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Molecular Cloning and Sequencing of the Edeine B_1 Amidinohydrolase Gene of *Bacillus brevis* TT02-8 and Its Expression in *Escherichia coli*

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The gene encoding edeine B_1 amidinohydrolase from *Bacillus brevis* TT02-8 was cloned into *Escherichia coli* and its nucleotide sequence was determined. An open reading frame was identified and was found to encode a polypeptide of 289 amino acid residues with a predicted molecular weight of 32,455, which was consistent with that previously calculated for edeine B_1 amidinohydrolase purified from this bacterium. Comparison of the deduced amino acid sequence of this enzyme with other amidinohydrolases revealed the highest homology to *B. subtilis* agmatine ureohydolase. The enzymatic activity of the protein produced in *Escherichia coli* was analyzed. Three histidine residues, H-112, H-137 and H-151 in the edeine B_1 amidinohydrolase, which are highly conserved in amidinohydrolases, were changed to alanine by site-directed mutagenesis. Analysis of each of these mutants revealed that three histidine residues are important but not essential for the enzyme activity.

Edeine A_1 and B_1, produced by *Bacillus brevis* TT02-8, are peptide antibiotics, composed of five amino acids and an organic base; spermidine in edeine A_1 and guanylspermidine in edeine B_1 (Fig. 1). These antibiotics inhibit growth of Gram-positive and Gram-negative bacteria, mycoplasmas, fungi and mammalian neoplasmic cells in tissue culture. The mechanism of the action of these antibiotics is *via* inhibition of DNA and protein synthesis by binding to nucleic acids.[1)]

We purified the edeine B_1 amidinohydrolase (EB-hydrolase), which converted edeine B_1 to A_1, by successive chromatographic separations from *B. brevis* TT02-8, and characterized the enzyme activity of this protein and determined the partial *N*-terminal amino acid sequence.[2)] Analysis of this enzyme indicated significant differences from arginase,[3)] which is another amidinohydrolase of the same bacteria.

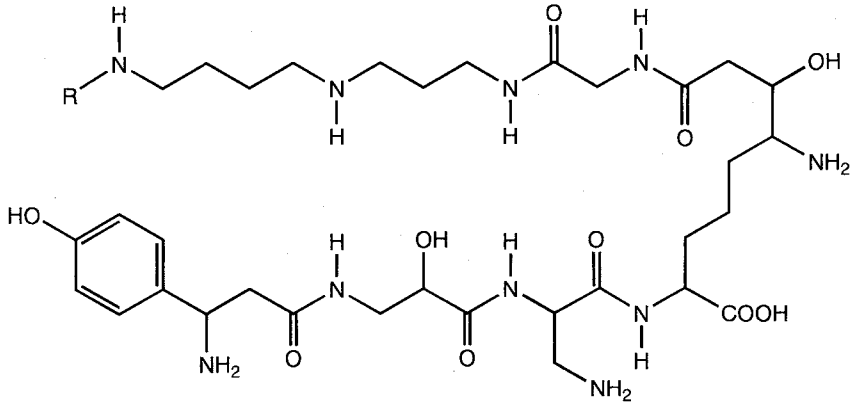
To further characterize this enzyme features, we molecularly cloned the EB-hydrolase gene by PCR using the partial amino acid sequence of this enzyme.[2)] Recombinant EB-hydrolase was ectopically expressed in *E. coli* and analyzed. Furthermore, as there is considerable evidence to suggest that a histidine or tryptophan residue plays a critical role in active-site chemistry in rat liver arginase,[29,30)] we examined which amino acid residue is critical for enzymatic activity by site-directed mutagenesis.

Materials and Methods

Strains, Plasmids and Culture Conditions

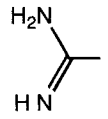
B. brevis TT02-8 strain was used as the source of EB-hydrolase and the chromosomal DNA for construction of a gene library. Culture conditions of this organism were described previously.[4)] *E. coli* JM109 [*recA1*, *Δ(lacproAB)*, *endA1*, *gyrA96*, *thi1*, *hsdR17*, *relA1*, *supE44{F′ traD36*, *proAB*, *lacI^{q}ZΔM15}*] and DH5 (*supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi1*, *relA1*) (both Toyobo Co. Ltd., Osaka) were used as host strains for expanding plasmid from the gene libraries. Plasmids pTZ19R[5)] and pUC19[6)] (both Toyobo) were used as vectors for cloning. *E. coli* JM109 and DH5 transformants bearing clones were grown on LB agar plates with 50 μg/ml of ampicillin. Plasmid pKK223-3 (Pharmacia Biotechnology, Uppsala, Sweden), which contains a *tac* promoter Shine-Dalgarno (SD) sequence,[7)] was used as a vector for the expression of the cloned EB-hydrolase gene.

