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Purification and Properties of "N^{α}-Benzyloxycarbonyl Amino Acid Urethane Hydrolase III" from *Lactobacillus casei* ε ATCC 7469[1)]

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"N^{α}-Benzyloxycarbonyl amino acid urethane hydrolase III" was purified from the cell-free extract of *Lactobacillus casei* ε ATCC 7469. The enzyme was purified 583-fold with an activity yield of 50%, and its homogeneity was confirmed by polyacrylamide gel electrophoresis. The molecular weight of the native enzyme is about 230000, and the isoelectric point is 4.60. The enzyme activity was inhibited by *p*-chloromercuribenzoic acid (PCMB) and dithiothreitol (DTT). The presence of divalent cations (*i.e.*, Co^{2+} or Zn^{2+}) is essential for its activity. The enzyme catalyzed the hydrolysis of the urethane bonds of Z–Gly, Z–Ala and Z–Ser.

Keywords——urethane hydrolase III; urethane bond; hydrolytic enzyme; Z–Ser; Z–Ala; Z–Gly; *Lactobacillus casei* ε ATCC 7469; purification; property

Previously we have reported that a new enzyme, designated "N^{α}-benzyloxycarbonyl amino acid urethane hydrolase (urethane hydrolase) I and II", catalyzes the hydrolysis of the urethane bond of Z-amino acids to give equimolar benzyl alcohol and amino acid. Urethane hydrolase I, which catalyzes the hydrolysis of Z–Gly and Z–Ala, was purified from *Streptococcus faecalis* R ATCC 8043.[2)] Urethane hydrolase II, which catalyzes the hydrolysis of Z–Arg, was purified from *Lactobacillus fermenti* 36 ATCC 9338.[3)]

Subsequent investigations revealed that *Lactobacillus casei* ε ATCC 7469 has hydrolytic activity toward the urethane bonds of Z–Gly, Z–Ala, Z–Ser and Z–Arg. The hydrolysis of the four Z-amino acids was catalyzed by two enzymes; one showed hydrolytic activity toward Z–Gly, Z–Ala and Z–Ser, and the other toward Z–Arg. In this paper, we deal with the purification of the enzyme from *Lactobacillus casei* ε ATCC 7469 which catalyzes the hydrolysis of Z–Gly, Z–Ala and Z–Ser. The enzyme was named "N^{α}-benzyloxycarbonyl amino acid urethane hydrolase III (urethane hydrolase III)", and was characterized by comparing it with urethane hydrolase I.

Materials and Methods

Materials——*Lactobacillus casei* ε ATCC 7469 used in the present experiments was a stock culture maintained in this laboratory. Bactotryptone (Difco Laboratories, U.S.A.) and casamino acids (Nissui Seiyaku, Japan) were used as medium components. Z(*p*-OCH_3)–Gly and Bz(*o,m,p*-OH)–Gly were synthesized as described previously.[2c)] The other substrates were purchased from the Protein Reseach Foundation (Osaka, Japan) or Sigma. Reference proteins (thyroglobulin, catalase and aldolase), DEAE-Sephadex A-50, Sephadex G-150 and Sephacryl S-300 were products of Pharmacia Fine Chemicals. AH-Sepharose 4B was prepared according to the method of Cuatrecasas.[4)] Other chemicals were commercial products.

Determination of Enzyme Activity——Urethane hydrolase activity was determined by the ninhydrin method,[5)]

measuring the amount of released amino groups. The reaction was performed in a mixture containing 1 μmol of Z–Gly, 1 μmol of Co^{2+} and about 10^{-9} unit of the enzyme in a total volume of 1.0 ml buffered with 0.05 M phosphate buffer, pH 6.2. The reaction was carried out at 50 °C for 10 min. The K_m and V_{max} values for substrates were estimated by means of Lineweaver–Burk plots.[6] The substrate concentrations used in the kinetic study ranged from 0.05 to 5 mM. One unit of enzyme activity was defined as the amount of the enzyme which catalyzes the hydrolysis of 1 mol of substrate (Z–Gly) per min under the standard conditions.

