Inactivation of Blasticidin S by *Bacillus cereus*. V. Purification and Characterization of Blasticidin S-Deaminase Mediated by a Plasmid from Blasticidin S Resistant *Bacillus cereus* K55-S1

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Blasticidin S (BS) deaminase (BSR) from a BS-resistant strain, *Bacillus cereus* K55-S1, was purified to homogeneity. Molecular weights determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by gel filtration on HPLC are about 15500 and 35000, respectively, indicating the enzyme is a homodimer. The amino acid composition and N-terminal sequence of BSR are the same as those deduced from the nucleotide sequence of the BS-resistant gene, *bsr*. The optimum temperature and pH for enzyme activity are 60–65 °C and near 10.0, respectively. The activity of BSR is inhibited by Cu^{2+} , Hg^{2+} , and *p*-chloromercuric benzoate (PCMB). Inhibition by PCMB or HgCl_2 is reversible by the addition of SH reagents. The enzyme catalyzes the deamination of BS and its derivatives, but not cytosine nucleosides.

Key words *Bacillus cereus*; blasticidin S; blasticidin S-deaminase; *bsr*

Blasticidin S (BS), elaborated by *Streptomyces griseochromogenes*,¹⁾ is an aminoacyl nucleoside antibiotic that inhibits protein synthesis in prokaryotic and eukaryotic cells. The antibiotic has been used to control rice blast disease caused by *Pyricularia oryzae* (complete stage *Magnaporthe grisea*). BS is inactivated by some soil microorganisms such as *Aspergillus fumigatus*,²⁾ *A. terreus*,³⁾ and *Bacillus cereus* K55-S1,⁴⁾ which are protected against antibiotic action by the production of BS-deaminase (BSR, BS-aminohydrolase, EC 3.5.4.23), which converts BS to an inactive derivative.^{3,4)} We have isolated the BS-resistant gene, *bsr*, carried on the plasmid pBSR 8 (10.5 kb), from *Bacillus cereus* K55-S1.⁵⁾ The gene is encoded in the 640 bp *Nde*I fragment and contains a promoter region.^{6,7)} Cloning experiments in which *bsr* was utilized as a vector marker showed a wide range of expression including bacteria,^{6,8)} fungi,⁹⁾ plant¹⁰⁾ and mammalian cells.¹¹⁾

In this paper, we describe the purification and properties of BSR from *Bacillus cereus* K55-S1 and compare them with the enzyme from *A. terreus* (BSD).

MATERIALS AND METHODS

Culture Conditions *Bacillus cereus* K55-S1 was grown in peptone broth (polypeptone 1%, NaCl 0.5%, pH 7.4) supplemented with BS (200 µg/ml) at 37 °C for 18 h and used as an inoculum. Cells, transferred (2%) to the same medium containing BS (200 µg/ml), were grown at 37 °C until late log phase on a reciprocal shaker.

Enzyme Purification 1) Preparation of Crude Enzyme Extract: Bacterial cells were harvested by centrifugation at 16000 × *g* for 10 min and washed twice with saline solution. Cells, suspended in TD buffer (10 mM Tris-HCl pH 7.5–2 mM dithiothreitol (DTT) containing 20% (w/v) glycerin) (wet cells 0.35 g/ml of TD buffer), were sonicated with an ultrasonic disintegrator for 15 min at 4 °C and centrifuged at 16000 × *g* for 30 min to remove cell debris. The supernatant was used in the following purification

steps.

2) 30–65% (w/v) Ammonium Sulfate Precipitation: Solid ammonium sulfate was added gradually to the supernatant obtained as above to give a 30% (w/v) saturated solution, and the mixture was stored for 30 min. After removal of the precipitate by centrifugation (16000 × *g*, 30 min), the supernatant was adjusted to 65% (w/v) saturation by the further addition of ammonium sulfate. The pellet, collected by centrifugation, was dissolved in a minimal volume of TD buffer and dialyzed overnight against several changes of the same buffer.