Fig. 1. Structures of edeine A_1 and B_1.



Edeine A_1: R = H—

Edeine B_1: R =



DNA Isolation and Preparation of Plasmid DNA

Chromosomal DNA from *B. brevis* TT02-8 was obtained as follows. *B. brevis* TT02-8 was grown in Borowska medium without Mn^{2+} at 27°C for 48 hours, and the cells were collected by centrifugation and washed once with 100 ml of TES buffer (30 mM Tris-HCl [pH 8.0], 5 mM EDTA, and 50 mM NaCl). The cell pellet was suspended in TES buffer containing 25% sucrose and lysozyme at a final concentration of 3.3 mg/ml, and the mixture was kept at 0°C for 30 minutes. After a final concentration of 40 μg/ml proteinase K and 0.1% SDS were added, the suspension of lysed cells was gently mixed and incubated at 37°C for 3 hours. The lysate was extracted twice with an equal volume of a 24 : 24 : 1 mixture of phenol : chloroform : isoamylalcohol and finally with chloroform to remove the protein. Nucleic acids were precipitated with 0.1 volume of 2 M sodium acetate and 2.5 volumes of ethanol, dissolved in TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA), and treated with RNase A (40 μg/ml).

Plasmids used here were prepared by alkaline extraction procedure.[8] In brief, a cell suspension was treated with lysozyme (final concentration: 1 mg/ml) at room temperature for 5 minutes. SDS was added to the final concentration of 0.7% to complete lysis, and NaOH was added to the the final concentration of 0.13 M. The lysate was neutralized with potassium acetate buffer (pH 5.2) at 0°C for 10 minutes and then centrifuged for 10 minutes at 13,000×*g*. After the supernatant was treated with phenol and chloroform, nucleic acids were precipitated with an equal volume of isopropanol and the RNA in the fluid was digested with RNase A (40 μg/ml). The DNA in the fluid was analyzed for plasmids by agarose gel electrophoresis.

PCR for Isolation of the EB-Hydrolase Gene

Nucleotide sequence corresponding to the partial amino acid sequence, FDEAY (residues 3 to 7) and GNYEE (residues 17 to 21) in the *N*-terminal sequence of EB-hydrolase, identified by direct sequencing of the purified EB-hydrolase protein,[2] were synthesized by Sawady Technology Co., Ltd. (Tokyo, Japan) and used as PCR primers. The 5′TT(T/C)GA(T/C)GA(A/G)GCNTA(T/C)3′ and 5′(C/T)TC(C/T)TC(G/A)TA(G/A)TTNCC3′ oligonucleotides , in where N is a mixture of A, C, G and T, were sense and antisense primer, respectively, used to generate probe-1. Amplification by PCR was done in a reaction mixture (50 μl) containing 1 μg of the chromosomal DNA as a template, both primers (1 μM each), four deoxyribonucleoside triphosphates (NTPs, 100 μM each), about 3 units of Taq DNA polymerase (Takara Shuzo Co., Ltd., Tokyo) in 10 mM Tris-HCl buffer (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatine. The reaction was done for 30 cycles (30 seconds at 94°C;

20 seconds at 45°C; 2 minutes at 72°C) using a Program Temp Control System (Astec Co., Ltd., Tokyo). A 57-base pair DNA fragment, the predicted size for the *N*-terminal region, was obtained. The PCR amplified DNA fragment, labeled with [α-^{32}P]dCTP by a random primer labeling method, was used as a probe (probe-1).[10] to screen DNA library constructed as described below.

Construction of DNA Library

To generate a library of *B. brevis* TT02-8 genomic DNA, chromosomal DNA from *B. brevis* TT02-8 was partially digested with *Mbo*I restriction endonuclease and ligated into the dephosphorylated-*Bam*HI site of pTZ19R, and then transformed into *E. coli* JM109 (library 1).

Another library, library 2, was constructed as follows. The chromosomal DNA from *B. brevis* TT02-8 was digested with *Hind*III. DNA fragment 7.5 to 8.5 kb in length were ligated into the *Hind*III site of pUC19, and transformed into *E. coli* DH5 (library 2).