Analytical Determinations——Protein concentration was estimated spectrophotometrically by measuring the absorbance at 280 nm, assuming that the absorbance at the concentration of 1.0 mg/ml is 1.0 (light path: 10 mm). The molecular weight of the enzyme was estimated by the methods of Andrews,[7] using a column (2.6 × 66 cm) of Sepharose 6B in 0.05 M sodium phosphate buffer, pH 6.8, containing 0.1 M NaCl. The molecular weight of the denatured and reduced enzyme was determined by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn.[8] Electrophoresis in polyacrylamide gel was carried out according to the method of Davis.[9] Isoelectric focusing was carried out on Agarose IEF (Pharmacia Fine Chemicals, pH 3—10) in the presence of Pharmalyte (Pharmacia Fine Chemicals) carrier. Focusing was run at 8—10 W and 10 °C.

Results

Cultivation and Harvesting of the Microorganisms

For the preparation of cells, a semisynthetic medium,[10] consisting of casamino acids, Bactotryptone, vitamins, purine bases, glucose and inorganic compounds, was incubated with 1% of an active culture of *Lactobacillus casei* ε ATCC 7469 cultured in the same medium. The organisms was grown anaerobically at 37 °C for 16 h. The cells were harvested by centrifugation (5000 rpm, 10 min).

Purification of Urethane Hydrolase III

All purification procedures were performed at 0—5 °C, unless otherwise specified. Washed cells (wet weight 1.16 kg), obtained from 100 l of culture broth, were suspended in 1 l of the 0.1 M sodium phosphate buffer, pH 6.8, and then continuously disrupted for 60 min in a Vibrogen cell-mill (Edmund Bühler, Tübingen, West Germany) with 0.1 mm glass beads. The cell debris was removed by centrifugation (6000 rpm, 10 min). The resulting supernatant was purified as follows. The cell-free extracts (1500 ml) were saturated with ammonium sulfate (40—70% saturation). The precipitate was collected by centrifugation (9000 rpm, 20 min), then dissolved in 100 ml of the 0.01 M sodium phosphate buffer, pH 6.8, and dialyzed against the same buffer. The enzyme solution (110 ml) was applied to a column (7 × 86 cm) of DEAE-Sephadex A-50, previously equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. The enzyme was eluted with a linear gradient of NaCl concentration up to 1 M in the same buffer. The flow rate was 40 ml/h and 2.5 ml fractions were collected. This chromatography separated two activities, one for the hydrolysis of Z–Gly and the other for the hydrolysis of Z–Agr (Fig. 1).

The fractions showing hydrolytic activity toward Z–Gly were pooled and concentrated by ultrafiltration with an Amicon PM-10 membrane. The enzyme solution (145 ml) was applied to a column (3 × 58 cm) of AH-Sepharose 4B, equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. After purging of unadsorbed materials with the same buffer, elution was done with a linear gradient of NaCl concentration up to 0.5 M in the same buffer. The enzyme activity was detected in the fractions of 0.1 M sodium phosphate buffer, pH 6.8, containing 0.2 M NaCl. The active fractions were pooled, concentrated and then thoroughly dialyzed against 0.01 M sodium phosphate buffer, pH 6.8. The enzyme solution (29 ml) was applied to a column (5 × 88 cm) of Sephadex G-150 equilibrated with 0.01 M sodium phosphate buffer, pH 6.8, containing 0.1 M NaCl. The active fractions were pooled and then concentrated. The concentrated enzyme solution (34 ml) was again chromatographed on a column (5 × 70 cm) of DEAE-Sephadex A-50, by the same method as used in the first DEAE-Sephadex A-50 chromatography. The active eluates were pooled, concentrated and then dialyzed against 0.01 M sodium phosphate buffer, pH 6.8. The dialyzed enzyme solution

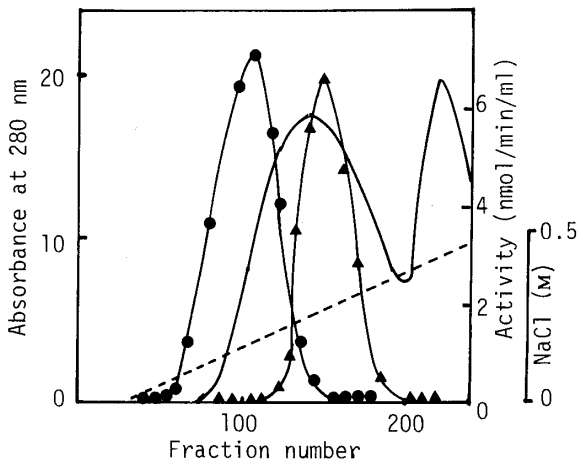

Fig. 1. First DEAE-Sephadex A-50 Column Chromatography of Urethane Hydrolase of *Lactobacillus casei* ε ATCC 7469

●—●, enzyme activity toward Z–Gly; ▲—▲, enzyme activity toward Z–Arg; ——, absorbance at 280 nm; ------, NaCl concentration.

