Molecular Cloning of the Gene for Edeine B₁ Amidinohydrolase in Addition to the Agmatinase Activity in *Bacillus subtilis*

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A gene with a high-nucleotide sequence homology to the edeine B₁ amidinohydrolase gene of *Bacillus brevis* was identified in the database of the *Bacillus subtilis* genome. The gene was isolated, expressed in *Escherichia coli*, and the gene product was analyzed with regard to the characteristics of its enzyme activity. A 32-kDa protein encoded by the *ywhG* gene showed a 69.8% amino acid sequence-homology to the edeine B₁ amidinohydrolase of *B. brevis*. Among various guanidino-compounds, edeine B₁ and agmatine were both efficiently hydrolyzed by the protein encoded by the *ywhG* gene, although edeine B₁ was a more potent substrate than agmatine in this assay system. These data indicate that the protein encoded by the *ywhG* gene is an agmatinase that is essential for polyamine biosynthesis in *B. subtilis*.

Key words edeine B₁ amidinohydrolase; agmatinase (agmatine ureohydrolase); *ywhG* (*spe B*); *Bacillus subtilis*; *Bacillus brevis*

Bacillus brevis TT02-8 produces edeine A₁ and B₁, two peptide antibiotics that inhibit the growth of Gram-positive and Gram-negative bacteria, mycoplasmas, fungi and mammalian cells.¹⁾ Conversion from edeine B₁ to edeine A₁ is conducted by edeine B₁ amidinohydrolase (EB-hydrolase) (Fig. 1). Previously, we purified EB-hydrolase from *B. brevis* TT02-8 using chromatography followed by acrylamide gel electrophoresis²⁾ and determined the amino acid sequence of the N-terminal region.²⁾ Using this sequence, we molecularly cloned the EB-hydrolase gene using a DNA fragment amplified by polymerase chain reaction (PCR) as a probe.³⁾ The gene thus obtained was characterized by predicting its amino acid sequence, and the enzymatic activity encoded by the gene was analyzed.³⁾ We searched the database of the *Bacillus subtilis* genome⁴⁾ using DNASIS (HITACHI Software, Yokohama, Japan) for a sequence that was homologous to the EB-hydrolase gene and identified the largest open reading frame (ORF), *ywhG*, in the genome. The nucleotide sequence of the ORF region was 66.7% homologous to the EB-hydrolase gene and, moreover, the deduced amino acid sequence of the ORF region was 69.8% identical to the EB-hydrolase produced by *B. brevis*. Thus, we analyzed the enzyme activity of the protein encoded by the ORF, *ywhG*.

We first tried to isolate the *ywhG* gene of *B. subtilis* using PCR. For this purpose, the chromosomal DNA of the *B. subtilis* 168 (Trp[−]) strain was prepared. As PCR primers, nucleotide sequences corresponding to the N- and C-terminal regions of the putative EB-hydrolase gene in *ywhG* were synthesized (Sawady Technology Co. Ltd., Tokyo, Japan). The sense primer, 5'-GGATCCGAATTCATGAGATTTGATGAAGCTTATTCAG3', contained the *Bam*HI-*Eco*RI cloning site followed by the region including the first ATG codon; the anti-sense primer, 5'-GTCGACTGCAGCTATTATTCA-CAAACCAAGCAGCATTTTCAC3', contained the *Sal*I-*Pst*I cloning site plus the region containing the terminator codon of the gene. Thirty-five cycles (one cycle consists of a set of 45 s at 94 °C, 60 s at 57 °C, and 2 min at 72 °C) of PCR using *Pfu* DNA polymerase (Stratagene, U.S.A.) were conducted using the Program Temp. Control System (Astec Co. Ltd., Tokyo, Japan). An amplified DNA fragment consisting of approximately 900 base pairs (bp) was obtained. The PCR