DNA Sequencing and Sequence Analysis

DNA sequence analysis was done by the dideoxynucleotide chain termination method,[10] using alkali-denatured plasmid as a template and a synthetic oligonucleotide as a primer. The oligonucleotides (5′GAGCTGACCCGCGACTA3′, 5′ATTCCACTTCCTTTTGGAAA3′, 5′TGTATACTCTTTCGGAATCC3′ and 5′CTGTGTATGACCACAGTGAA3′) were synthesized as DNA sequencing forward primers and oligonucleotides (5′ATTCAATCTCTCACCAATA3′, 5′TTCTGTTGTACCTGTTCCTGG3′, 5′GTGGGAGTATTCGTATCCC3′ and 5′ATTTGATGTCTTCCAACAGC3′) as reverse primers for determination of the EB hydrolase gene region. The sequence was determined from both strands. Nucleotides and amino acid sequences were analyzed with the programs of GENETYX MAC Software (Software Development Co., Ltd., Tokyo).

Construction of an EB-Hydrolase Expression Plasmid

A 228-bp fragment from pEB-1 digested with *Bgl*II and *Bam*HI was used as probe-2 to screen for the full-length EB-hydrolase gene as described. Three positive clones containing the complete ORF of the entire EB-hydrolase gene were obtained; pEB-2, which has insert of 4.6 kb, pEB-3, which has insert of 6.3 kb, and pEB-4 with an insert of 4.0 kb. To construct an EB-hydrolase expression plasmid, the entire coding region of the EB-hydrolase gene was recloned by PCR from pEB-2. The sense primer, 5′GGATCCGAATTCATGCGTTTTGACGAAGC3′ (Eco5′ATG), which contains *Bam*HI-*Eco*RI cloning sites and the ATG initiation region of the gene, and the antisense primer, 5′AAGCTTCTGCAGTTATTTTACAAAGCTCA3′ (Pst3′TAA), consisting of *Hind*III-*Pst*I cloning sites and the terminator region of the gene were synthesized by Sawady Technology. Thirty cycles (30 seconds at 94°C; 20 seconds at 45°C; 2 minutes at 72°C) of PCR were done using a Program Temp Control System (Astec). The PCR product with an 891-bp fragment was digested with *Pst*I and *Eco*RI, and the corresponding 873-bp fragment was extracted from an agarose gel after electrophoresis. The fragment was ligated to pKK223-3, which was previously digested with *Eco*RI and *Pst*I, and dephosphorylated. The resultant plasmid, designated pEB-5, was transformed into *E. coli* JM109 and plasmid DNA was prepared and the sequence of the insert determined.

Culture of *E. coli* Carrying the Expression Plasmid

E. coli JM109 carrying the EB-hydrolase expression plasmid was grown in 3 ml of LB medium containing ampicillin (50 μg/ml) at 37°C for 4 hours with IPTG (added after incubation for 1 hour, 3 mM final concentration). The cells were harvested by centrifugation at 10,000 rpm at room temperature for 10 minutes 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 (0.260 g wet cell/ml) and disrupted by sonication. Intact cells and cell debris were removed by centrifugation at 10,000 rpm at 4°C for 10 minutes. The resulting supernatant was used directly for characterization of the EB-hydrolase activity and a sample for SDS-PAGE as well.

Enzyme and Protein Assay

EB-hydrolase activity was measured by the method described by J. M. GEYER *et al.*[11] with a slight modification. One unit was defined as 1 μmol of urea liberated per minute from L-arginine. Protein was assayed by the Bradford methods using bovine serum albumin as a standard.

Inactivation by Chemical Modifiers

To determine which amino acid residues are essential for enzyme activity, wild-type EB-hydrolase was chemically modified. Treatment of the enzyme was carried out by addition of the indicated concentration of chemical modifiers (Table 1) such as phenylmethylsulfonylfluoride (PMSF), diethyl pyrocarbonate (DEPC), phenylglyoxal (PGO) and *N*-bromosuccinimide (NBS) to 250 μl reaction mixture containing 10 μg enzyme protein, reacted at 25°C for 30 minutes in appropriate buffer.

Mutagenesis

Site-directed mutagenesis was carried out by the method of HIGUCHI *et al.*[12] to prepare three mutants, H112A, H137A and H151A, which have mutation of His-112, -137 or -151 to Ala, respectively. The two primary rounds of PCR were carried out using a primer set of the most 5′ nucleotide sequence oligomer tagged with *Eco*RI site at the 5′ end, Eco5′ATG, and an antisense mutagenic oligonucleotide primer, 5′GAAACCAAGGCCTCTCCGCCA3′ for H-112, 5′AGGTCTGTAGCCGCGTCAAA3′ for H-137, and 5′CGGGGTGGAGGCGGAGTATT3′ for H-151, and a primer set of the most 3′ end antisense nucleotide sequence tagged with a *Pst*I site at the 3′ end, Pst3′TAA, and a mutagenic oligonucleotide primer, 5′TGGCGGAGAGGCCTTGGTTTCCT3′ for H112A, 5′TTTGACGCGGCTACAGACCT3′ for H137A, and 5′AATACTCCCACTCCACCCCG3′ for H151A, using pEB-2 as a template. The underlines show nucleotides mutated to convert His to Ala. PCR fragments were each isolated by electrophoresis on 1.2% low melting agarose gels and recovered by Geneclean II (BIO 101, INC., USA). The second round of PCR was performed using Eco5′ATG and Pst3′TAA as primers using each set of the primary PCR products as a template. The resulting full-length mutated DNA was digested with *Eco*RI and *Pst*I and ligated into *Eco*RI- and *Pst*I-digested pKK223-3, followed by transformation into *E. coli* JM109. All clones were sequenced to confirm the mutation and correct reading frame and to ensure that the PCR had not introduced any random mutations.