3) Sephadex G-75 Column Chromatography: The enzyme solution was applied to a Sephadex G-75 column (3.0 × 90 cm) and eluted with TD buffer. Fractions containing BSR activity were collected and concentrated in a dialysis tube with Aquacide II (Calbiochem Corp.).

4) DEAE-Cellulose Column Chromatography: The enzyme solution was applied to a DEAE-cellulose column (2.8 × 45 cm) and eluted with a linear gradient of NaCl (0–1.0 M) solution. The active fractions were concentrated in the same manner and dialyzed overnight against several changes of TD buffer.

5) Affinity Column Chromatography: Based on the method of Yamaguchi *et al.*,¹²⁾ the above concentrate was applied to a CH-Sepharose 4B column containing pyrimidino-BS (BSP) as an affinity ligand. The column was washed with TD buffer and eluted with 10 mM BS solution. Fractions containing BSR activity were pooled and concentrated with Centricon 10 (Amicon Corp. Ltd.).

Assay of BSR Activity The enzyme activity was determined from the rate of decrease in OD₂₈₂ corresponding to the transformation of a cytosine moiety in the substrate to a uracil moiety in the product. The reaction mixture, containing various amounts of enzyme preparation and 1 mg/ml BS (substrate) in a final volume of 125 µl of 10 mM Tris-HCl buffer (pH 7.5), was incubated at 30 °C for various periods. At the end of each reaction period, 25 µl of reaction mixture was diluted in 1.25 ml of

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0.1 N HCl to terminate the reaction and the optical density was measured. The initial velocity was measured in quartz cuvettes with a 1 cm light path with a Hitachi UV-220 type recording spectrophotometer at 30 °C in a final volume of 0.5 ml. One unit of enzyme activity is defined as the amount of enzyme that deaminates 1 μ mol BS to deaminohydroxy-BS per min under the assay condition.

Effect of Temperature and pH on the Activity and Stability of BSR The BSR activity of samples in 10 mM Tris-HCl buffer (pH 7.5) incubated at various temperatures between 25 and 80 °C for 1 min was measured to determine the effect of temperature on the reaction rate. The thermostability of the enzyme was investigated by measuring the residual activity of the enzyme after incubation at various temperatures for 10 min in the same buffer.

To determine the effect of pH, the reaction rates of samples incubated for 2 min at 30 °C at various pH values between 4 and 10.5 were measured. Citrate phosphate buffer was used for pH 4 to 7, phosphate buffer for pH 6 to 8, Tris-HCl buffer for pH 7.5 to 9, and glycine-NaOH buffer for pH 8.6 to 10.5. All buffers were 5 mM in concentration.

Effect of Metal Ions and Inhibitors on the Activity of BSR Metal ions and inhibitors were added to the enzyme solutions in 10 mM Tris-HCl buffer (pH 7.5) before the addition of substrate. The activity was expressed as the rate relative to a control sample (without inhibitors).

Protein Determination Protein contents were determined by the method of Lowry *et al.*¹³⁾ or with a Bio-Rad Protein Reagent Concentrate Microassay kit with bovine serum albumin as the standard.

Molecular Weight Determinations To determine the molecular weight of the purified enzyme, the enzyme solution was subjected to HPLC chromatography on a TSK gel G3000SW column (0.75 $\times$ 60 cm) at a flow rate of 0.65 ml/min, using 50 mM sodium phosphate buffer (pH 7.5)-2 mM DTT containing 0.1 M NaCl. Marker proteins were bovine serum albumin (66000), ovalbumin (43000), chymotrypsin (25000), and myoglobin (18000).

The molecular weights of the subunits were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 18% gels according to the method of Laemmli.¹⁴⁾ Proteins were stained with Coomassie brilliant blue R-250 or Silverstain reagent Daiichi. Marker proteins for SDS-PAGE were obtained from BRL Co.

