Purification, Crystallization and Some Properties of β -Cyano-L-alanine-degrading Enzyme in *Pseudomonas* sp. 13[†]

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A β -cyano-L-alanine (β -CNAIa)-degrading enzyme in *Pseudomonas* sp. 13 was purified and isolated as a crystalline preparation. The purification procedure involved ethanol fractionation, ammonium sulfate fractionation, column chromatography on DEAE-cellulose and crystallization in the presence of ammonium sulfate, resulting in 660-fold purification with a yield of 21%.

The crystalline enzyme was homogeneous as judged by ultracentrifugation and SDS-polyacrylamide gel electrophoresis. The enzyme has a molecular weight of approximately 1,000,000, and consists of about 30 apparently identical subunits, each of a molecular weight of approximately 35,000.

The enzyme catalyzed the hydrolysis of β -CNAIa to form asparagine and aspartic acid. The enzyme reaction was inhibited strongly by thiol reagents.

β -Cyano-L-alanine (β -CNAIa) is known as an intermediate in the first step of cyanide assimilation in higher plants^{1~5)} and microorganisms,^{6~8)} and is known to be converted to amino acids in some organisms.^{3~8)} Castric *et al.*⁵⁾ have reported the detection, partial purification and determination of some properties of an enzyme hydrolyzing β -CNAIa to asparagine in lupine. As for microorganisms, *Bacillus megaterium*^{6,7)} and *Chromobacterium violaceum*⁸⁾ are known to incorporate labeled cyanide into asparagine and aspartic acid. In *E. coli*,^{9,10)} β -CNAIa is known to be converted to aspartic acid and ammonia, without formation of asparagine, by the action of asparaginase. Thus, some studies have been done on enzymes catalyzing the degradation of β -CNAIa in microorganisms. Based on the results of these studies, asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) has been used in the conversion of β -CNAIa. However, an enzyme specifically degrading β -CNAIa has not yet been found.

We previously demonstrated that

Pseudomonas sp. 13 that utilized β -CNAIa as a sole nitrogen source produced a β -CNAIa-degrading enzyme which showed neither asparaginase nor nitrile hydratase activity.¹¹⁾

The present paper describes the purification, crystallization, and some properties of a β -CNAIa-degrading enzyme from *Pseudomonas* sp. 13.

MATERIALS AND METHODS

Chemicals. All chemicals unless otherwise noted were products of Wako Pure Chemical Industries, Ltd. (Osaka), and were of certified reagent grade. β -Cyano-L-alanine (β -CNAIa) and standard proteins for the determination of molecular weight of the enzyme were obtained from Sigma Chemical Co. (St. Louis). DEAE-cellulose was obtained from Whatman (Kent). Toyopearl® HW 65 was from Toyo Soda Manufacturing Co., Ltd. (Tokyo).

Microorganism and culture conditions. *Pseudomonas* sp. 13 was used as the source of the enzyme. An aliquot from a seed culture of the organism was inoculated into 100 ml of medium containing 0.01% β -CNAIa, 1% meat extract, 1% peptone and 0.5% NaCl, pH 7.0. Incubation was carried out for 24 hr in 500 ml flasks at 30°C on a reciprocal

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shaker. The cells were harvested by centrifugation, washed twice with 0.85% NaCl solution, and suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM 2-mercaptoethanol.

Enzyme assay. β -CNAIa-degrading activity was assayed at 37°C in a mixture containing 20 μ mol of β -CNAIa, a suitable amount of the enzyme, 10 μ mol of 2-mercaptoethanol and 50 μ mol of Tris-HCl buffer, pH 8.5, in a total volume of 1.0 ml. The reaction was initiated by the addition of the enzyme solution, and terminated by heating for 1 min in a boiling water-bath. The amount of β -CNAIa was determined colorimetrically¹²⁾ or with an amino acid analyzer. One unit of activity is defined as the activity which degrades 1 μ mol β -CNAIa in 1 hr, and specific activity is the activity of 1 mg of enzyme protein.

Electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Davis.¹³⁾ Electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was performed according to the method of Weber and Osborn,¹⁴⁾ in 10% gel with the normal amount of cross-linkage at 6 mA per gel. After the run, the gel was stained with 0.1% Coomassie Brilliant Blue G-250 and destained in 7% acetic acid.

Determination of molecular weight. The molecular weight of the enzyme was determined by the method of Andrews,¹⁵⁾ using Toyopearl® HW 65 gel filtration, with ovalbumin (MW 45,000), aldolase (MW 158,000), catalase (MW 240,000), ferritin (MW 450,000) and thyroglobulin (MW 669,000) as standard proteins. The molecular weight of the subunit was determined by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn¹⁴⁾ with α -lactalbumin (MW 14,400), soybean trypsin inhibitor (MW 20,000), carbonic anhydrase (MW 30,000), ovalbumin (MW 45,000), bovine serum albumin (MW 67,000) and phosphorylase (MW 94,000) as standard proteins.

Ultracentrifugal analysis. Sedimentation behavior of the

enzyme preparation was observed at 20°C with a Beckman model E ultracentrifuge equipped with a schlieren cylindrical lens system, operating at 36,000 rev/min.

Chromatofocusing chromatography. The isoelectric point of the enzyme was determined with chromatofocusing kits (Pharmacia Fine Chemicals AB., Uppsala). The enzyme was applied to a PBE® 94 gel column equilibrated with 0.025 M imidazole-HCl buffer, pH 7.4. Then the enzyme was eluted with Polybuffer® 74-HCl buffer with a pH gradient from 7.4 to 4.0.

Other methods. The various nitriles were estimated by GLC according to the method described by Kuwahara *et al.*¹⁶⁾ Ammonia was determined with a Determiner® NH₃ kit (Kyowa Hakkô Kôgyô Co., Ltd. Tokyo). Protein was assayed by the method of Lowry *et al.*¹⁷⁾ with bovine serum albumin as the standard protein, or by absorbance at 280 nm.

RESULTS

Stability of the enzyme

The β -CNAIa-degrading enzyme from *Pseudomonas* sp. 13 was very unstable. However, some reagents such as dithiothreitol, glutathione (reduced form) and 2-mercaptoethanol were found to protect the enzyme from inactivation almost completely. Therefore, 10 mM 2-mercaptoethanol was added to the buffer used in this experiment.

Purification procedure

Table I summarizes the procedure used. All operations were performed at 4°C, unless otherwise noted, and the buffer contained 10 mM 2-mercaptoethanol.

Washed cells were suspended in 50 mM po-

TABLE I. PURIFICATION OF β -CNAIa-DEGRADING ENZYME FROM *Pseudomonas* sp. 13 CELLS

Step	Volume (ml)	Protein (mg)	Activity		Fold	Yield (%)
			(units)	(units/mg)		
Cell-free extract	2,270	68,100	340,500	5	1	100
Ethanol-fractionation (50 ~ 75% satd.)	1,110	5,100	357,000	70	14	105
(NH ₄) ₂ SO ₄ -fractionation (0 ~ 45% satd.)	140	180	277,200	1,540	308	81
DEAE-Cellulose	52	40	156,000	3,900	780	46
1st Crystallization	8	31	114,700	3,700	740	34
2nd Crystallization	8	22	72,160	3,280	656	21

tassium phosphate buffer, pH 7.5, and disrupted in a sonic oscillator (20 kHz, Kaijo Denki, Osaka). Debris was removed by centrifugation at $10,000 \times g$ for 20 min. The resultant supernatant, called the cell-free extract, was used as the crude enzyme preparation.

The cell-free extract was fractionated with ethanol and the precipitate which formed at concentrations of between 50~70% ethanol was collected as follows.