Enzymes and Chemicals

Restriction enzymes, calf intestine alkaline phosphatase, T_4 DNA ligation kit, and Sequenase kit were obtained from Takara, Toyobo, Amersham and Stratagene, respectively. All other reagents were of highest quality commercially available.

Results and Discussion

Cloning and Nucleotide Sequencing of the EB-Hydrolase Gene from *B. brevis* TT02-8

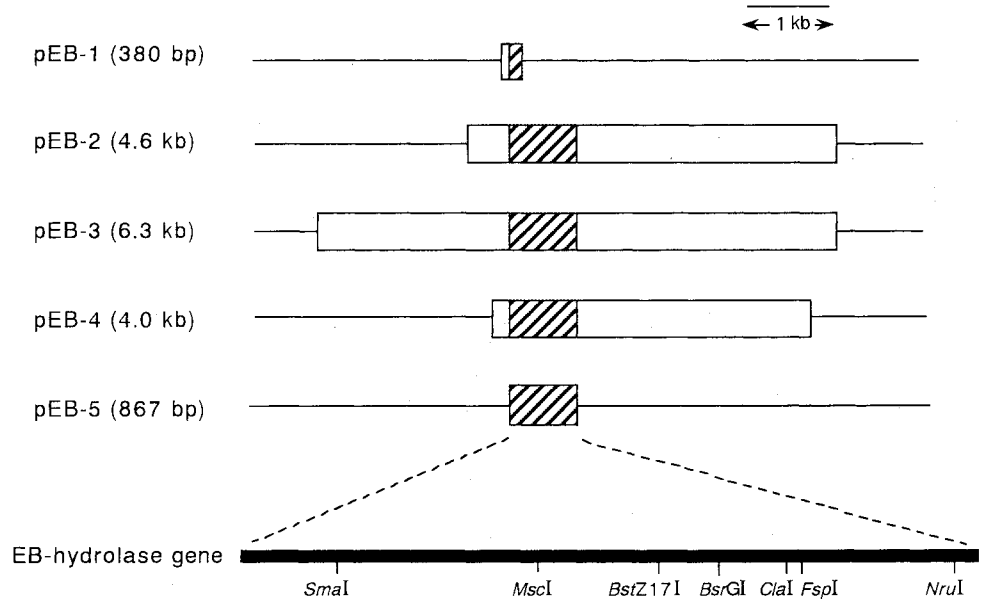
To clone the EB-hydrolase gene by PCR amplification, degenerate oligonucleotide primers were synthesized based on the partial amino acid sequences of peptide fragments derived from the purified enzyme.[2] The resulting PCR-amplified DNA fragment of 57-bp which was shown to correspond to nucleotide sequence of the *N*-terminal region of EB-hydrolase gene was used as a probe (probe-1) to screen a *B. brevis* DNA library, which was prepared from *Mbo*I partial digestion of *B. brevis* TT02-8 DNA and ligation into the *Bam*HI site of pTZ19R. One positive clone, designated pEB-1, containing an insert of 380 bp was isolated from approximately 100,000 ampicillin-resistant transformants. The pEB-1 insert was sequenced in both directions,[10] and found to cover the 5′ end of the gene, including some upstream region, the initiation codon, and 112 bp of open reading frame.

A 230-bp fragment corresponding to the *N*-terminus of EB-hydrolase (pEB-1) was prepared as a second probe (probe-2) by digestion of pEB-1 with *Bgl*II and *Bam*HI. This probe was used to isolate the entire EB-hydrolase gene and for Southern blot analysis. As we only observed a single 8-kb band in the genomic DNA digested with *Hind*III by Southern blot analysis using probe-2, the DNA fragments corresponding to 7.5~8.5 kb in size obtained by *Hind*III digestion of *B. brevis* DNA were extracted from agarose gel after electrophoresis, and cloned into pUC19 vector to construct library II.

