ISOLATION AND PURIFICATION OF BLASTICIDIN S DEAMINASE
FROM *ASPERGILLUS TERREUS*

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(Received for publication September 6, 1974)

An enzyme catalyzing the deamination of the cytosine moiety of blasticidin S was extracted from a fungal strain that belongs to *Aspergillus terreus*. The enzyme was purified with ammonium sulfate fractionation, Sephadex G-100 column and DEAE cellulose column chromatography, followed by preparative polyacrylamide gel electrophoresis. Blasticidin S deaminase could be separated easily from co-existing cytidine deaminase by DEAE column chromatography or gel electrophoresis, and preliminary study on the substrate specificity showed that this enzyme acts on blasticidin S derivatives, such as cytomycin and acetylblasticidin S, but not on cytosine, cytidine, purine bases or their nucleosides. Blasticidin S deaminase could be induced by the addition of blasticidin S to the culture, and sulfhydryl compounds, such as mercaptoethanol, were effective in protecting the enzyme from inactivation. The homogeneity of the enzyme was examined by both sedimentation analysis and polyacrylamide gel electrophoresis. The molecular weight and isoelectric point were found to be around 30,000 and 4.35, respectively. Some other properties were also examined.

In the course of an investigation on the fate of blasticidin S,[1] an agricultural antibiotic, in the environment, we found that a fungal strain, isolated from a soil of paddy field, was able to decrease significantly the bioactivity of the antibiotic.[2]

The structure of blasticidin S was determined by Ōtake *et al.*[3]; it consists of cytosinine, a new nucleoside, and a β-amino acid designated as blastidic acid. As previously reported,[2] the enzymatic deamination product of blasticidin S was identified as deaminohydroxyblasticidin S[4] by TLC and UV spectra; it differs from blasticidin S in the pyrimidine base, *i.e.*, uracil instead of cytosine (Fig. 1).
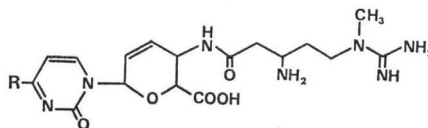
Many enzymes are known which deaminate purine or pyrimidine compounds, and the deamination of cytosine and its derivatives has been observed in various tissues and microorganisms.[5,6] Blasticidin S deaminase (blasticidin S—cytosine aminohydrolase) is also considered a deaminase of cytosine derivatives.

Fig. 1. Enzymatic transformation of blasticidin S.

Blasticidin S : R=NH₂
Deaminohydroxyblasticidin S: R=OH

In this paper, the purification and some properties of blasticidin S deaminase from *Aspergillus terreus* are described.

Materials and Methods

Identification of fungus: Colonies on Czapek-Dox's solution agar grow rather rapidly, reaching a diameter of 3.4~4.0 cm in 10 days at 25~26°C; they have shallow radial furrows, velvety, becoming floccose, with margins usually thin and irregular; heavy sporing is observed

throughout, with massed columnar heads giving the colonies their characteristic texture and color ranging from pale yellow to pale brown; abundant pale amber exudate is produced; odor is absent; the reverse is dark yellow orange to dark orange. Conidial heads are long, columnar, compact, of uniform diameter throughout their length, commonly ranging from 30 to 50 μ in diameter and 150~500 μ or more in length in about 15 days. Conidiospores are more or less flexuous, smooth, colorless, commonly 170~200 μ in length and 4.5~5.0 μ in diameter, approximately uniform in diameter throughout; vesicles are hemispherical, dome-like, commonly 10~16 μ in diameter; sterigmata are in two series: the primary sterigmata are crowded, parallel, 5.0~6.0 μ by 2.0~2.5 μ; the secondary sterigmata are closely packed, 5.5~6.0 μ by 1.5 ~2.0 μ; conidia are globose to slightly elliptical, smooth, commonly 1.5~2.0 μ in diameter.