Amino Acid Composition and N-Terminal Amino Acid Sequence For amino acid analysis, the purified enzyme was hydrolyzed with 6 N HCl at 110 °C for 22 h. HCl was removed by evaporation and samples were analyzed with a Model L8500 Hitachi Amino Acid Analyzer.

The N-terminal amino acid sequence of purified BSR was analyzed by automatic Edman degradation using a Model 477A-120A Protein Sequencer System (Applied Biosystem Inc.).

RESULTS

Purification of BSR BSR was purified from *Bacillus cereus* K55-S1 cells by successive sonication, ammonium sulfate fractionation, gel filtration, DEAE-cellulose col-

Fig. 1. BSP-CH-Sepharose 4B Affinity Column Chromatography of BSR

BSR was eluted by 10 mM BS.

TABLE I. The Purification Steps of BSR from *B. cereus* K55-S1

Purification steps	Total activity (10 ⁷ U)	Total protein (mg)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Cell-free extract	3.66	654.9	5.59×10^4	1.0	100
30–65% (NH ₄) ₂ SO ₄	4.10	423.1	9.69×10^4	1.7	112
Sephadex G-75	2.47	132.4	1.87×10^5	3.3	67
DEAE-cellulose	1.25	62.7	1.99×10^5	3.6	34
BSP-(CH)-sepharose 4B	1.16	3.7	3.14×10^6	56.2	32

Fig. 2. SDS-PAGE of the Purified BSR from *B. cereus* K55-S1

Molecular weight markers include ovalbumin (43000), α -chymotrypsinogen (25700), β -lactoglobulin (18400), lysozyme (14300), bovine trypsin inhibitor (6200), and insulin A and B chains (3400 and 2300).

umn chromatography, and affinity chromatography. The elution profile of BSR from CH-Sepharose 4B containing BSP as a ligand is shown in Fig. 1. The enzyme was eluted efficiently from the affinity column with 10 mM BS (94% on the basis of activity). The result of a typical purification is summarized in Table I. The BSR was purified about 56-fold with a 32% recovery. The purified enzyme was unstable; after storage for one week at -20 °C in Tris-HCl

Fig. 3. Effects of Temperature (A) and pH (B) on BSR Activity

(B) —□—, citrate-phosphate buffer; —◇—, phosphate buffer; —○—, Tris-HCl buffer; —△—, glycine sodium buffer.

(pH 7.5)–2 mM DTT containing 50% glycerin, the remaining activity was 34%.

Purification of BSR from *E. coli* TK121, a BS-resistant transformant carrying a plasmid pTK17 encoding the *bsr* gene, was carried out by the same procedures as for *B. cereus* K55-S1, except the Sephadex G-75 column chromatography step was omitted. A 24.3-fold purification was obtained.

The molecular weight of native BSR purified from *B. cereus* and *E. coli* was estimated to be an approximately 35000 by HPLC gel filtration on TSK gel G3000 SW (Tosoh). SDS-PAGE analysis revealed a single band with a molecular weight of approximately 15500, indicating that the enzyme is a homodimer (Fig. 2). The isoelectric point of the enzyme was found to be 5.5 as compared with commercial IEF standards (Bio-Rad) using a Bio-Rad model 111 mini IEF system (results not shown).

The effect of temperature on enzyme reactivity was examined. The reaction proceeded most rapidly at 60–65 °C (Fig. 3a). The thermostability of BSR was tested by measuring the activity remaining after incubation of the enzyme at various temperatures for 10 min in 10 mM Tris-HCl buffer (pH 7.5). More than 70% of the activity remained after incubation at 35 °C, but the activity was completely lost following incubation at 45 °C. Figure 3b shows that BSR displays optimum activity at pH near 10. At pH values below 6, rapid inactivation of the enzyme occurs.