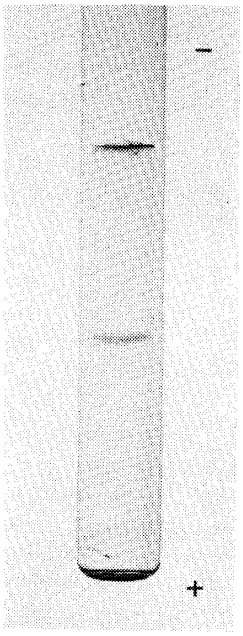

Fig. 2. Disc Electrophoresis of the Urethane Hydrolase from *Lactobacillus casei* ε ATCC 7469 at the Final Purification Step

Electrophoresis was carried out on 7% (w/v) polyacrylamide gel with 0.05 M Tris–HCl buffer, pH 8.0, and at 2 mA per tube for 60 min. The gel was stained with 0.2% Coomassie Brilliant Blue R-250 for protein detection.

TABLE I. Summary of the Purification of "N^{α}-Benzyloxycarbonyl Amino Acid Urethane Hydrolase III" from *Lactobacillus casei* ε ATCC 7469

	Total protein (mg)	Total activity ($\times 10^3$ U)	Specific activity ($\times 10^3$ U/mg)	Recovery (%)
Cell-free extract	15100	—	—	—
Salting out	10300	125	0.01	100
DEAE-Sephadex A-50 (1st)	1260	117	0.09	93.5
AH-Sepharose 4B	460	97	0.21	77.5
Sephadex G-150	280	95	0.34	76.2
DEAE-Sephadex A-50 (2nd)	18	80	4.44	64.2
Sephacryl S-300	9	63	7.00	50.1

(12 ml) was applied to a column (2.6 × 91 cm) of Sephacryl S-300 equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. The active eluates were pooled, and dialyzed against deionized water to yield pure enzyme solution.

Overall, 583-fold purification with 50% activity yield from cell-free extracts of *Lactobacillus casei* ε ATCC 7469 was achieved, as shown in Table I.

Purity of Urethane Hydrolase III

The homogeneity of the purified enzyme was determined by polyacrylamide gel electrophoresis. As shown in Fig. 2, the enzyme preparation was proved to be homogeneous.

Table II. Effect of Inhibitors on Enzyme activity

Inhibitor (1 mM)	Residual activity (%)
None	100
o-Phenanthroline	44
EDTA	15
DTT	0
PCMB	0
PMSF	52
TLCK	51
TPCK	38
ZPCK	26
Iodoacetate	35
Iodoacetamide	54

The enzyme was preincubated at 50 °C with various inhibitors for 10 min, and then the residual activity was assayed in the presence of 1 mM Co^{2+} by the standard assay method.

Table III. Effect of Various Metal Ions on Enzyme Activity

Reagent (concentration, mM)		Relative activity (%)
None		0
$CoCl_2$	4	37
	2	60
	1	100
	0.4	42
$ZnCl_2$	1	29
$MnCl_2$	1	9
$CaCl_2$	1	2
$MgSO_4$	1	0
$PbAc_2$	1	0
$FeCl_3$	1	1
$FeSO_4$	1	6
$CdCl_2$	1	0
$BaCl_2$	1	0
$CuSO_4$	1	0
$HgCl_2$	1	0

The enzyme was incubated at 50 °C with various metal ions in 0.05 M phosphate buffer (pH 6.2). After 10 min, the enzyme activity was assayed.