Colonies on malt extract agar grow rapidly, attaining a diameter of 6.0~6.4 cm in 10 days at 25~26°C; they are thin plane, heavily sporulating throughout; the conidial heads are in shade of pale brown; the reverse is in pale yellowish brown shades.

From the characters described above and by comparison with an authentic strain, the fungus was identified as *Aspergillus terreus*.

Preparation of crude enzyme extract: Spores of *A. terreus* on a yeast-starch slant (0.5 % yeast extract, 2.0% soluble starch and 1.5 % agar) were inoculated into a yeast-glucose medium (0.5 % yeast extract and 2 % glucose, pH 7.0) containing blasticidin S (1 mg/ml) and incubated at 28°C for 40 hours, unless otherwise stated. A portion of the pre-cultured mycelia was transferred to a fresh medium, free of blasticidin S, and cultured at 28°C on a reciprocal shaker for about 50 hours. The cultured mycelia were collected by filtration, and washed twice with distilled water and once with 0.015 M phosphate buffer (pH 6.5).

The washed mycelia (1 volume) were ice-cooled, suspended in cold phosphate buffer (2 volumes), homogenized in a Waring blendor, and disrupted by passage through a French pressure cell (1,000 kg/cm^2) or by two treatments in a Manton Gaulin homogenizer (300 kg/cm^2).

Enzyme assay: A spectrophotometric assay, based on the differential absorption of the substrate and product, was generally used.[5] The reaction mixture contained, in a final volume of 250 μl, 0.01 M Tris-HCl buffer (pH 7.5), various amounts of enzyme preparation and substrate; the reactions were carried out at 30°C for various periods of time, and terminated by the addition of 4.75 ml of 0.1 N HCl. The decrease in optical density at 282 nm was measured against a reference in which the enzyme preparation was replaced by the buffer. Sometimes the bioassay was performed in parallel with the spectrophotometric assay; the enzyme reaction was stopped by dipping the reaction mixture in boiling water for 5 minutes; then the residual antibiotic activity was determined by the disc plate method using *Bacillus cereus* as the test organism.

Protein was determined by the method of LOWRY et al.,[7] using crystalline bovine serum albumin as a standard, or by the absorbance method (OD_{280}).

Enzyme purification: All the procedures were conducted at 4°C or below. The crude enzyme extract was centrifuged at 1,500 g for 15 minutes, and the supernatant was again centrifuged at 15,000 g for 20 minutes. To this supernatant, designated as S-15 fraction, solid ammonium sulfate was added gradually to achieve 25 % (w/v) and the precipitate was removed by centrifugation; a second precipitate was obtained by further addition of ammonium sulfate, up to 50 % (w/v); the pellet was solubilized in a minimal volume of 0.01 M Tris-HCl buffer (pH 7.5). This fraction was applied to a Sephadex G-100 column, and developed with Tris-HCl buffer containing 5 mM mercaptoethanol. In this procedure, the enzyme protein was separated from most of the foreign proteins and residual ammonium sulfate. Active fractions were combined and concentrated, if necessary, by vacuum ultrafiltration through a collodion bag, then adsorbed on a DEAE cellulose column equilibrated with the buffer described above, and fractionated by elution with linear NaCl gradient up to 0.5 M. Enzyme fractions were pooled, concentrated by ultrafiltration and are referred to as the DEAE cellulose fraction. Subsequent steps in the purification procedure were carried out by polyacrylamide gel electro-

phoresis. The DEAE cellulose fraction was dialyzed, the dialyzate applied with sucrose (20 % w/v) on a stacking gel, and a constant current of 50 mA applied, using a Toyo CD-50 preparative polyacrylamide gel electrophoresis apparatus. Tris-glycine buffer (pH 8.4) was used for the first electrophoresis, then active fractions were combined and concentrated as described above; the second electrophoresis was performed with Tris-barbital buffer (pH 7.0). Enzyme fractions were again pooled and concentrated in a collodion bag, and then dialyzed overnight against 0.01 M Tris-HCl buffer (pH 7.5). The purified enzyme preparation was usually stored at $-20°C$ in 50 % glycerin in the presence of 5 mM mercaptoethanol. No appreciable loss of the activity was observed in the preparation after 3 months of storage.