To the cell-free extract, ethanol was added slowly, keeping the temperature below 0°C , to a final concentration of 45%. After the mixture had stood at 0°C for 2 hr, the precipitate was removed by centrifugation at $15,000 \times g$ for 30 min. Ethanol was then slowly added to the supernatant to a final concentration of 75%. The precipitate was collected and dissolved in 50 mM potassium phosphate buffer, pH 7.5, containing 20% ethanol.

Solid ammonium sulfate was added to the enzyme solution to 0.45 saturation and the pH was adjusted to 7.5. After this had stood for 2 hr, the precipitate which formed was collected by centrifugation at $15,000 \times g$ for 20 min, and dissolved in 50 mM potassium phosphate buffer, pH 7.5, containing 20% ethanol.

The dialyzed enzyme solution was applied to a column (2.5×25 cm) of packed DEAE-cellulose equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 20% ethanol. The column was then washed thoroughly with the same buffer, and then the enzyme was eluted with a linear gradient of potassium chloride of from 0 to 0.9 M. Chromatography was performed at a flow-rate of 6 ml/hr and 5.7-ml fractions were collected.

The fractions containing enzyme activity were pooled and dialyzed against 50 mM potassium phosphate buffer, pH 7.5. The dialyzed enzyme solution was concentrated to 7.6 ml in a Collodion bag®, and ammonium sulfate was added gradually to the concentrated enzyme solution until faint turbidity was seen while maintaining the pH of the solution around 7.5. On standing for several days, needle-like crystals formed.

After the crystals were collected by centrifugation at $10,000 \times g$ for 20 min, they were dissolved in 50 mM potassium phosphate buffer, pH 7.5. Second crystallization was performed in the same manner as described above. A photomicrograph of the second crystals of the enzyme is shown in Fig. 1.

By these procedures the enzyme was purified about 656-fold with an about 21% yield from the cell-free extract (Table I).

Homogeneity of the enzyme

The crystalline enzyme was homogeneous according to the criteria of ultracentrifugal and electrophoretic analyses. The enzyme showed a single symmetrical peak during ultracentrifugation in 0.2 M sodium chloride, as shown in Fig. 2. In polyacrylamide gel electrophoresis, many protein bands appeared, as shown in Fig. 3A. However, in SDS-polyacrylamide gel electrophoresis, there was a

FIG. 1. Photomicrograph of Crystals of β -CNAIa-degrading Enzyme.

FIG. 2. Sedimentation Pattern of the Crystalline β -CNAIa-degrading Enzyme.

Sedimentation patterns were obtained at 4.0 mg/ml of protein in 10 mM potassium phosphate buffer, pH 7.5, containing 200 mM potassium chloride and 10 mM 2-mercaptoethanol. The pictures were taken 8, 14 and 20 min after achieving the top speed (36,000 rev/min).

FIG. 3. Disc Gel Electrophoresis of the Crystalline β -CNAIa-degrading Enzyme.

(A) Polyacrylamide gel electrophoresis. A sample of approximately 20 μ g of crystalline enzyme was applied to a 7.5% gel column and run at pH 9.4 for 40 min at 5 mA per column.

(B) SDS-polyacrylamide gel electrophoresis. The crystalline preparation (10 μ g) treated with SDS at 100°C for 5 min was applied on a 10% gel containing 0.1% SDS and run at 7 mA per column for 5 min.

FIG. 4. Determination of Sedimentation Coefficient of the β -CNAIa-degrading Enzyme.

The enzyme was used at concentrations of 1.75, 2.75 and 4.0 mg of protein per ml of 10 mM potassium phosphate buffer, pH 7.5, containing 200 mM potassium chloride and 10 mM 2-mercaptoethanol.

single band, as shown in Fig. 3B.

Physical and chemical properties of the enzyme

Sedimentation coefficient. The crystalline enzyme was centrifuged under the conditions described in the legend to Fig. 4. By extrapolation of the linear plot of $1/s$ vs. protein concentration to the intercept, the corrective sedimentation coefficient, $s_{20,w}^0$, was calculated to be 46.1S, assuming a partial specific volume of protein of 0.75.

