Isolation and Characterization of a Novel Enzyme, *N*^α-Benzyloxycarbonyl Amino Acid Urethane Hydrolase, from *Streptococcus faecalis* R ATCC 8043

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A novel enzyme, which was named *N*^α-benzyloxycarbonyl amino acid urethane hydrolase, was purified from a cell-free extract of *Streptococcus faecalis* R ATCC 8043, using *N*^α-benzyloxycarbonyl glycine as substrate. The enzyme was purified 1300-fold with an activity yield of 8%. The purified enzyme was homogeneous by disc electrophoresis. The molecular weight of the native enzyme is about 220,000 by gel filtration, and a molecular weight of 32,000 was determined for the reduced and denatured enzyme by gel electrophoresis in sodium dodecyl sulfate. The isoelectric point was 4.48. The enzyme was inhibited by *p*-chloromercuribenzoate. The presence of divalent cations (*i.e.*, Co²⁺ or Zn²⁺) is essential for its activity.

We have reported that a new enzyme, named *N*^α-benzyloxycarbonyl amino acid urethane hydrolase, hydrolyzed the urethane bond in *N*^α-benzyloxycarbonyl glycine to yield glycine and benzyl alcohol.¹⁾

The benzyloxycarbonyl group, a urethane type protecting group, is widely employed as an amino protecting group in amino acids or peptides. However, an effective enzyme for releasing benzyloxycarbonyl moieties from *N*^α-protected amino acids and/or peptides has not been reported. We searched for an effective enzyme in microbes and found such an enzyme activity in various lactic acid bacteria. This paper describes the isolation of urethane hydrolase from *Streptococcus faecalis* R ATCC 8043, together with its properties.

MATERIALS AND METHODS

Materials. Bactotryptone (Difco Laboratories, U.S.A.) and casamino acids (Nissui Seiyaku, Japan) were used in the medium. Substrates for the enzyme assay were obtained from the Protein Research Foundation (Osaka, Japan) or Tokyo Kasei Kogyo Co., Ltd. Reference pro-

teins (thyroglobulin, catalase, and aldolase), Sepharose 4B, DEAE-Sephadex A-50, and Sephadex G-150 were products of Pharmacia Fine Chemicals. Hydroxyapatite and AH-Sepharose 4B were prepared by the methods of Tiselius²⁾ and Cuatrecasas,³⁾ respectively.

All other chemicals were of commercially available grade.

Microorganisms and cultivation. *Streptococcus faecalis* R ATCC 8043, used in these experiments, was the stock culture of this laboratory. The composition of the basal medium, used for studying the optimum conditions for the production of the enzyme, was: 5.5 g yeast extract, 12.5 g polypeptone, 11.0 g glucose, 0.25 g KH₂PO₄, 0.25 g K₂HPO₄, 10.0 g sodium acetate, 0.1 g MgSO₄ · 7H₂O, 5.0 mg MnSO₄ · nH₂O, and 5.0 mg FeSO₄ · 7H₂O in 1 liter of tap water (final pH 6.8). The seed culture medium for the evaluation of culture conditions was: 20 g glucose, 10 g sodium acetate, 0.5 g KH₂PO₄, 0.5 g K₂HPO₄, 10 mg FeSO₄ · 7H₂O, 10 mg MnSO₄ · nH₂O, and 10 mg MgSO₄ · 7H₂O in 1 liter of tap water (final pH 6.8), unless otherwise noted. The organisms were anaerobically grown at 37°C for 16~24 hr.

Preparation of cell-free extracts. The cells, harvested after about 16 hr of cultivation, were washed with distilled water and then disrupted by a Vibrogen Cell-Mill (Edmund Bühler, Tübingen, West Germany) with 0.1 mm glass beads in 0.05 M phosphate buffer (pH 6.8). The cell

debris was removed by centrifugation (6000 rpm, 10 min), the resulting supernatant was used for purification of the enzyme.