Homogeneity of the enzyme: The purified enzyme in 0.01 M Tris-HCl buffer containing 0.1 M NaCl was applied to sedimentation analysis employing a Hitachi UCA-1 analytical ultracentrifuge equipped with a synthetic boundary cell and an RA-60 rotor. The rotor speed was 55,430 rev./min. Homogeneity of the enzyme was also examined by disc-gel electrophoresis according to the method of DAVIS et al.[8] The electrophoresis was carried out at 4°C for 50 minutes at 3 mA/tube and protein bands were stained by amideblack 10 B.

Molecular weight and isoelectric point: The molecular weight was determined by ultracentrifugation according to the ARCHIBALD method[9] and by SDS polyacrylamide gel electrophoresis.[10]

The isoelectric focusing technique[11] was used to examine the isoelectric point of the enzyme. The purified enzyme protein was dialyzed against 1 % glycine overnight at 4°C before charging into an electrofocusing apparatus equipped with an LKB 8100 column. Ampholine carrier ampholites, pH 3 to 10, in sucrose density gradient were used, and focusing was carried out at a constant potential of 300V for 50 hours. Each 2-ml portion was pumped out of the column, and enzyme activity was measured at around pH 7.15.

Substrates and reagents: Blasticidin S hydrochloride and free base were kindly supplied by Kaken Chemical Co., Ltd., Tokyo. Pyrimidine or purine bases and their nucleosides were obtained either from Sigma Chemical Co. or Seikagaku Kogyo Co., Tokyo. Sephadex G-100, G-200 and blue dextran 2000 were purchased from Pharmacia Fine Chemicals and protein markers were obtained from M & S Instruments Inc. DEAE cellulose and reagents for gel electrophoresis were purchased from Seikagaku Kogyo Co. Other chemicals were of reagent grade or of the highest quality available.

Results

Aspergillus terreus produced far more enzyme when grown in the presence than in the absence of blasticidin S in the culture medium; growth was not much affected by the antibiotic, as shown in Fig. 2. Such inducing effect was also obtained with gougerotin, a similar aminohexose pyrimidine nucleoside antibiotic, but not with other

Fig. 2. Effect of blasticidin S on mycelial growth and Bc S deaminase production of *A. terreus*.

   A. terreus was grown in the presence (1,000 μg/ml) or absence of blasticidin S in yeast-glucose medium (A).

   Enzyme activity of each S-15 fraction was examined and calculated from $-\Delta OD_{282}$; one unit is that amount of enzyme which deaminates one micromole of substrate per minute (B).