Effects of Enzyme Inhibitors A number of metal ions and inhibitors were tested for their effects on the activity of the purified enzyme, and the results are summarized in Table II. The enzyme activity was partially inhibited by 1 mM NiCl₂, 1 mM AlCl₃, 1 mM FeCl₃, and 10 mM monoiodo acetic acid (MIA), and almost completely inhibited by 0.1 mM CuCl₂, 1 mM ZnCl₂, 10 μM HgCl₂, and 0.1 mM *p*-chloromercuric benzoate (PCMB), but the activity was not inhibited by 1 mM EDTA or 10 mM *N*-ethylmaleimide (NEM). The inhibitions by PCMB and HgCl₂ were reversible by the addition of β-mercaptoethanol (2-ME), DTT, or glutathione (GSH). However, the

TABLE II. Effect of Metal Ions and SH Inhibitors on BSR Activity

Metal ions (1 mM)	Relative activity (%)
None	100
NaCl	107
KCl	107
LiCl	105
MgCl ₂	100
MnCl ₂	106
BaCl ₂	101
CaCl ₂	103
FeCl ₂	92
CoCl ₂	61
NiCl ₂	27
ZnCl ₂	5.2
CuCl ₂ (0.1 mM)	5.4
HgCl ₂ (0.01 mM)	6.6
AlCl ₃	11
FeCl ₃	11
EDTA	100
EDTA (10 mM)	3.8
PCMB (0.1 mM)	1.9
MIA	82
MIA (10 mM)	26
NEM	100

TABLE III. Substrate Kinetics of BSR

Substrate	K_m	V_{max}	Wave length (nm)
BS	4.76×10^{-5}	1.11	282
5-F-BS	9.10×10^{-6}	4.65	290
5-Cl-BS	7.41×10^{-5}	0.25	295
5-Br-BS	7.14×10^{-5}	0.03	302
5-CH ₃ -BS	5.00×10^{-5}	0.02	285
Leu-BS	1.72×10^{-4}	4.35	282

inhibitions by 1 mM CuCl₂ and 10 mM MIA were not reversible.

Substrate Specificity The enzyme deaminates BS and BS-derivatives (Fig. 5) to various extents as shown in Table III, while cytidine and arabinosyl-cytidine are not

Fig. 4. Structures of BS and BS Derivatives

TABLE IV. Effect of BS Derivatives on BSR Activity

BS derivative	K _i (μM)	BS derivative	K _i (μM)
5-Cl-BS	584	Deamino-BS	96.6
5-Br-BS	103	4-Thio-BS	2.2
5-CH ₃ -BS	990	N-Ethyl-BS	214
5-CH ₂ OH-BS	200		

deaminated. Table III shows the K_m and V_{max} values for BS derivatives. Some BS-derivatives with substitutions at the C-5 or N⁴ positions inhibited the deamination of BS. The K_i values of BS-derivatives were calculated as shown in Table IV.

DISCUSSION

BSR was purified from *B. cereus* cells by procedures that included affinity chromatography on Sepharose 4B coupled with the substrate analog, BSP, as a ligand.

Up to now, the enzyme has been considered to be unstable, but the purified enzyme was stable for 3 weeks at 4°C at concentrations above 200 μg/ml protein. However, at protein concentrations under 200 μg/ml in Tris-HCl (pH 7.5) buffer, the enzyme activity was only 46% of the initial level after 24 h at 4°C, and was completely lost after one week. The addition of 2 mM DTT provided partial stabilization, with 54.1% of the enzyme activity recoverable after 24 h and 18.5% after 2 weeks. These conditions were applied for short term storage at 4°C or -20°C. Enzyme stored in Tris-HCl (pH 7.5) buffer with 50% glycerol at -20°C retained 23% of its initial activity after two weeks. After storage for several days, a minor band appeared below the main band on SDS-PAGE, suggesting some conformational change or cleavage of the peptide chain, which remains to be clarified.

