we suspected the cutting sites were not specific. In another protocol, we subcloned *pabS* cDNA into the pET28a vector and purified the soluble *Ab*ADCS with an N-terminal His tag. As a long enzyme digestion procedure will result in enzyme activity loss, we compared the biochemical characters of *Ab*ADCS with N-terminal GST tag and N-terminal His tag directly. The result was that they had no obvious difference, which suggested that the N-terminal GST tag or N-terminal His tag had no significant effects on *Ab*ADCS biochemical character.

Table 2. Steady-state kinetic parameters for His-*Ab*ADCS.

Substrate	V_{max} (nmol/min·mg)[a]	K_m (1/mmol)	k_{cat} (1/s)	k_{cat}/K_m (1/mol·s)
Glutamine	9.42 ± 0.27	2.55 ± 0.2	0.013	5.06
Chorismate	12.68 ± 0.07	0.12 ± 0.005	0.017	143

[a]The rates of the reaction are expressed as nmol of PABA produced. Results are the means ± standard deviation for triplicate determinations.

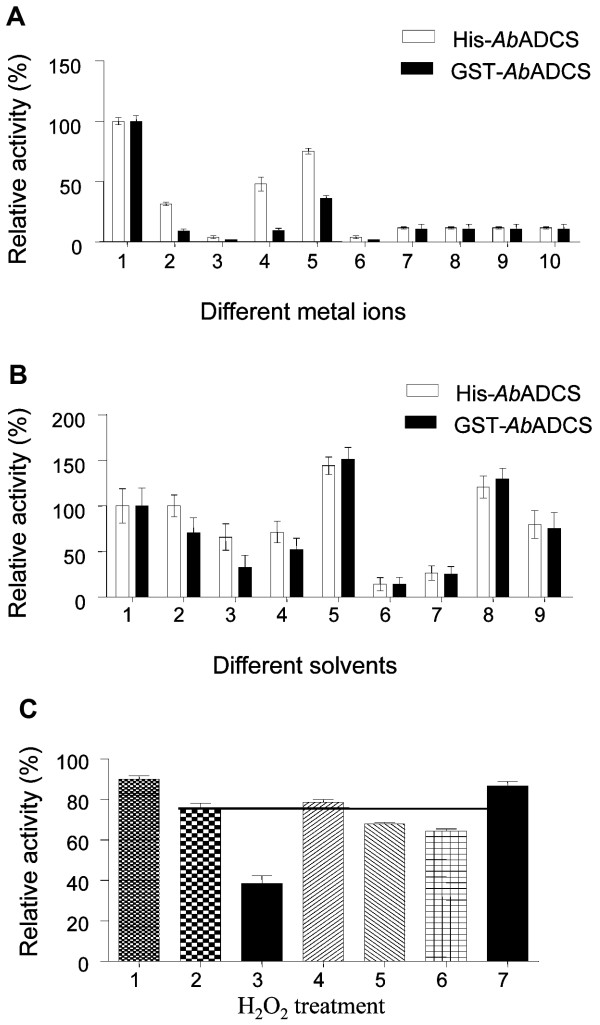

Fig. 4. Effects of metal ions, solvents, and H_2O_2 on *Ab*ADCS enzyme activity.

(**A**) Effects of different metal ions on enzyme activity. 1, Mg^{2+}; 2, Ca^{2+}; 3, Cu^{2+}; 4, Co^{2+}; 5, Mn^{2+}; 6, Zn^{2+}; 7, K^+; 8, Li^+; 9, Na^+; and 10, H_2O. (**B**) Effects of different solvents on enzyme activity. 1. None; 2, DMSO; 3, ethanol; 4, isopropanol; 5, mercaptoethanol; 6, SDS; 7, EDTA; 8, DTT; and 9, PMSF. (**C**) Effects of H_2O_2 on His-*Ab*ADCS enzyme activity. 1, 5 μmol/l H_2O_2; 2, 50 μmol/l H_2O_2; 3, 500 μmol/l H_2O_2; 4, 50 μmol/l H_2O_2 + 0.9 mg/ml Hsp20; 5, 50 μmol/l H_2O_2 + 0.2 mg/ml PPI; 6, 50 μmol/l H_2O_2 + 0.25 mg/ml BCAT; and 7, 50 μmol/l H_2O_2 + 250 mmol/l DTT.

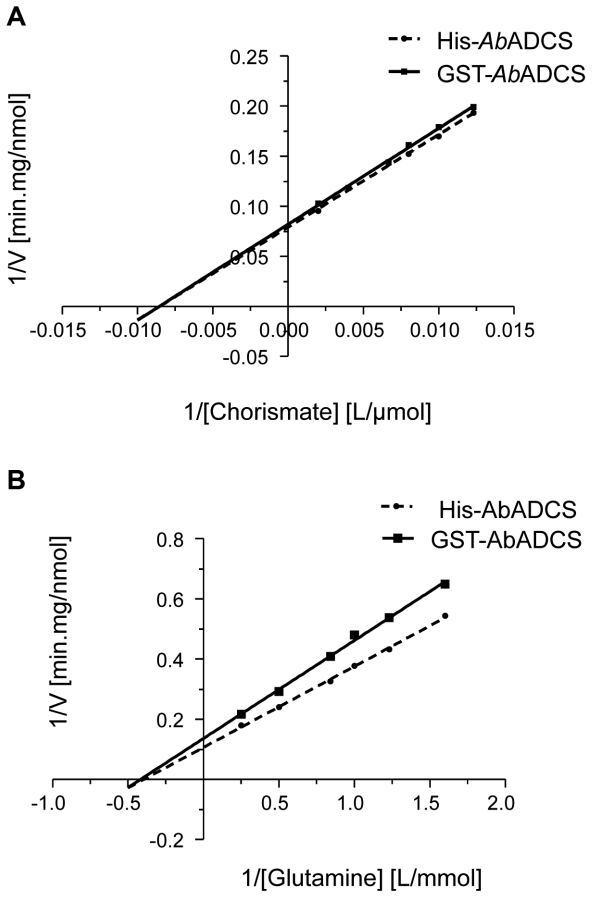

Fig. 5. Lineweaver-Burk plot of *Ab*ADCS for the kinetic analysis of the reaction rates.

(**A**) At a series of concentrations for chorismate and 5 mM glutamine. (**B**) At a series of concentrations for glutamine and 200 μM chorismate.

In our experiment, we found *Ab*ADCS to be a thermosensitive and low-efficient enzyme for binding with chorismate. The K_m value of recombinant *Ab*ADCS for chorismate was 116.8~117.0 μM, whereas its congeners from *Arabidopsis thaliana* and *E. coli* were 1.3 ± 0.2 μM and 4.2 ± 1.4 μM, respectively [21, 24]; however, their K_m values were calculated at 37°C, a different temperature. To investigate the effect of heat stress, the enzyme was incubated with H_2O_2 and recovered with other upregulated proteins. Results showed that H_2O_2 did obvious damage on the enzyme; Hsp20 only recovered a little activity of the enzyme, whereas PPI and BCAT did not recover any activity.

In conclusion, we postulate that, during the mild heat stress, the enzyme may be damaged by the ROS generated from heat stress; however, upregulated heat shock proteins cannot completely recover the activity of the enzyme. To sustain the housekeeping function of the enzyme, the mRNA of *pabS* has to be upregulated and the protein overexpressed. During further heat stress, the upregulated *Ab*ADCS cannot match the harm from heat stress, and organisms cannot survive. To disclose the relationship of expression of *Ab*ADCS with the survival rate of mushroom after heat stress, further experiments are required.

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