   Presence of blasticidin S: (1) in the pre-culture medium, (2) in the culture medium, (3) in both media and (4) no blasticidin S.
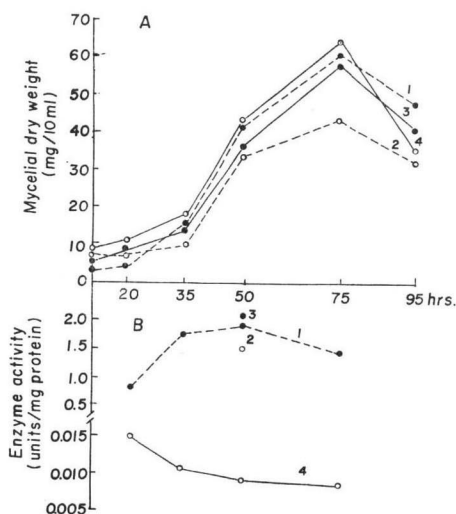
antibiotics, such as streptomycin, polyoxins, antimycin A or aabomycin, or with antimicrobial substances, such as bromouracil or phenylmercuriacetate. Neither cytosine, cytidine, nor cytosinine and cytomycin, which are derivatives of blasticidin S, gave rise to enzyme induction. The effect of the addition of blasticidin S was also observed in subcultures grown in a fresh medium free of the inducer.
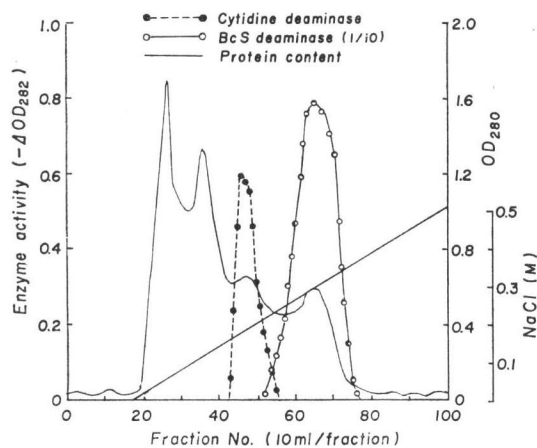
The elution profile of blasticidin S deaminase (abbreviated Bc S deaminase) from a DEAE cellulose column is shown in Fig. 3. The enzyme was eluted at a concentration of *ca.* 0.3 M sodium chloride, and clearly separated from cytidine deaminase. Though most of the inactive proteins were removed, the purification factor at this step was not so high unless the eluting buffer contained mercaptoethanol; this suggests some inactivation to have occurred.

The protein content, specific activity and recovery of the enzyme at each stage of a typical purification run are summarized in Table 1. An overall 200-fold purification was achieved, and the recovery of enzymic activity was 14%.

The homogeneity of the purified Bc S deaminase was examined by disc-gel electrophoresis and sedimentation analysis. A single band of protein, which corresponds to the active band, was observed when the enzyme was subjected to analytical disc electrophoresis in pH 9.4,[8]

Fig. 3. Elution profile of Bc S deaminase and cytidine deaminase on DEAE cellulose column chromatography.

Column size, 2.1×45 cm; flow rate, 24 ml/hr. The column was equilibrated and eluted with 0.01 M Tris-HCl buffer (pH 7.5) containing 5 mM mercaptoethanol.

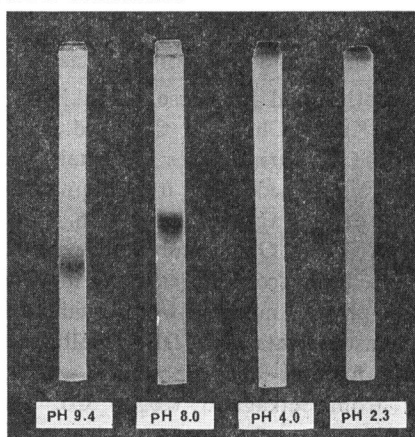Fig. 4. Polyacrylamide gel electrophoretograms of Bc S deaminase.

Table 1. Purification of Bc S deaminase from *A. terreus.**

| Fraction	Protein (mg)	Specific activity (units/mg protein)	Total activity (units)
Crude extract	13,200	1.10	14,500
S-15	5,200	1.85	9,620
Ammonium sulfate	2,200	3.10	6,820
Sephadex G-100	515	7.20	3,710
DEAE cellulose	74.5	39.50	2,940
Preparation I**	21.0	106.00	2,230
Preparation II**	9.0	220.50	1,980

* The procedures were described under Materials and Methods.
** Preparations by the first and second polyacrylamide gel electrophoresis.

8.0,[12] 4.0,[13] and 2.3 gels, as shown in Fig. 4. The enzyme moved quickly at high pH but not under acidic conditions. The homogeneity of the enzyme was also investigated by sedimentation velocity experiments using an analytical ultracentrifuge; a single symmetrical sedimentation boundary corresponding to $S_{20,w}$ value of 2.98 was observed (Fig. 5).