Determination of enzyme activity. *N*^α-benzyloxycarbonyl amino acid urethane hydrolase activity was determined by the ninhydrin method,⁴⁾ measuring the amount of released amino groups as described previously.¹⁾ One unit of enzyme activity was defined as the amount of the enzyme which catalyzes hydrolysis of 1 mol substrate (Z-Gly) per min under the standard conditions.

Other analytical methods.

i) **Estimation of protein.** 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).

ii) **Molecular weight determination of protein.** The molecular weight of the enzyme was estimated by the methods of Andrews,⁵⁾ using a column (2.6 × 66 cm) of Sepharose 6B with 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.⁶⁾

SDS-PAGE marker I (RNA polymerase b, Seikagaku Kogyo Co., Ltd.) was employed as a standard marker protein.

iii) **Electrophoresis.** Electrophoresis in polyacrylamide gel was done by the method of Davis.⁷⁾

iv) **Determination of isoelectric point.** Isoelectric focusing was done using an electrophoresis column (110 ml vol., LKB Produkter), equipped with a cooling mantle, as in the method of duckter, equipped with a cooling mantle, as in the method of Vesterberg and Svensson.⁸⁾

RESULTS

Cultural conditions for the production of *N*^α-benzyloxycarbonyl amino acid urethane hydrolase

The effect of the nitrogen source on the production of the enzyme was studied. Table I shows that the activity appeared when the strain was grown on casamino acid and bactotryptone. Accordingly casamino acid and bactotryptone were used as nitrogen sources and glucose was used as the carbon source.

Figure 1 shows the change in the activity of enzyme in *S. faecalis* R cells during growth on the production medium. The maximum activity of enzyme was at the middle of the logarithmic growth phase. The glucose was consumed sluggishly during the cultivation,

TABLE I. EFFECTS OF MEDIA ON PRODUCTION OF ENZYME

Medium	Wet cell weight (g/liter)	Total protein (mg)	Total activity (units × 10 ⁷)
A	3.5	102	10.3
B	4.5	42	1.5
C	4.4	74	3.6
D	1.9	75	1.6

Compositions of media:

Medium A; 5 g casamino acid, 5 g bactotryptone, 0.2 g L-cystine, 0.1 g L-aspartic acid, 0.5 g DL-alanine, 20 g glucose, 10 g sodium acetate, 20 g sodium succinate, 0.5 g KH₂PO₄, 0.5 g K₂HPO₄, 10 mg FeSO₄·7H₂O, 10 mg MnSO₄·nH₂O, 10 mg MgSO₄·7H₂O, 10 mg NaCl, 1 mg thiamine, 1 mg riboflavin, 2 mg pyridoxine, 0.25 mg pyridoxal, 1 mg niacin, 1 mg Ca pantothenate, 0.25 mg folic acid, 0.5 mg *p*-aminobenzoic acid, 5 μg biotin, 5 mg adenine sulfate, 5 mg guanine hydrochloride, 5 mg uracil, and 1 ml Tween 80, in 1000 ml volume (pH 6.8). Medium B; 0.2 g DL-alanine, 0.2 g L-arginine hydrochloride, 0.4 g DL-aspartic acid, 0.1 g L-cystine, 0.5 g L-glutamic acid, 0.1 g glycine, 0.1 g L-histidine, 0.2 g DL-isoleucine, 0.2 g DL-leucine, 0.2 g L-lysine hydrochloride, 0.2 g DL-methionine, 0.2 g DL-phenylalanine, 0.1 g L-proline, 0.1 g DL-serine, 0.2 g DL-threonine, 0.1 g DL-tryptophan, 0.1 g DL-tyrosine, 0.2 g DL-valine, 10 mg adenine sulfate, 10 mg uracil, 10 mg guanine hydrochloride, 20 g glucose, 0.1 mg thiamine hydrochloride, 1 mg riboflavin, 1 mg pyridoxine, 1 mg pyridoxal, 1 mg Ca pantothenate, 1 mg niacin, 0.2 mg *p*-aminobenzoic acid, 10 μg biotin, 10 μg folic acid, 20 g sodium citrate, 0.5 g KH₂PO₄, 0.5 g K₂HPO₄, 0.8 g MgSO₄·7H₂O, 40 mg FeSO₄·7H₂O, 0.16 g MnSO₄·nH₂O, 40 mg NaCl, in 1000 ml (pH 6.8). Media C and D; 5.5 g yeast extract, 12.5 g peptone, 11.0 g glucose, 0.25 g KH₂PO₄, 0.25 g K₂HPO₄, 10 g sodium acetate, 0.1 g MgSO₄·7H₂O, 5.0 mg MnSO₄·nH₂O, 5.0 mg FeSO₄·7H₂O and (medium D; Z-glycine 10 g) in 1000 ml volume (pH 6.8).