product was cloned into a pT7Blue T-Vector (Novagen, Inc., U.S.A.) using a ligation high kit (Toyobo Co. Ltd., Osaka, Japan), and the inserted DNA of the resulting plasmid was confirmed by sequencing. To construct an expression plasmid for the obtained gene, the plasmid was digested by *Eco*RI and *Pst*I, and the 870-bp fragment containing the *ywhG* sequence was ligated to the *Escherichia coli* expression plasmid, pKK223-3 (Pharmacia Biotech, Uppsala, Sweden), which had been previously digested with *Eco*RI and *Pst*I and dephosphorylated. The ligated plasmid, designated as pKSEb-14, was transformed into *E. coli* JM109 (Toyobo Co. Ltd., Osaka, Japan). The transformed *E. coli* JM109 containing pKSEb-14 was grown in an LB broth containing ampicillin (50 µg/ml) for 1 h at 37 °C; isopropylthio galactoside (IPTG; 3 mM, final concentration) was then added to the medium, and incubation was continued for another 4 h. An aliquot of the culture medium was harvested by centrifugation at 8000×*g* for 10 min at room temperature, and the cell pellet was washed twice with 50 mM Tris-HCl buffer (pH 8.0). The cell pellet was resuspended in the same buffer

Fig. 1. Chemical Structure of Edeine B₁ and A₁ and the Conversion of Edeine B₁ to A₁ by EB-Hydrolase

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(0.26 g wet cell/ml) and then disrupted by sonication at 4 °C. Unbroken cells and cell debris were removed by centrifugation at 8000×g for 10 min at 4 °C. The resulting lysate was used in the EB-hydrolase assay and was also analyzed using sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE).

A protein band of about 32-kDa was detected by SDS-PAGE analysis in all of the cells carrying pKSEb-14, regardless of IPTG induction (lanes 4, 5, Fig. 2); corresponding bands were not observed in the lysates of host *E. coli* JM109 cells (lane 1), cells transformed with pKK223-3 (lane 2), or cells cultured in the presence of IPTG (lane 3). The production of the 32-kDa protein was enhanced when IPTG was added to the culture medium (lane 5). To confirm that the 32-kDa protein was the predicted protein, the corresponding protein band was blotted onto a nylon membrane filter, and N-terminal amino acid sequencing was performed using Edman degradation and an Applied Biosystems Model 477A sequencer. The first 20 amino acids sequence of the protein were determined and confirmed to be identical to the N-ter-

минаl sequence of the deduced protein encoded by *ywhG* in *B. subtilis* (Fig. 3). The observed molecular mass, 32-kDa, of the expressed protein coincided with the molecular mass, 32.4-kDa, calculated from the amino acid composition of the deduced protein encoded by the ORF.

Next, we analyzed the enzymatic activity of this protein. Bacterial strains transformed by pKSEb-14 or an empty vector were cultured with IPTG, and the cells were pelleted by centrifugation and resuspended in 50 mM Tris-HCl buffer (pH 8.0). The crude protein lysates were prepared by sonication and analyzed for EB-hydrolase activity. To measure enzyme activity, the same amount of protein from each lysate was used. The lysates of the untransformed *E. coli* JM109 strain and of the bacteria that was transformed using an empty plasmid did not show any EB-hydrolase activity (Table 1). On the other hand, the lysate from the bacterial strain carrying pKSEb-14 exhibited EB-hydrolase activity (Table 1). This lysate was then further characterized for the specificity of the substrate. The guanidino-compounds listed in Table 1 were tested as potential substrates for the enzyme activity. The highest level of enzyme activity was observed when edeine B₁ was used as the substrate; activity was also observed when agmatine, L-arginine and L-homoarginine were used as substrates (relative activity levels to edeine B₁ of 81%, 8% and 4%, respectively). No activity was detected in lysates containing the other evaluated substrates (Table 1). These data indicate that the protein coded in the ORF, *ywhG*, exhibits agmatine ureohydrolase as well as EB-hydrolase activity. EB-hydrolase produced by *B. brevis* was most active when edeine B₁, which is a secondary metabolic product of *B. brevis*, was provided as a substrate (Table 1). The lysate from pKSEb-14-transformed *E. coli* also exhibited the highest level of enzyme activity when edeine B₁ was provided as the substrate, compared to the other substrates examined, even though *B. subtilis* is not an edeine-producing organism. Interestingly, the EB-hydrolase produced by *B. brevis* had a specific activity that was 3.0 times higher for edeine B₁ (100%) than for agmatine (33%), although the activity of the enzyme produced by *B. subtilis* was similar for edeine B₁ (100%) and agmatine (81%). Thus, the enzyme with EB-hy-