Fig. 5. Ultracentrifugal sedimentation patterns of Bc S deaminase.
    The concentration of the enzyme protein was 0.52% in 0.01 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 1.0 mM mercaptoethanol. The temperature was 25°C. The photographs were taken at the indicated times after reaching 55,430 rev./min. The direction of sedimentation was toward right.

10 min.   20 min.   30 min.   40 min.
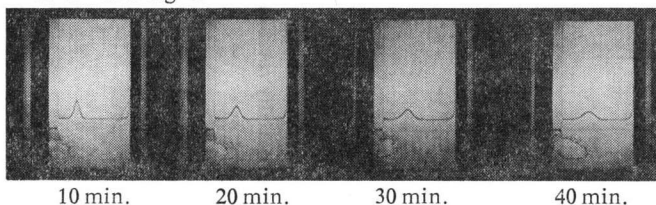
The molecular weight of the enzyme estimated from the sedimentation analysis by the ARCHIBALD method[9] was approximately 29,600 on the assumption that $1-v\rho$ is 0.2523. This value is in good agreement with that obtained by SDS polyacrylamide gel electrophoresis by the method of WEBER et al.[10] Gel filtration on a Sephadex G-200 column, however, did not afford coincidence with the value described above (see discussion). A mixture of enzyme preparation and marker proteins of known molecular weights was applied to a column (2.5 × 72 cm), equilibrated with 0.01 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, and then eluted with the same buffer, at a flow rate of 7 ml/hour. The apparent molecular weight of the enzyme was estimated to be 46,000. The elution volume corresponding to Bc S deaminase varied significantly depending on the presence or absence of NaCl; if sodium chloride was omitted from the buffer, the molecular weight was estimated to be 55,000 (Fig. 6).

The isoelectric point of the enzyme was estimated according to the electrofocusing method described in Materials and Methods, and the value obtained was approximately 4.35 (Fig. 7). Therefore this enzyme is charged with anion, and this finding coincides with the

Fig. 6. Effect of ionic strength on the elution profile of Bc S deaminase from a Sephadex G-200 column.

o——o Eluted by 0.01 M Tris-HCl buffer
•——• Eluted by 0.01 M Tris-HCl buffer with 0.1 M NaCl
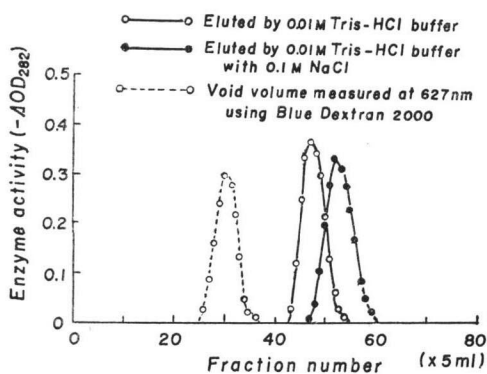o------o Void volume measured at 627nm using Blue Dextran 2000

Fig. 7. Isoelectric focusing pattern of Bc S deaminase.
    Gradient of pH and enzyme activity under pH conditions of assay were measured according to Materials and Methods.
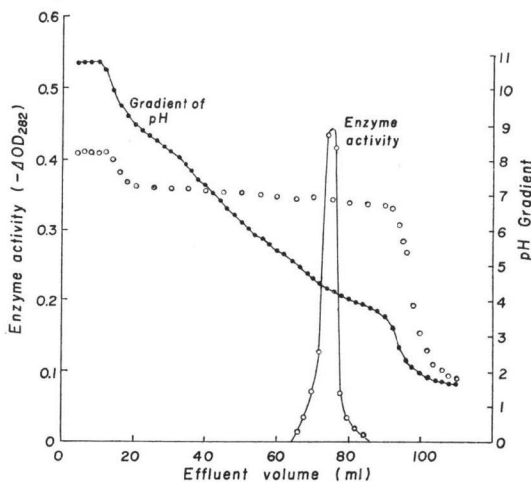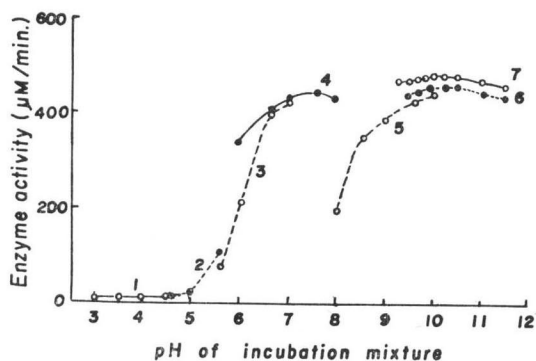
Fig. 8. Effect of pH on Bc S deaminase activity.
Buffers used: (1) formate, (2) acetate, (3) cacodylate, (4) phosphate, (5) Tris-HCl, (6) glycinate, (7) bicarbonate.