After transformation of this DNA library into *E. coli* DH5, an approximately 44,000 ampicillin-resistant transformants were obtained, and were screened using probe-2. Three positive clones were obtained; pEB-2 (4.6-kb), pEB-3 (6.3-kb), and pEB-4 (4.0 kb) (Fig. 2). The total nucleotide sequence of the insert derived from pEB-2 was determined and the entire open reading frame encoding EB-hydrolase was identified (Fig. 3). The other two clones, pEB-3 and pEB-4, were shown to encode the full-length EB-hydrolase gene by restriction analysis.

A partial nucleotide sequence of the pEB-2 insert and deduced amino acid sequence of the *B. brevis* EB-hydrolase are shown in Fig. 3. We could identify an open reading frame which began with an ATG codon at position +1 and ended with a TAA codon at position 868, that encoded a predicted protein of 289 amino acids, with an estimated molecular mass of 32,455 Da, similar to that estimated of the purified protein on SDS-PAGE (32,000, see ref. 2 and Fig. 4). The deduced *N*-terminal 25-amino acid sequence corresponded exactly to that of purified EB-hydrolase. At −9~−17 bases upstream to the putative ATG initiation codon, there was an AG-rich region. GGAG in this AG-rich region, preceding the putative initiator ATG with a 10-bp space, was considered to be the Shine-Dalgarno sequence of the ORF. Sequences resembling the *E. coli* consensus for −35 (TTGACA) and −10 (TATAAT) promoter regions were analyzed using GENETYX MAC Software, however no such typical promoter sequences could be identified in the region from −260 nt to the initiation codon of the EB-hydrolase gene. In the termination area of the coding

Fig. 2. Schematic structure of the inserts containing EB-hydrolase gene.



Boxes and lines represent the DNA insert and the vector DNAs, respectively. Shaded boxes indicated DNA for the gene and open boxes are its flanking sequences. The protein coding region as predicted from the DNA sequence is indicated by a thick line at bottom. Restriction sites and their relative position were shown. Numbers in parantheses indicate sizes of the inserts.

sequence, there were inverted repeat sequences at positions 886~897 and 907~918. Although we did not analyze the functional role of this region, it is presumed to be involved in regulation of the termination of translation.

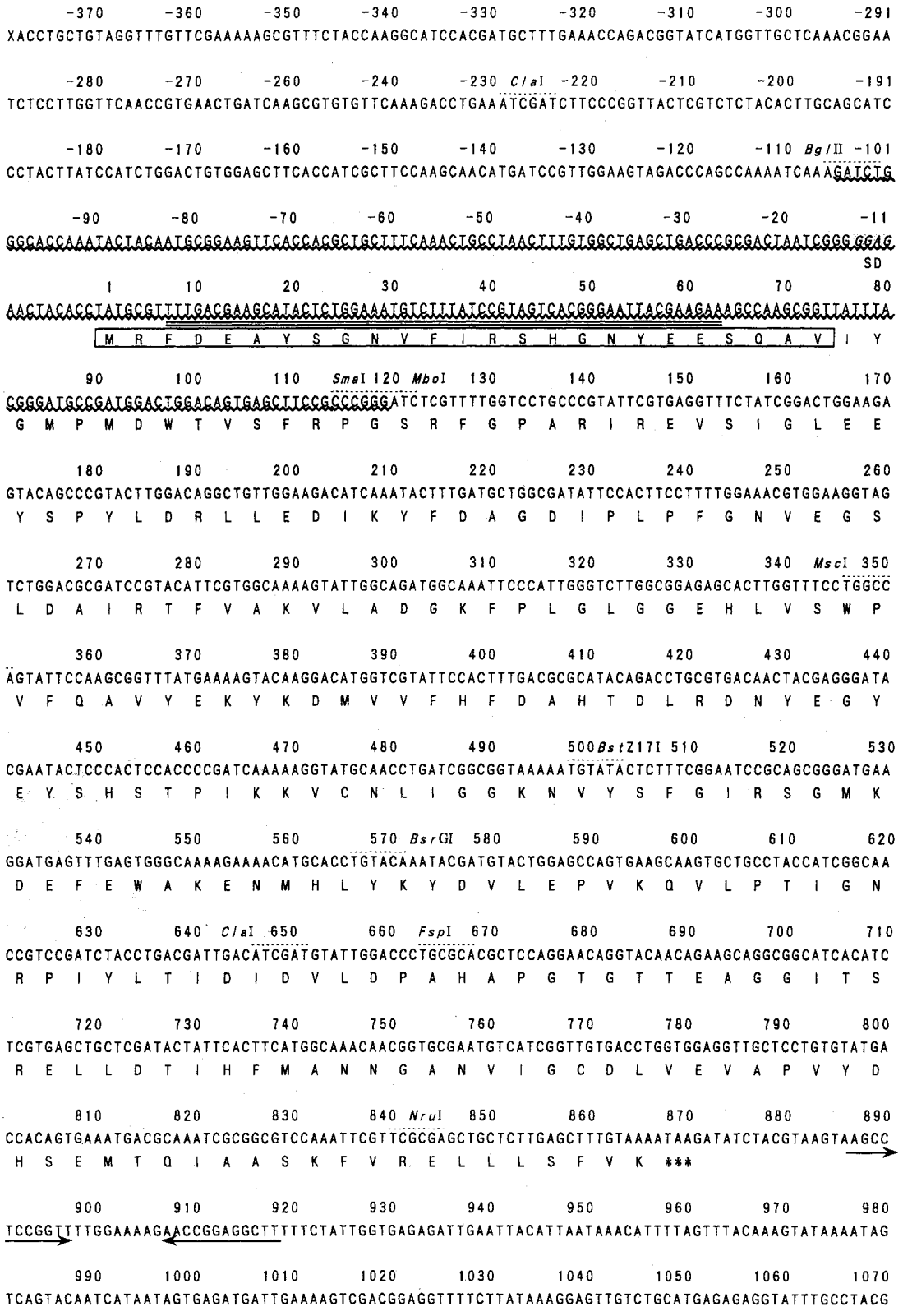
Expression of Recombinant EB-Hydrolase in *E. coli*