Fig. 2. SDS-PAGE Analysis of Crude Lysates from *E. coli* Transformants

Soluble protein fractions from *E. coli* were prepared as described in the text. Equal amounts of proteins (20 µg) were resolved by 10% SDS-PAGE and stained by Coomassie Brilliant Blue. Lane 1: *E. coli* JM109 carrying no plasmid; lane 2: *E. coli* JM109 carrying an empty plasmid, pKK223-3; lane 3: *E. coli* JM109 carrying pKK223-3 induced by IPTG; lane 4: *E. coli* JM109 carrying pKSEb-14; lane 5: *E. coli* JM109 carrying pKSEb-14 induced by IPTG; lane 6: Marker proteins, α-lactalbumin (14400), trypsin inhibitor (20100), carbonic anhydrase (30000), ovalbumin (43000), albumin (67000) and phosphorylase b (94000). The arrow indicates the position of EB-hydrolase.

Table 1. Amidinohydrolase Activity of Lysates Derived from *E. coli* Transformed by pKSEb-14

Substrate	OD ₅₂₀				Relative EB-hydrolase activity (%)	
	No <i>E. coli</i> lysate	<i>E. coli</i> lysate	pKK223-3- transformed <i>E. coli</i> lysate	pKSEb-14- transformed <i>E. coli</i> lysate	pKSEb-14- transformed <i>E. coli</i> lysate	EB-hydrolase of <i>B. brevis</i>
None	0.077	0.074	0.085	0.077	0	0
Edeine B ₁	0.082	0.094	0.098	1.626	100	100
L-Arginine	0.105	0.117	0.111	0.235	8	0
D-Arginine	0.109	0.109	0.106	0.105	0	0
Agmatine	0.097	0.336	0.376	1.584	81	33
Blasticidin S	0.104	0.099	0.114	0.104	0	0
L-Canavanine	0.078	0.081	0.070	0.081	0	0
Creatine	0.084	0.095	0.085	0.081	0	0
Glycocyamine	0.068	0.070	0.076	0.071	0	0
Guanidinobutyric acid	0.080	0.089	0.090	0.097	0	0
Guanidinopropionic acid	0.091	0.091	0.092	0.097	0	0
L-Homoarginine	0.389	0.401	0.405	0.458	4	2

The hydrolysis reaction solution consisted of 50 mM Tris-HCl (pH 8.0), 5 mM MnCl₂, 25 mM of each substrate, and 10 µg of the lysate protein. The reaction was carried out for 1 h at 37 °C. After stopping the reaction, the produced urea was measured using the method described by J.W. Geyer *et al.*¹⁴ The activity of amidinohydrolase in the cell lysates obtained from untransformed cells was measured and subtracted from the values obtained for the pKSEb-14-transformed lysate. The relative activity to edeine B₁ is shown as a percentage. The amidinohydrolase activity of purified EB-hydrolase is listed for reference in the right column.