Fig. 9. Effect of mercaptoethanol and bovine serum albumin on thermal inactivation of Bc S deaminase.

The incubation mixture were held at 50°C for given periods, rapidly chilled, and remaining activity was assayed with 250 mμM blasticidin S as a substrate in the presence of 10 mM mercaptoethanol (ME) or 1 mM bovine serum albumin (BSA).
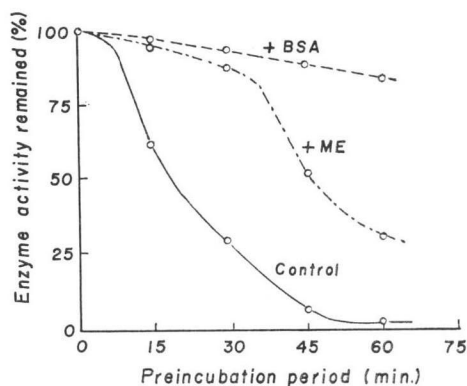

results obtained by disc gel electrophoresis.

The effect of incubation temperature on enzyme reaction was examined. The reaction was conducted at 20, 25, 30, 35, 40, 50, 60, 70 and 80°C. The reaction proceeded most rapidly at 60~70°C, and it occurred only during a short initial period at 80°C.

Fig. 8 shows the reaction rate as a function of the hydrogen ion concentration. The mixture contained 25 μl of enzyme preparation (10 μg/ml), 25 μl of 10 mM blasticidin S and 200 μl of each buffer. Buffers at constant ionic strength of 0.05 μ were used. The pH optimum was found to be near 10. At pH values lower than 4.5, rapid inactivation of the enzyme occurred.

Heat sensitivity of Bc S deaminase was tested in Tris-HCl buffer and the results are shown in Fig. 9. The enzyme was partially protected from thermal inactivation by the presence of bovine serum albumin of mercaptoethanol.

Bc S deaminase from *A. terreus* is highly sensitive to sulfhydryl inhibitors, such as mercurial

Table 2. Inhibition and reactivation of Bc S deaminase.

SH inhibitor (mM)		SH protective reagent (20 mM)			
		None	GSH	ME	DTT
None		100.0%	99.5%	100.0%	99.7%
pCMB	(0.01)	25.5	75.2	97.8	91.0
	(0.1)	0.0	58.3	58.3	59.3
HgCl₂	(0.01)	0.0	75.2	95.4	91.0
	(0.1)	0.0	69.2	91.0	81.3
CuCl₂	(0.1)	32.8	70.4	93.4	80.0
	(1.0)	0.0	20.6	37.6	47.3
NEM	(1.0)	97.1	—	—	—
	(10.0)	75.2	—	—	—
MIA	(1.0)	96.9	—	—	—
	(10.0)	81.3	—	—	—

ion, p-chloromercuribenzoate (pCMB) or cupric ion, but fairly resistant to N-ethylmaleimide (NEM) or monoiodoacetate (MIA); the activity could be recovered by the addition of sulfhydryl compounds, e.g. glutathione (GSH), mercaptoethanol (ME) or dithiothreitol (DTT) as shown in Table 2.