Molecular weight. The molecular weight of the enzyme was determined by gel filtration with Toyopearl® HW 65. As shown in Fig. 5, the molecular weight of the enzyme was approximately 1,000,000. Furthermore, determination of the molecular weight of the enzyme with Sepharose 4B also gave a value of approximately 1,000,000.

The molecular weight of the enzyme subunit was determined by SDS-polyacrylamide gel electrophoresis. As shown in Figs. 6 and 3B, the molecular weight of the subunit was approximately 35,000.

Absorption spectrum. The enzyme exhibits an absorption maximum at 279 nm with a shoulder around 291 nm and a minimum at 253 nm, as shown in Fig. 7.

Isoelectric point. In order to clarify the isoelectric point of the enzyme, the purified enzyme was applied on a chromatofocusing

FIG. 5. Estimation of the Molecular Weight of the β -CNAIa-degrading Enzyme by Toyopearl® HW 65 Gel Filtration.

A column of Toyopearl® HW 65 (1.5 $\times$ 90 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 200 mM potassium chloride and 10 mM 2-mercaptoethanol was standardized with (1) ovalbumin, (2) aldolase, (3) catalase, (4) ferritin and (5) thyroglobulin.

FIG. 6. Estimation of the Molecular Weight of the β -CNAla-degrading Enzyme by SDS-gel Electrophoresis. Details of the experiment are described in METHODS. Standard proteins: (1) phosphorylase, (2) bovine serum albumin, (3) ovalbumin, (4) carbonic anhydrase, (5) soybean trypsin inhibitor and (6) α -lactalbumin.

FIG. 7. Absorption Spectrum of the β -CNAla-degrading Enzyme.

The absorption spectrum of the crystalline enzyme was measured with a Hitachi 124 spectrophotometer. The enzyme (0.75 mg of protein/ml) was first dialyzed against 50 mM potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol. The absorbance was measured using the dialyzed fluid as a blank.

column, and eluted with a pH gradient from 4.0 to 7.4. However, the enzyme was not eluted by the above treatment but was eluted with 1.0N potassium chloride, which suggests that

FIG. 8. Amino Acid Analysis of Reaction Mixtures with an Amino Acid Analyzer.

the isoelectric point of the enzyme is lower than pH 4.0, and thus the enzyme appears to be an acidic protein.

Enzyme properties

Stoichiometry of the enzyme reaction. In our previous paper,¹¹⁾ we reported that partially purified β -CNAla-degrading enzyme catalyzed the formation of aspartic acid and asparagine from β -CNAla. In order to confirm the reaction products from β -CNAla, reaction mixtures obtained with different incubation periods were analyzed with an amino acid analyzer, and the patterns are shown in Fig. 8. On the chart from the amino acid analyzer, two new peaks were observed in addition to that of

TABLE II. STOICHIOMETRY OF THE β -CNAla-DEGRADING REACTION

The reaction mixture was composed of 20 μ mol of β -CNAla, 50 μ mol of Tris-HCl buffer, pH 8.5, and the purified enzyme in a final volume of 1.0 ml. The reaction was carried out at 37°C, and the substrate and products were assayed with an amino acid analyzer.

Reaction time (min)	β -CNAla consumed (μ mol/ml)	Asparagine formed (μ mol/ml)	Aspartic acid formed (μ mol/ml)	Asparagine formed/Aspartic acid formed
0	0	0	0	
15	3.8	2.8	1.3	2.15
30	7.7	5.4	2.5	2.16
45	12.0	8.3	3.8	2.18
60	16.2	11.4	5.3	2.15

β -CNAla. These peaks were identified with authentic aspartic acid and asparagine.