<i>B. subtilis</i> SPEB	:	MRFDEAY-SGKV-FI	: 13
<i>B. brevis</i> EB-hydrolase	:	MRFDEAY-SGNV-FI	: 13
<i>E. coli</i> agmatinase	:	MSTLGHQYDNSLVSNFAGFL	: 20
<i>N. meningitidis</i> agmatinase	:	MQYSTLAGQTDNSLVSNFAGFL	: 22
Human agmatinase	:	MLRLLASGCARGPGPGVGARPAAGLFHPGRRQSRQASDAPRNQPPSPFVARPVGVCSMM	: 60
		+	+
<i>B. subtilis</i> SPEB	:	ASRPEWE--EADA--ILYGMPMDWTSYRPGSRFGPSRTREVSIQLE--EYSPYLDRLA	: 67
<i>B. brevis</i> EB-hydrolase	:	RSHGNYE--ESQA--VIYGMPMDWTSYRPGSRFGPARTREVSIQLE--EYSPYLDRLLE	: 67
<i>E. coli</i> agmatinase	:	RLPMNFQPYDSADWVITGVFDMATSGRAGGRHGPAATROVSTNLAWEHNRFPWFDMR	: 80
<i>N. meningitidis</i> agmatinase	:	RLPLNFMPYESHADWVITGVFDMATSGRAGGRHGPAATROVSTNLAWEHNRFPWFDFVR	: 82
Human agmatinase	:	RLPVQTSP-EGLDAAFI-GVPLDTGTSNRPGARFGPRRTREESVMER--TVNPSTGALPF	: 116
		+	++
<i>B. subtilis</i> SPEB	:	D-LNFFDAGDIPLPGNPQRSLDIEEYVDSILEKGFPMGMGGEHLVSWPVKAMYKKY	: 126
<i>B. brevis</i> EB-hydrolase	:	D-IKYFDAGDIPLPGNVEGSLDAIRTFVAKVLADGKFPLGLGGEHLVSWPVFAVYKEY	: 126
<i>E. coli</i> agmatinase	:	ERLNVVDCGDLVYAFGDAREMSEKLQAHAELLAAGKRMLSGGDFHVTPLLLRAHAKHF	: 140
<i>N. meningitidis</i> agmatinase	:	ERLNIIDCGDLVFSFGDSRDFVEKMEAHAGKLLSSGKRCLSLGGDFHVTPLLLRAHARYF	: 142
Human agmatinase	:	QSLMVADLGDVNVNLYNLQDSCRQIQEAYEKIVAAGCIPLTLGGDHITITPILOAMAKKH	: 176
		+	++
<i>B. subtilis</i> SPEB	:	PDLAIHFDAHTDLRVDYEGEPLSHSTPIRKAA-E-LIGPHNVYSFGTRSGMKKEFEWAK	: 184
<i>B. brevis</i> EB-hydrolase	:	KDMVVFHDAHTDLRVDYEGEYSHSTPIKKVC-N-LIGKNVYSFGTRSGMKDEFWAK	: 184
<i>E. coli</i> agmatinase	:	GKMLVHFDAHTDTYAN--GCEFDAGTMFYAPKEGLIDPNHSVQIGTIRT---EFD--K	: 192
<i>N. meningitidis</i> agmatinase	:	GKLALHFDAHTDTYDN--GCEYDAGTMFYAPKEGLIDPSRSVQIGTIRT---EHS--K	: 194
Human agmatinase	:	GPVGLLVHDAHTDTTKALGEKLYGAPFRRCDVDEGLDCKRVVQIGTRGSSTTLDPYRY	: 236
		++++*	++
<i>B. subtilis</i> SPEB	:	ENG-----MHISKFEVLEPLKEVLPLKLAGRPVYVTIDIVLDPAAHAPGTGTVDAGGI	: 236
<i>B. brevis</i> EB-hydrolase	:	EN-----MHLKYDVLEPVKQVLTIGNRPIYLTIDIVLDPAAHAPGTGTTEAGGI	: 235
<i>E. coli</i> agmatinase	:	DN--GFTVLDAQVNDRSVDDVIAQVKQIVGDMPVYLTFDIDCLDPAFAPGTGTPVIGGL	: 250
<i>N. meningitidis</i> agmatinase	:	--KLPTVLSAPKVNDSVEETVRKIKETVGNMPVYLTFDIDCLDPSFAPGTGTPVCGGL	: 252
Human agmatinase	:	NRSQGRFVLAEDCNMKSLLVPLMGEVRQMGGKPIISFDIDALDPAYAPGTGTPEIAGL	: 296
		++	++
<i>B. subtilis</i> SPEB	:	TSKELLASVHEIARSEVNVKGAADLVEVAPVYDHSQNTANTASKIIREMLLGFVK	: 291
<i>B. brevis</i> EB-hydrolase	:	TSRELLDTIHFMANNGANVIGCDLVEVAPVYDHSQNTAASKFVRELLLSFVK	: 290
<i>E. coli</i> agmatinase	:	TSDRAIKLVRGL--KDLNIVGMDVVEVAPYDQSEITALAATLALMEYIQAACKGE	: 306
<i>N. meningitidis</i> agmatinase	:	SSDRALKILRGL--TDLDIVGMDVVEVAPSYDQSDITALAGATIALEMYLQGAKKD	: 307
Human agmatinase	:	TPSQALEIIRGC--QGLNVMGCDLVEVSPPYDLSGNTALLAANLIFEMCALPKVTTV	: 352
		+	++