A preliminary study of substrate specificity was also conducted. The reaction mixture contained, in a final volume of 500 μl, different amounts of the enzyme and 250 mμM of substrate in 0.01 M Tris-HCl buffer (pH 7.5). The rate of deamination of each substrate was followed by spectrophotometric assay with a Shimazu UV 200 absorbance recording spectrophotometer. Besides blasticidin S, monoacetylblasticidin S* and cytomycin were good substrates for the enzyme, while gougerotin, cytidine or deoxycytidine were only slightly deaminated even at higher concentrations of the enzyme. In contradistinction, cytosinine, cytosine, CMP, d-CMP, adenine, adenosine, AMP, guanine, guanosine and GMP were not deaminated. Details of the substrate specificity and reaction kinetics will be reported elsewhere.

Discussion

The data presented indicate the presence in *Aspergillus terreus* of an enzyme capable of deaminating blasticidin S, an aminohexose pyrimidine nucleoside antibiotic. Blasticidin S deaminase, which is considered to be a nucleoside aminohydrolase, is found to exist in several other microorganisms, especially in several species of the genus *Aspergillus*: *A. flavipes* NHL 5013, *A. fumigatus* NHL 5009, *A. terreus* NHL 5037, *A. flavus* ATCC 9643, *A. ochraceus* 67-MI-87 and *A. ustus* 1004.

The enzyme in *A. terreus* was induced by blasticidin S and also by gougerotin, a similar pyrimidine nucleoside antibiotic, which was only slightly deaminated by the enzyme. In contradistinction, cytomycin, which is a non-antimicrobial derivative of blasticidin S and a good substrate for the enzyme, did not induce the enzyme. It has often been recognized that substances other than the normal enzyme substrate may act as enzyme inducers in microorganisms, and it appears that the specificity of enzyme induction is related to, but distinct from, the specificity of enzyme function. While many studies on the induction of penicillinase,[14,15] on microorganisms carrying R-factor resistance to chloramphenicol, streptomycin, kanamycin *etc.*,[16] and on the regulatory mechanism of protein synthesis[17] have been reported, a satisfactory interpretation of resistance and related enzyme induction in fungi has not yet been provided. In this connection, the mechanism of synthesis of Bc S deaminase and its function in the organism is worth investigating. Other experiments indicated that a resistant clone of *Pyricularia oryzae*, which was obtained by selection with blasticidin S, could not deaminate the antibiotic (YAMAGUCHI, I.: Unpublished data).

Though Bc S deaminase resembles cytidine deaminase in many respects, *e.g.*, behavior on Sephadex gels, and sensitivity to mercurial agents and others,[6] the enzyme is well separated from cytidine deaminase by DEAE cellulose chromatography and substrate specificity, which will be reported in detail in the following paper; this may imply that the enzyme is a new aminohydrolase acting on specific nucleosides.

The molecular weight of Bc S deaminase was estimated to be *ca.* 30,000 by sedimentation analysis and SDS polyacrylamide gel electrophoresis, while the results of gel filtration on a Sephadex G-200 column were not in agreement with the value described above. Preliminary experiments suggest that the anomalous gel filtration behavior of Bc S deaminase may be attributed to the presence of carbohydrates in the enzyme molecules, as reported by ANDREWS.[18] Moreover, the elution profile of Bc S deaminase on Sephadex gel is much more influenced by ionic strength than marker proteins of known molecular weight, and this may indicate that

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* SETO, H.: To be published.

higher ionic strength causes considerable conformational changes of the enzyme molecules and/or the decrease of ionic interaction of enzyme molecules and the acidic groups of Sephadex gels. The possibility that the enzyme may dissociate into subunits under conditions of higher ionic strength was ruled out, since the apparent molecular weight of Bc S deaminase, which was estimated at 46,000 from the elution volume in the presence of 0.1 M NaCl, increased to 55,000 when rechromatographed in the absence of NaCl (lower ionic strength).

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