The characteristics of the cloned enzyme were the same as those of the original enzyme. This suggests that the drastic deletion of pBSR8 on the first cloning that occurs with the *B. subtilis* cloning system has no effect on the *bsr* gene product, BSR.

The molecular weight of the purified BSR was estimated to be approximately 35000 by gel-filtration chromatography on HPLC TSKgel G3000SW and the single band on SDS-PAGE was estimated to have a molecular weight of 15000. These values agree well with the molecular weight of 15500 deduced from the *bsr* nucleotide sequence. These results suggest the homodimeric characteristics of BSR. The isoelectric point was found to be 5.5. These properties are different from those of the enzyme from *A. terreus* (BSD).³⁾ BSD is a glycoprotein with a molecular weight of ca. 30000 as determined by sedimentation analysis and gel-filtration, and a pI of 4.35. The amino acid compositions of BSR and BSD were compared with some resemblances observed, even though BSR is richer in Glu/Gln and Pro while BSD is richer in Ser, Gly and Ala. The structural gene for BSD has been cloned and sequenced by Kimura *et al.*¹⁵⁾ who deduced 130 amino acid residues with a sequence homology to BSR from *B. cereus* of 27.2%.

The optimum temperature and pH, and the thermostability profiles of the *B. cereus* enzyme are similar to those of the *A. terreus* enzyme, *i.e.*, optimum temperature of 60–65°C, optimum pH of about 10, and complete loss of enzyme activity after heating at 45°C for 10 min.

The *B. cereus* enzyme is sensitive to sulfhydryl inhibitors, as is the case for the *A. terreus* enzyme and cytidine deaminase. The activity is significantly inhibited by mercuric ion, cupric ion, and PCMB, but this is fairly resistant to NEM and MIA. The inhibitions by these compounds are reversible by the addition of sulfhydryl compounds including GSH, 2-ME, and DTT. This suggests that a thiol group may be involved in enzyme activity, although not directly at the catalytic site.

The *B. cereus* enzyme is easily dissolved by nonspecific proteases such as actinase E, but is resistant to trypsin hydrolysis at arginine and lysine residues. This suggests that Arg and Lys residues are not located at sites exposed to catalysis nor at sites that affect the tertiary structure.

The importance of the His residue at the catalytic site has been described for rat adenosine deaminase¹⁶⁾ and *E. coli* cytidine deaminase.¹⁷⁾ Yamaguchi *et al.* assumed the participation of a histidine residue at the active site from kinetic studies on the *A. terreus* enzyme.¹²⁾ In the case of BSR from *B. cereus*, some participation of a His residue in the activity can be assumed from the fact that when the enzyme is treated with diethylpyrocarbonate, the modification of a His residue can be observed in the differential UV spectrum and the enzyme activity is lost.

Among cytosine-containing compounds, several BS derivatives and cytosine (Fig. 4) are good substrates for the enzyme, while cytidine, arabinosylcytosine, and 5-CH₂OH-BS are not substrates, suggesting that the *B. cereus* enzyme only catalyzes the deamination of BS and related derivatives, but not cytosine nucleoside deamination.

Most studies on the inactivation of antibiotics have

focussed on the mechanisms of resistance and the biosynthetic sequences. For BS, a BS-acetylating enzyme has been reported by Sugiyama *et al.*¹⁸⁾ from *Streptomyces morookaensis* JCM 4673 (*Streptomyces morookaensis* KCCS-0673), a BS-producing strain. The enzyme was presumed to protect BS activity during the biosynthetic process. The BS-deaminases (BS-deaminohydrolase) from *B. cereus* and *A. terreus*, non-producers of BS, represent cases in which a resistance mechanism or antibiotic degradation occurs in nature. The gene that confers resistance to BS in *B. cereus*, *bsr*, is encoded on a plasmid DNA, pBSR 8 (10.5 kb), while that in *A. terreus* is located in the chromosomal DNA.

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