The stoichiometry of the enzyme reaction was investigated with various incubation periods. When β -CNAla was subjected to the enzyme under the standard assay conditions, the sum of aspartic acid and asparagine formed was equimolar to the amount of β -CNAla degraded during the course of the reaction, as shown in Table II. Moreover, the ratio of asparagine to aspartic acid formed was constant at 2.2 during the course of the reaction. On the other hand, asparagine was not a substrate for the β -CNAla-degrading enzyme. An experiment was performed using a reaction mixture containing 20 μ mol of ammonium sulfate, 20 μ mol of L-aspartic acid, 10 μ mol of 2-mercaptoethanol, 50 μ mol of Tris-HCl buffer, pH 8.5, and enzyme in a final volume of 1.0 ml, which was incubated at 37°C for 12 hr, and amino acid analysis of the reaction mixture showed that asparagine is not formed under these conditions.

Substrate specificity. The activity of the enzyme on various substrates is shown in Table III. The degrading activity toward compounds containing nitrile groups was assayed by GLC, except that for β -CNAla which was determined by a chromatographic method, and the degrading activity toward compounds containing amide groups was assayed by determining the amount of ammonia formed. Only β -CNAla served as a substrate for the enzyme reaction.

The apparent Michaelis constant for β -

TABLE III. SUBSTRATE SPECIFICITY OF THE β -CNAla-DEGRADING ENZYME

Activities were determined according to the assay methods described in METHODS, except for the substrate changes indicated.

Substrate (20 mM)	Relative activity (%)
H ₂ NHOOCCHCH ₂ CN ^a	100
Nitrile compounds ^b	
CH ₃ CN	0
CH ₃ CH ₂ CN	0
CH ₃ CH ₂ CH ₂ CN	0
CH ₃ CH ₂ CH ₂ CN	0
CH ₂ CHCN	0
H ₂ NCH ₂ CH ₂ CN	—
NCCH ₂ CN	—
NCCH ₂ CH ₂ CN	0
NCCH ₂ CH ₂ CH ₂ CN	0
NCCH ₂ CH ₂ CH ₂ CH ₂ CN	0
C ₆ H ₅ CN	0
Amide compounds ^c	
CH ₃ CONH ₂	0
CH ₃ CH ₂ CONH ₂	0
CH ₃ CH ₂ CH ₂ CONH ₂	0
H ₂ NOCCH ₂ CONH ₂	0
H ₂ NOCCH ₂ CH ₂ CONH ₂	0
H ₂ NHOOCCHCH ₂ CONH ₂	0
H ₂ NHOOCCHCH ₂ CH ₂ CONH ₂	0
HNH ₂ NCNHCH ₂ CH ₂ CH ₂ CHNH ₂ COOH	0
H ₂ NCONH ₂	0

^a β -CNAla degraded was determined by paper chromatography.

^b Nitrile degraded was determined by GLC.

^c Ammonia formed was determined by the enzymatic method.

CNAla was determined by a Lineweaver-Burk plot to be 83 mM, as shown in Fig. 9.

Effect of pH on the activity and stability of the enzyme. As shown in Fig. 10A, the optimum pH of the β -CNAla-degrading enzyme reaction was around 9.5 in Tris-HCl buffer.

The purified enzyme in various buffers (50 mM) containing 10 mM 2-mercaptoethanol was incubated at various pHs for 3 hr at 37°C, and then residual activity was assayed. The results shown in Fig. 10B indicate that the enzyme was stable between pH 7 to 11.

FIG. 9. Effect of Substrate Concentration on the β -CNAla-degrading Activity.

The β -CNAla-degrading activity was assayed as described in METHODS except that the concentration of β -CNAla was varied.

However, in the absence of 2-mercaptoethanol the enzyme was inactivated completely under the same conditions.

Effect of temperature on the activity and stability of the enzyme. Enzyme activity was determined at various temperatures under the standard conditions. The optimum temperature of the enzyme reaction is shown in Fig. 11A, and the highest reaction rate was observed around 55°C.