Cultivations were performed at 37°C for 16 hr. The cells were harvested by centrifugation (6000 rpm, 10 min), the supernatant was used for determining the enzyme activity by the standard method.

and about 65% of the glucose remained in the medium when the enzyme activity was maximum.

Purification of *N*^α-benzyloxycarbonyl amino acid urethane hydrolase

S. faecalis R was grown in 100 liters of the production medium (Table I, medium A) in jar fermentor. All purification procedures were performed at 0~5°C, unless otherwise speci-

FIG. 1. Course of Enzyme Production.

Ten ml of seed culture, as indicated in MATERIALS AND METHODS, was inoculated into 100 ml of medium A (production medium) and incubated at 37°C. At certain times, 5 ml of cultured broth was withdrawn. The cells were harvested by centrifugation (3000 rpm, 10 min). The supernatant was used for estimation of residual glucose by the glucose oxidase method. After the cells were washed with 0.05 M phosphate buffer (pH 6.8) twice, and suspended in 5 ml of the same buffer, they were used for determining enzyme activity by the standard method.

●—●, enzyme activity; ○—○, bacterial growth; □—□, residual glucose; △—△, pH of the broth.

fied. Sodium phosphate buffer (pH 6.8) was used throughout the purification.

Step 1. Preparation of cell-free extract. Washed cells (wet weight 240 g), obtained from 100 liters of culture broth, were suspended in 200 ml of the buffer (0.1 M), and then continuously disrupted for 15 min by a Vibrogen Cell-Mill. The cell debris was removed by centrifugation (6000 rpm, 10 min), the resulting supernatant was used as the starting material for the following purification.

Step 2. Ammonium sulfate fractionation. The cell-free extract (1500 ml) from step 1 was brought to 0.4 saturation with solid ammonium sulfate, and then the precipitate formed was removed by centrifugation (6000 rpm, 20 min). The ammonium sulfate concentration of the supernatant was then brought up to 0.7 saturation. The precipitate was collected by centrifugation (9000 rpm, 20 min), dissolved in 150 ml of the buffer (0.1 M), and then dialyzed against the same buffer.

Step 3. Removal of precipitate at pH 4.8. The

dialyzed enzyme solution was acidified with Britton–Robinson buffer (pH 3.0) to a final pH 4.8 and left for 30 min in an ice-bath; the resulting precipitate was removed by centrifugation (6000 rpm, 20 min). About 106% of the initial activity was found in the supernatant, which was thoroughly dialyzed against 0.01 M buffer. The dialyzed solution was concentrated by ultrafiltration with an Amicon PM-10 membrane.

Step 4. DEAE-Sephadex A-50 column chromatography. The concentrated enzyme solution (65.5 ml) from step 3 was put onto a column (3.3 $\times$ 76 cm) of DEAE-Sephadex A-50, previously equilibrated with 0.01 M buffer. The enzyme was eluted by an increasing linear gradient of NaCl concentration to 0.5 M in the same buffer. The enzyme activity was detected in the fractions at about 0.3 M NaCl; the active fractions were combined and then concentrated by ultrafiltration with an Amicon PM-10 membrane.