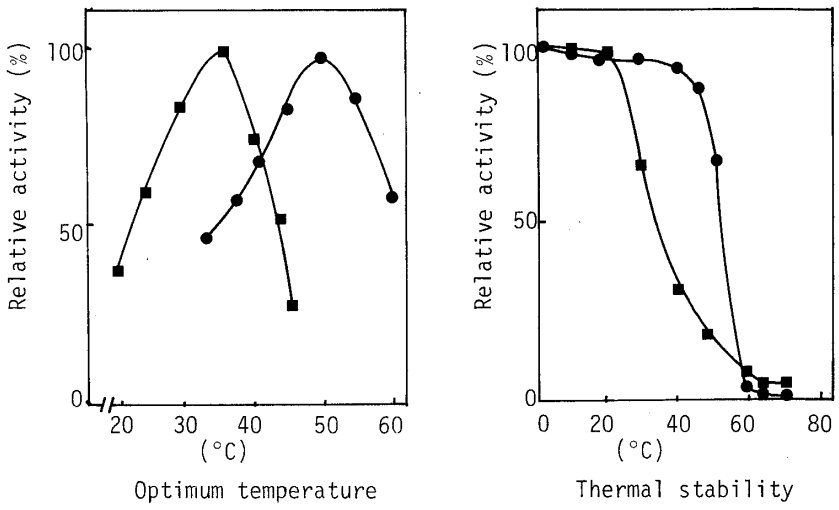

Fig. 3. Effects of Temperature on Enzyme Activity

Optimum temperature, the enzyme activity was determined at various temperatures. Other conditions were those of the standard assay method. Thermal stability, the enzyme was preincubated at the indicated temperature at pH 6.2 for 16 h, then the residual activity was determined by the standard assay method.
●—●, urethane hydrolase III; ■—■, urethane hydrolase I.

Properties of Urethane Hydrolase III

The molecular weight of the native enzyme was determined to be approximately 230000 by gel filtration on a Sepharose 6B column. Estimation of the molecular weight of reduced and denatured enzyme by SDS-polyacrylamide gel electrophoresis gave a value of 56000. These results suggest that the enzyme is composed of four subunits or that the enzyme tends to aggregate under the conditions used for gel filtration. The isoelectric point was determined by the method of flat bed isoelectrofocusing (Pharmacia Fine Chemicals). The enzyme showed

TABLE IV. The Rates of Hydrolysis of Various Substrates

	Reaction rate (nmol/min/mg[a])		Reaction rate (nmol/min/mg[a])
Z–Gly	2.00	Bz–Gly	30.20
Z(p-OCH_3)–Gly	0.40	Bz(o-OH)–Gly	0.40
Z–Gly–Gly	0.40	Bz(m-OH)–Gly	18.20
Z–Ala	0.52	Bz(p-OH)–Gly	11.20
Z–Ser	0.44	Bz–Gly–Gly	0.02
		Bz–Ala	4.80

Inert substrates:[b]

Z–Val, –Leu, –Ile, –β-Ala, –Thr, –Tyr, –Trp, –Phe, –Pro, –Met, –Cys, –Arg, –Lys, –His, –Asp, –Asn, –Gln, –Glu; Boc–Gly, –Ala, –Ser, –Phe, –Pro, –Arg, –Asp; Bz–β-Ala, –DL-Leu, –Phe, –Arg, –DL-Met; Ac–Gly, –Ala, –DL-Ser, –Leu, –Phe, –Arg-OMe, –Tyr, –Trp, –DL-Met, *O*-Ac–Ser; For–Gly, –Met; phenyl–Gly, *p*-nitrophenylacetyl–Gly, phenylacetyl–Gly, DNP–Gly, –Ala, –Ser; Tos–Gly, –Ala, –Ser; Z–D-Ala, Bz–D-Ala, Ac–D-Ala, Pht–D-Ala, Tos–D-Ala; Z–Gly–NH_2, Z–Gly–ONp, Bz–Ala–OMe, Gly–NH_2, Ala–NH_2, benzamide, Val–Gly, Leu–Gly, Tyr–Gly, Gly–Ala, Gly–Met, Gly–Gly, Gly–Ser, Ala–Ser, Z–Gly–Gly–Gly, Z–Gly–Gly–Ser, Z–Gly–Gly–Phe, Z–Ala–Ser–OMe, casein, hemoglobin.

Initial velocity was measured by the standard assay method. *a*) Protein was determined spectrophotometrically by measuring the absorbance at 280 nm, assuming that the absorbance at the concentration of 1 mg/ml is 1.0. *b*) The reaction period was 24 h, and other conditions were the same as in the standard assay method.

TABLE V. K_m and V_{max} Values for the Hydrolysis of Z-Amino Acids and Bz-Amino Acids

	Urethane hydrolase III		Urethane hydrolase I[2c]	
	K_m (mM)	V_{max} (nmol/s/mg)	K_m (mM)	V_{max} (nmol/s/mg)
Z–Gly	0.61	505	0.36	164
Z–Ala	0.78	12	0.80	11
Z–Ser	0.30	352	No	No
Bz–Gly	0.03	545	0.10	93
Bz–Ala	0.35	72	0.35	1.5