The enzyme in 50 mM potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol was incubated for 10 min at various temperatures, and then the residual activity was measured. As shown in Fig. 11B, the enzyme was stable up to 60°C.

Effect of enzyme inhibitors. The effects of some compounds generally used as enzyme inhibitors were examined in the presence of 0.2 mM and 10 mM 2-mercaptoethanol, and the results are shown in Table IV. Mercuric compounds such as pCMB (*p*-chloromercuribenzoate), pCMPSA (*p*-chloromercuriphenyl sulfonic acid) and mersalyl acid appeared to inhibit the enzyme reaction. The effects of various metal ions on the enzyme activity were also examined, and the results are shown in Table V. The enzyme activity was inhibited by some metal ions, such as Ag^+ ,

FIG. 10. Effect of pH on the β -CNAla-degrading Activity.

(A) Optimum pH. Enzyme activity was assayed according to the procedures described in METHODS, except for changes in pH as indicated in the figure.

(B) pH stability. The enzyme solution was incubated at 37°C for 3 hr at the pHs indicated in the figure. The residual activity was determined as described. The buffers used were: (—○—), phosphate; (—△—), Tris-HCl; (—□—), borate.

FIG. 11. Effect of Temperature on the β -CNAla-degrading Activity.

(A) Optimum temperature. The reaction was allowed to proceed at the indicated temperatures for 5 min, and the amount of β -CNAla degraded was assayed.

(B) Thermal stability. The enzyme, dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM 2-mercaptoethanol, was incubated for 10 min at the temperatures indicated in the figure. The residual β -CNAla-degrading activity was assayed as described.

TABLE IV. EFFECT OF INHIBITORS ON THE β -CNAla-DEGRADING ENZYME

The enzyme, dissolved in 50 mM Tris-HCl buffer, pH 8.5, containing 10 or 0.2 mM 2-mercaptoethanol, was incubated for 5 min at 37°C with the listed reagents. The residual β -CNAla-degrading activity was assayed as described.

Compound (1 mM)	Residual activity (%)	
	Containing 2-mercaptoethanol 10 mM	0.2 mM
None	100	100
NaNO ₂	100	60
NaF	100	70
NaN ₃	100	98
α,α' -Dipyridyl	100	63
Iodoacetic acid	103	70
<i>o</i> -Phenanthroline	103	88
2-Mercaptoethanol	97	88
<i>N</i> -Ethylmaleimide	103	74
pCMB	39	0
pCMPSA	52	14
Mersalyl acid	103	14
Na ₂ HA ₃ O ₄	94	74
EDTA	90	94

TABLE V. EFFECT OF METAL SALTS ON THE β -CNAla-DEGRADING ENZYME

The enzyme, dissolved in 50 mM Tris-HCl buffer, pH 8.5, containing 10 or 0.2 mM 2-mercaptoethanol, was incubated for 5 min at 37°C with the compounds indicated, and then the substrate was added. The residual β -CNAla-degrading activity was assayed as described.

Metal salt (1 mM)	Residual activity (%)	
	Containing 2-mercaptoethanol 10 mM	0.2 mM
None	100	100
AgNO ₃	103	0
BaCl ₂	88	91
CaCl ₂	78	103
CdCl ₂	83	18
CoAc ₂	58	53
CuSO ₄	50	26
FeCl ₂	90	100
FeCl ₃	80	138
HgAc	83	12
HgCl ₂	80	6
MgCl ₂	90	85
MnCl ₂	120	100
NiCl ₂	40	82
PbCl ₂	58	97
SnCl ₂	95	106
KCl	90	138
NaCl	75	106

Cd²⁺, Cu²⁺ and Hg²⁺. However, their inhibiting activities were decreased in the presence of 10 mM 2-mercaptoethanol.