E. coli JM109 clones harboring pEB-5 plasmid had EB-hydrolase activity, but no EB-hydrolase activity was detected in *E. coli* JM109 cells with no plasmid or with pKK223-3 alone (data not shown). Sonicated extracts of bacterial cells harboring pEB-5 were studied by SDS-PAGE and a 32-kDa protein is clearly demonstrated. The level of the protein was increased upon treatment by IPTG (Fig. 4, lanes 1 and 2).

Comparison of Amino Acid Sequence of EB-Hydrolase with Other Amidinohydrolases

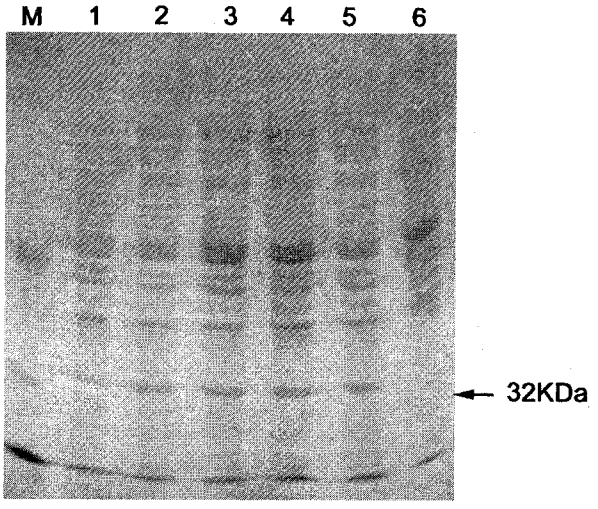
We compared the deduced amino acid sequence of EB-hydrolase with amidinohydrolases from other origins, including the arginase from *B. brevis*, which was also purified, characterized and molecularly cloned by us.[3,4] *B. brevis* EB-hydrolase shared 24% amino acid identity with *B. brevis* arginase,[4] 34% identity with agmatine ureohydrolase (AUH) from *E. coli*,[13] 32% identity with proclavaminic acid amidinohydrolase (PAH) from *Spreptomyces clavuligerus*,[14,15] 24 to 29% identity with arginases from eukaryotes and prokaryotes,[16~23] and 17 to 18% identity with creatine amidinohydrolases from *Bacillus* sp.,[24] *Flavobacterium*[25] and *Pseudomonas*.[26] We compared the sequence of the *B. brevis* EB-hydrolase gene to the complete genomic sequence of *B. subtilis*, the genome of which was fully sequenced in 1997.[27] The *B. brevis* EB-hydrolase gene showed the highest similarity to a sequence located downstream of thrZ of *B. subtilis*. It showed the highest homology (69.4%) at the amino acid level to a corresponding gene of *B. subtilis*. This *B. subtilis* gene seems to correspond *speB*,[28] relevant to *ywhG*, which was involved in polyamine biosynthesis as an agmatine ureohydrolase (AUH). EB-hydrolase, AUH, PAH and arginases from many organisms are 289 to 333 amino acid residues long, whereas creatinases are much longer ranging from 403 to 411 amino acid residues in length. The three highly homologous regions, which were suggested by SZUMANSKI,[13] between AUH and arginases, were also

Fig. 3. Nucleotide and deduced amino acid sequences of the EB-hydrolase from *Bacillus brevis* TT02-8.