Step 5. Sephadex G-150 column chromatography. The concentrated enzyme solution (38 ml) from step 4 was put onto a column (5.0 $\times$ 100 cm) of Sephadex G-100, equilibrated with 0.01 M buffer. The active fractions were combined and then precipitated by solid ammonium sulfate (0.7 saturation).

Step 6. AH-Sepharose 4B column chromatography. The active enzyme solution (12.8 ml) from step 5, after dialysis and concentration, was put onto a column (2.7 $\times$ 91 cm) of AH-Sepharose 4B, equilibrated with 0.01 M buffer. After purging unadsorbed materials with the buffer, elution was done by a linear gradient of NaCl concentration from 0.1 to 1 M. Active fractions were pooled, concentrated by ultrafiltration, and then thoroughly dialyzed against 1 mM buffer.

Step 7. Hydroxyapatite column chromatography (1st). The enzyme solution (9.4 ml) from step 6 was put onto a column (2.0 $\times$ 54 cm) of hydroxyapatite, previously equilibrated with 1 mM buffer. Elution was by a linear gradient of buffer concentration from 1 mM to 0.3 M. The combined active eluate (80 ml) was concentrated and dialyzed against deionized

water.

Step 8. Isoelectric focusing. The dialyzed enzyme solution from step 7 was further purified by isoelectric focusing using Ampholine (pH 3.5~5.0) in a sucrose gradient. The separation profile is shown in Fig. 2. The active fractions were pooled.

Step 9. Hydroxyapatite column chromatography (2nd). The pooled eluate from step 8 was concentrated by ultrafiltration and then dialyzed thoroughly against 1 mM buffer. The dialyzed enzyme solution was again chromatography on a column (1.6 × 30 cm) of hydroxyapatite, by the same elution methods as in step 7. The active eluates were pooled and dialyzed against deionized water to yield pure enzyme solution.

A summary of the purification of the en-

FIG. 2. Isoelectric Focusing of Enzyme.

●—●, enzyme activity; △—△, absorbance at 280 nm; ○—○, pH.

zyme shows that 1300-fold purification with a 8% activity yield from a cell-free extract of *S. faecalis* R was achieved, as shown in Table II.

Purity of *N*^α-benzyloxycarbonyl amino acid urethane hydrolase

The homogeneity of the purified enzyme preparation was determined by polyacrylamide gel electrophoresis. As shown in Fig. 3, the purified enzyme preparation was homogeneous.

Molecular weight of the enzyme

The molecular weight of the native enzyme, measured by gel filtration on a Sepharose 6B

FIG. 3. Disc Electrophoresis of the Enzyme.

Approximately 10 μg of protein was put on a 7.0% polyacrylamide gel column and electrophoresed at pH 8.0 and 2 mA per tube for 60 min. The gels were stained with 0.2% Coomassie Brilliant Blue R-250.

TABLE II. SUMMARY OF THE PURIFICATION OF *N*^α-BENZYLOXYCARBONYL AMINO ACID URETHANE HYDROLASE

Steps	Total protein (mg)	total activity (units × 10 ⁴)	Specific activity (units × 10 ⁴ /mg)	Recovery (%)
Cell free extract				
Salting out	4140	26	0.6	100
pH precipitation	1212	27	2	106
DEAE-Sephadex A-50	919	29	3	114
Sephadex G-150	437	31	7	120
AH-Sepharose 4B	31.5	15	47	58
Hydroxyapatite (1st)	5.4	6	106	22
Isoelectric focusing	1.3	5	344	17
Hydroxyapatite (2nd)	0.3	2	780	8

column, was approximately 220,000. Estimation of the molecular weight of reduced and denatured enzyme on SDS-polyacrylamide gel electrophoresis gave a value of 32,000, suggesting that the enzyme is composed of eight subunits or that the enzyme molecules tend to aggregate in the gel filtration conditions.

Isoelectric point of enzyme

From the separation profile in the isoelectric focusing experiment (Fig. 2), the isoelectric point of the enzyme was 4.48.