Fig. 3. Comparison of the Deduced Amino Acid Sequences of Proteins Corresponding to EB-Hydrolases and Agmatinase from Various Sources of *Bacillus subtilis* (PIR Accession No. H70057), *Bacillus brevis* TT02-8,³⁾ *Escherichia coli* (PIR Accession No. C42604), *Neisseria meningitidis* (PIR Accession No. B81196), and Human⁸⁾

The alignment was generated using the alignment program in the "GENETYX-MAC" ver 10.1 (Software Development Co. Ltd., Tokyo, Japan). Gaps were introduced to increase the similarity, and matching amino acids are shaded. Asterisks (*) indicate amino acid residues that are identical in all five proteins and plus signs (+) indicate conservative substitutions. The conserved histidine residue at the catalytic site is highlighted in italics. The underlines show the amino acid sequences determined by Edman degradation.

drolase activity from *B. subtilis* might have evolved differently from that of the enzyme produced in *B. brevis*. A comparative analysis of the higher order structure of these two proteins may shed light on this matter.

The EB-hydrolase produced by *B. brevis* requires Mn^{++} for the optimal enzyme activity.²⁾ Thus, we analyzed the metal ion requirements for the EB-hydrolase activity of *B. subtilis*. After adding various metal ions to dialyzed cell lysates obtained from pKSEb-14-transformed *E. coli*, the enzyme activity for edeine B_1 was measured. Mn^{++} evoked hydrolytic activity (100%), as did other divalent ions such as

Co^{++} , Ni^{++} and Zn^{++} (relative hydrolytic activities of 98, 99 and 86% compared to that of Mn^{++} , respectively). Mn^{++} is essential for the catalytic activity of agmatinase in *E. coli*⁵⁾ as well as for arginases and other amidinohydrolases from various organisms.^{6,7)} In terms of metal ion requirements, the recombinant protein produced from *ywhG* was more similar to arginase,⁷⁾ which is activated not only by Mn^{++} but also by Co^{++} and Ni^{++} , than to *E. coli* agmatinase,⁵⁾ which is strongly inhibited by Co^{++} , Ni^{++} and Zn^{++} .

Recently, agmatinase from humans was molecularly cloned, and the amino acid sequence of the enzyme was pre-

dicted.^{8,9)} The alignment of the amino acid sequences of amidinohydrolases reveals a significant sequence homology. As shown in Fig. 3, the predicted amino acid sequence of the protein encoded by *ywhG* was 69.8, 36.3, 35.2 and 34.7% homologous to amidinohydrolases in *B. brevis*,³⁾ humans,^{8,9)} *E. coli*¹⁰⁾ and *Neisseria meningitidis*,¹¹⁾ respectively. Among the conserved amino acid residues in amidinohydrolases, the histidine residue shown by an italic in Fig. 3 is critical for catalytic activity. The relevant histidine residue, which is required for enzyme activity,¹²⁾ is present at the 151st residue of the deduced protein in the EB-hydrolase of *B. subtilis*. In fact, the mutation of His at the 151st residue to another amino acid abolishes enzyme activity in *B. brevis*.³⁾

Sekowska *et al.* predicted the *ywhG* (renamed *speB*) gene for agmatinase by comparing the deduced amino acid sequence and the results of a disruption experiment on the *speB* gene.¹³⁾ Their prediction is consistent with the biochemical data described here. Our data, together with those of Sekowska's group, indicate that the gene product of *ywhG* exhibits agmatinase activity and is essential for the biosynthesis of polyamine in *B. subtilis*.

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