The deduced amino acid sequence is given below the corresponding nucleotide sequence. Nucleotides are numbered in the 5′ to 3′ direction, beginning with the first residue of the ATG coding the initiation Met. The *N*-terminal 25 amino acid sequence which was identified from the purified protein is boxed. The predicted ribosomal binding site (SD) is shown by italic. A double underline is a sequence for probe-1 (57 nt) and a wavy line is for a probe-2 (230 nt). Restriction sites are indicated by the dotted lines. Arrows indicate inverted repeats.

Fig. 4. SDS-PAGE analysis of crude extracts from *E. coli* transformants.



Soluble protein extracts were prepared as described in Materials and Methods. Equal amounts of proteins (20 μg) were resolved by 10% SDS-PAGE and stained with 2D-Silver Stain II reagent (Daiichi, Tokyo). Lane 1: *E. coli* JM109 carrying pEB-5 before induction; lane 2: *E. coli* JM109 carrying pEB-5 induced by IPTG; lane 3: H112A mutant induced by IPTG; lane 4: H137A mutant induced by IPTG; lane 5: H151A mutant induced by IPTG; lane 6: *E. coli* JM109 carrying pKK233-3 induced by IPTG; lane M: Marker proteins; lysozyme (14,300), β-lactoglobulin (18,400), carbonic anhydrase (29,000) and ovalbumin (43,000). The arrow indicated the position of EB-hydrolase.

found in EB-hydrolase corresponding to residues 104 to 112, 134 to 141 and 223 to 236, respectively (Fig. 5). However, no such homologous sequence could be found in creatinases. Conversely the conserved sequences found in creatinases from families of *Bacillus* sp., *Flavobacterium* and *Pseudomonas*[24] could not be found in EB-hydrolase, suggesting that EB-hydrolase is more closely related to AUH, PAH and arginases, than to creatinases.