DISCUSSION

In the present work, the purification, crystallization and determination of some properties of the β -CNAld-degrading enzyme from a strain of *Pseudomonas* sp. 13 were performed. Judging from analyses by SDS-polyacrylamide gel electrophoresis and ultracentrifugation, the enzyme preparation obtained was a homogeneous protein. However, the specific activity of the β -CNAld-degrading enzyme was observed to be decreased at the crystallization and recrystallization steps in the presence of ammonium sulfate. These phenomena were presumed to be due to the following; judging from that thiol reagents such as AgNO_3 , HgCl_2 , pCMB and pCMPSA markedly inhibited the enzyme reaction, the enzyme seems to have a sulfhydryl group at the active site. Therefore, the enzyme was inactivated with the trace amount of a heavy metal ion contained in ammonium sulfate or by the oxidation at the crystallization step. The molecular weight of the enzyme was determined by gel filtration to be approximately 1,000,000. The enzyme exhibited a single protein band on SDS-polyacrylamide gel electrophoresis and was composed of apparently identical subunits of a molecular weight of 35,000. On the other hand, on polyacrylamide gel electrophoresis, the enzyme preparation exhibited many protein bands. These results suggested that the crystalline β -CNAld-degrading enzyme, which seems to be obtained by polymerization of a low molecular weight enzyme under the purifi-

cation procedure conditions, was disaggregated to form various molecular weight or various electrical charged proteins under the gel electrophoresis conditions, and that such the proteins were electrophoresed successively on the polyacrylamide gel.

The enzyme was observed to have strict substrate specificity for β -CNAld and catalyzed its degradation to form both asparagine and aspartic acid, but catalyzed neither the formation of aspartic acid from asparagine nor the condensation of aspartic acid and ammonia to form asparagine.

Two enzymes concerned in the degradation of β -CNAld have been found: One, called β -CNAld hydrolase,⁵⁾ has been found in blue lupine and has been partially purified. This enzyme catalyzes the degradation of β -CNAld to form asparagine, but shows neither asparaginase, glutaminase nor nitrilase activity. The other enzyme is an asparaginase produced by *E. coli*,^{9,10)} which catalyzes the hydrolysis of β -CNAld as well as the hydrolysis of asparagine. The products with this enzyme are aspartic acid and ammonia. Judging from its substrate specificity and reaction products, our enzyme seems to be a novel β -CNAld-degrading enzyme which should be called β -CNAld nitrilase.

Our enzyme catalyzes the hydrolysis of nitrile groups to form carboxylic groups and their amides. Similar phenomena have been found for recinine nitrilase, which catalyzes the hydrolysis of the nitrile group of recinine to form ammonia, carboxylic acid and amide (Hook

SCHEME 1. Possible Mechanism of the Degradation of Nitrile.

and Robinson¹⁸⁾). They have proposed a mechanism for recinine nitrilase, shown in Scheme 1: A nucleophilic attack by the enzyme (EXH, in which X might be either an oxygen or sulfur atom) on the nitrile would lead to the formation of an enzyme-imino ether which could be hydrated to form a tetrahedral intermediate (I). In the presence of basic (B^-) and acidic (HA) groups on the enzyme, ammonia would be liberated from (I) with the formation of an acyl-enzyme (Equation II). Hydrolysis of the acyl-enzyme would liberate the acid and enzyme. A second reaction which intermediate (I) might undergo is represented by Equation III. In this case, the enzyme, rather than ammonia, is the leaving group and the products of the reaction are the enzyme and an amide.

From our observation that our enzyme formed aspartic acid and asparagine as the reaction products from β -CNAla, and the thiol group of the enzyme participated in the enzyme reaction, the degradation of β -CNAla by our enzyme is presumed to be by a similar mechanism to that for recinine nitrilase. However, the reaction mechanism of the formation of aspartic acid and asparagine from β -CNAla at the ratio of 1:2.2 remained unclear. Detailed investigations of the mechanism for our enzyme reaction are now in progress and the results will be presented elsewhere.

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