Enzymatic hydrolysis by urethane hydrolase III was carried out in 0.05 M phosphate buffer, pH 6.2, at 50 °C. Enzymatic hydrolysis by urethane hydrolase I was carried out in 0.05 M phosphate buffer, pH 6.0, at 35 °C.

an isoelectric point of 4.60. The optimum reaction conditions were observed at pH 6.2 and 50 °C. The enzyme was stable below 40 °C, but gradual inactivation occurred above 45 °C. The effects of metal ions and inhibitors on the enzyme activity were studied. As shown in Tables II and III, the enzyme activity was strongly inhibited by PCMB and DTT at the concentration of 1 mM. The enzyme required divalent cations (*i.e.*, Co^{2+} or Zn^{2+}). The optimum concentration of Co^{2+} was about 1 mM.

The purified enzyme showed almost the same characteristics as urethane hydrolase I, except for the effect of temperature (optimum temperature and thermal stability). Figure 3 shows the effect of temperature in comparison with the results for urethane hydrolase I.

Substrate Specificity of Urethane Hydrolase III

The substrate specificity of urethane hydrolase III was studied in detail, as summarized in

Table IV.

The present enzyme catalyzes the hydrolysis of Z–Ser, which is not hydrolyzed by urethane hydrolase I.[2a,c] Z–Gly, Z–Ala, Z(*p*-OCH_3)–Gly, Bz–Gly, Bz–Ala and Bz(*o*, *m*, *p*-OH)–Gly were hydrolyzed, but the reaction rates were quite distinct from those with urethane hydrolase I.[2c] The Bz-moiety was preferred to the Z-moiety by the present enzyme. In addition, the present enzyme hydrolyzed Z–Gly–Gly and Bz–Gly–Gly. Kinetic studies by thin-layer chromatography (TLC) showed that the enzyme first releases the COOH-terminal Gly, then attacks Z– or Bz–Gly bonds. The rate parameters for the substrates tested (K_m and V_{max} values) are listed in Table V, together with those of urethane hydrolase I for comparison.

The following three observations show that the character of urethane hydrolase III is different from that of urethane hydrolase I. (a) Z–Ser was hydrolyzed by urethane hydrolase III, (b) urethane hydrolase III catalyzed the hydrolysis of Z–Gly–Gly and Bz–Gly–Gly, and (c) the reaction rate of urethane hydrolase III toward Bz–Gly was 15-fold faster than that toward Z–Gly.

Discussion

The substrate specificity of the present enzyme differs from that of urethane hydrolase I isolated from *Streptococcus faecalis* R ATCC 9043. Thus, the present enzyme appears to be a new type of urethane hydrolase, having unique hydrolytic activity toward Z–Ser and high activity toward Bz–Gly. We have tentatively named the enzyme from *Lactobacillus casei* ε ATCC 7469 "urethane hydrolase III."

As reported previously, we have successfully used urethane hydrolase I for analytical purposes; that is, for colorimetric determination of angiotensin I converting enzyme activity in a coupled assay system with urethane hydrolase I, laccase (EC 1.10.3.2) and (4-OH)Bz–Gly–His–Leu,[11] and for a new assay of carboxypeptidase A activity by using (4-OH)Bz–Gly–Phe[12] in the same coupled assay system as in the case of angiotensin I converting enzyme assay.

Since urethane hydrolase III shows a high activity toward Bz–Gly and is stable up to high temperature, it should prove to be more useful for analytical purposes than urethane hydrolase I.

References and Notes

1) This work was presented in part at the Annual Meeting of the Agricultural Society of Japan, Sendai, 1983, p. 195. Abbreviated designations of amino acids, peptides and their derivatives follow the tentative rules recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (1965). Except when specified, constituent amino acids were all of L-configuration. The following abbreviations are used: EDTA = ethylenediaminetetraacetic acid, PCMB = *p*-chloromercuribenzoic acid, DTT = dithiothreitol, PMSF = phenylmethylsulfonyl fluoride, TLCK = *p*-tosyl-L-lysine chloromethyl ketone, TPCK = *p*-tosyl-L-phenylalanine chloromethyl ketone, ZPCK = *N*-Z-L-phenylalanine chloromethyl ketone, SDS = sodium dodecyl sulfate.
2) *a*) S. Murao, E. Matsumura, T. Shin and T. Kawano, *Agric. Biol. Chem.*, **48**, 1673 (1984); *b*) *Idem, ibid.*, **49**, 967 (1985); *c*) E. Matsumura, T. Shin, S. Murao and T. Kawano, *ibid.*, **49**, 973 (1985).
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