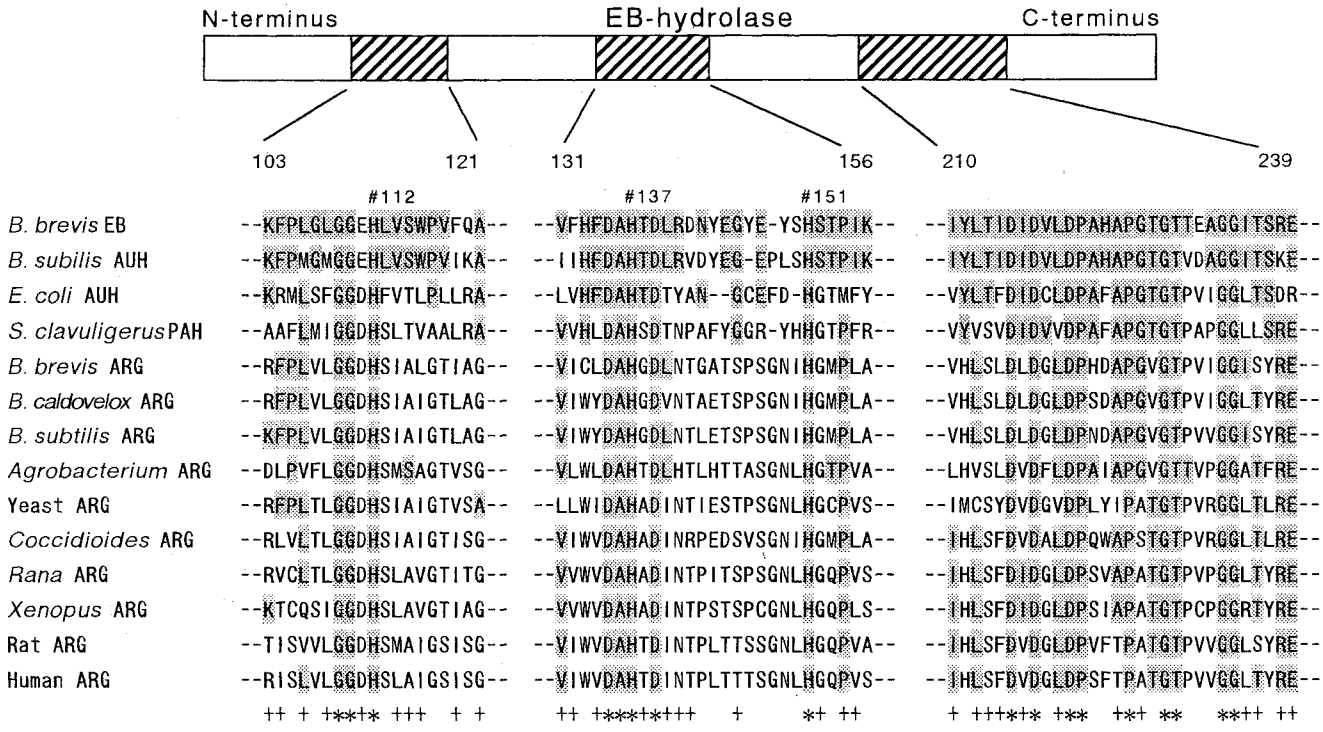
Site-directed Mutagenesis

Amidinohydrolases removes urea from substrate; edeine B_1 by EB-hydrolase, agmatine by AUH, 3-hydroxy-5-guanidino-2-(2-oxoazetidin-1-yl) pentanoic acid by PAH, and arginine by arginase. This suggests that there may be a conserved residue which binds the amidine residue of substrates and functions to produce urea as one end product. As these enzymes require metal ions such as manganese, it is assumed that another conserved residue might act as a cofactor binding as well as a substrate binding domain. BEWLEY *et al.*[23] suggested that the two histidine residues (H-101 and H-126 in the rat liver arginase) in the active site of arginase were potential ligands for the cofactor. Site-specific mutagenesis of rat liver arginase further suggested that these two histidines are involved in manganese ion binding.[29] DAGHIGH *et al.* also reported that the third histidine (H-141 in the rat liver arginase) was important for arginase function.[30] JENINSON compared the properties of arginases and their family proteins.[31] However, it is still unclear which conserved region is involved in substrate binding or in activation. To determine essential amino acid residues for enzyme activity, we used chemical modifiers which react with specific amino acid residues. DEPC, which modifies of histidine residues, and led to the loss of 94% of enzyme activity at 0.5 mM (Table 1). None of the other chemical modifiers, which variously reacted with serine/throsine, arginine or tryptophan, affected EB-hydrolase activity.

These results suggested that histidine residues play a critical role in activation of EB-hydrolase. To examine importance of histidine residues present in conserved regions of EB-hydrolase, we conducted mutagenesis analysis for this enzyme. EB-hydrolase contains three histidine residues at H-112, H-137 and H-151, which correspond to the active histidines in the rat liver arginase. Each of these histidine residues was modified to an alanine in three different EB-hydrolase mutant by site-directed mutagenesis. Each mutant was expressed in *E. coli*, resolved on SDS-PAGE (Fig. 4, lanes 3~5). Simultaneously, the enzyme activity of these enzymes were measured. The enzyme activity of the H112A, H137A and H151A mutant EB-hydrolases decreased to 20% of that of wild-type (data not shown), showed that these histidine residues are important, but are not essential, for EB-hydrolase activity. DEPC is known to a specific modifier to a histidine residue in a peptide, although tyrosine residue was also shown to be modified in some case.[32] EB-hydrolase treated with DEPC resulted in reduction of the activity to less than 10% (Table 1), suggesting that multiple histidine residues are simultaneously involved in the enzyme activity.

Additional mutagenesis analysis of the enzyme as well as a physico-chemical study including chemical modification experiment may help to clarify the role of these histidine residues of EB-hydrolase. Ultimately, three dimensional structures of EB-hydrolase and other amidinohydrolases may clarify the essential residues for enzyme activity.

Fig. 5. Comparison of conserved regions in deduced amino acid sequence of the EB-hydrolase with various amidinohydrolases.



Amino acid sequence of *Bacillus brevis* TT02-8 EB-hydrolase (*B. brevis* EB) is from Fig. 3, and that of AUH from *B. subtilis*,[27] AUH from *E. coli*,[13] PAH from *Streptomyces clavuligerus*,[14,15] arginases (ARG) from *B. brevis*,[4] *B. caldovelox*,[23] *B. subtilis*,[23] *Agrobacterium*,[21] *Coccidioides*,[22] yeast,[20] *Rana catesbeiana*,[19] *Xenopus*,[18] rat liver,[16] and human liver.[17] Gaps were introduced to increase the similarity, and matching amino acids are shaded. Asterisks (*) indicate amino acid residues identical in all amidinohydrolases and plus signs (+) indicate conservative substitutions. Histidine residues, H-112, H-137, and H-151, mutated in this work are indicated by #112, #137, and #151, respectively.

Table 1. Effects of various site-specific reagents on enzyme activity.

Reagents	Target amino acid	Buffer	Relative activity (%)	
			0.5 mM	2.0 mM
No addition	—		100	100
PMSF	Ser / Thr	10 mM Tris (pH7.4)	92	92
DEPC	His	100 mM Phosphate (pH6.5)	6	0.7
PGO	Arg	50 mM $NaHCO_3$ (pH8.8)	82	74
NBS	Trp	50 mM Acetate (pH5.1)	60	50

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