Ultraviolet absorption spectrum

The ultraviolet spectrum of the purified enzyme in 0.05 M phosphate buffer (pH 6.0) showed the maximum absorption at 278 nm. The absorbance of 1% enzyme solution at 278 nm was determined to be 9.3.

Stability

After the enzyme was incubated in 0.05 M phosphate buffer (pH 6.0) at various temperatures for 60 min, the remaining enzyme activity was determined. The enzyme was stable below 20°C, but above 25°C gradual inactivation occurred. The enzyme was stable around pH 6.0 for 16 hr at 25°C. The enzyme was stable on storage at 0°C for about 60 days at pH 6.0, and 80% of the activity remained after 3 months storage. Half of the initial activity was lost during storage at 0°C for 6 months (pH 6.0).

Effects of temperature and pH on the enzyme activity

The optimum temperature for the enzyme activity was about 35°C (pH 6.0). The optimum reaction was at about pH 6.0.

Effects of metal ions and various inhibitors on the enzyme activity

The effects of various metal ions and inhibitors on the enzyme activity was studied. As shown in Table III, the enzyme activity was strongly inhibited by *p*-chloromercuribenzoate. The inhibition by this reagent was re-

TABLE III. EFFECTS OF INHIBITORS ON THE ACTIVITY OF *N*^α-BENZYLOXYCARBONYL AMINO ACID URETHANE HYDROLASE

The enzyme was incubated at 35°C with various inhibitors in 0.05 M phosphate buffer (pH 6.0). After 10 min, the residual enzyme activity was assayed by the standard method.

Inhibitor (1 mM)	Relative activity (%)
Control	100
<i>o</i> -Phenanthroline	86
EDTA	49
DTT	20
PCMB	0
PMSF	89
TLCK	71
TPCK	85
ZPCK	72
Iodoacetate	80
Iodoacetoamide	86

EDTA, disodium ethylenediamine tetraacetate; DTT, dithiothreitol; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; TLCK, *p*-tosyl-L-lysine chloromethyl ketone; TPCK, *p*-tosyl-L-phenylalanine chloromethyl ketone; ZPCK, *N*-Cbz-L-phenylalanine chloromethyl ketone.

TABLE IV. EFFECTS OF METAL IONS ON THE ACTIVITY OF *N*^α-BENZYLOXYCARBONYL AMINO ACID URETHANE HYDROLASE

The enzyme was incubated at 35°C with various metal ions in 0.05 M phosphate buffer (pH 6.0). After 10 min, the residual enzyme activity was assayed by the standard method.

Reagents (1 mM)	Relative activity (%)
None	0
CoCl ₂	100
MnCl ₂	24
CaCl ₂	8
MgSO ₄	0
PbAc ₂	5
FeCl ₃	0
FeSO ₄	4
CdCl ₂	4
BaCl ₂	1
CuSO ₄	0
HgCl ₂	0

versible by the addition of 2-mercaptoethanol.

As shown in Table IV, the enzyme required

divalent cations (*i.e.*, Co^{2+} or Zn^{2+}) for the activity, and the optimum concentration for Co^{2+} was about 1 mM.

DISCUSSION

N^α-Benzyloxycarbonyl group (Z-), a urethane type protecting group, is widely employed for peptide synthesis. Deprotection of the Z-group under mild conditions by an enzymatic method may have various advantages.

As reported previously, we have successfully used this enzyme for analytical purposes (*e.g.* estimation of angiotensin I converting enzyme activity in biological fluids).⁹⁾

We now reported the purification of a novel enzyme, named *N*^α-benzyloxycarbonyl amino acid urethane hydrolase, from the cells of *S. faecalis* R. No other effective enzyme for hydrolysis of Z-amino acids has been reported, except for a preliminary report on a carboxypeptidase-like enzyme that catalyzed hydrolysis of Z-Ala.¹⁰⁾

A detailed description of the enzymatic

properties (*i.e.*, substrate specificity and identification of reaction products) will be presented in the accompanying paper.¹¹⁾

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