Communications to the Editor

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A NOVEL ENZYME "N^{α}-BENZYLOXYCARBONYL AMINO ACID URETHANE HYDROLASE II" FROM *LACTOBACILLUS FERMENTI* 36 ATCC 9338[1)]

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A novel enzyme, "N^{α}-benzyloxycarbonyl amino acid urethane hydrolase II", was isolated from the cell-free extract of *Lactobacillus fermenti* 36 ATCC 9338. The enzyme showed hydrolytic activity toward N^{α}-benzyloxycarbonyl arginine. The purified enzyme was homogeneous as indicated by disc gel electrophoresis. The molecular weight of the native enzyme is about 200,000 as estimated by gel filtration method. The isoelectric point was 5.0. The enzyme activity was inhibited by *p*-chloromercuribenzoic acid and ethylenediaminetetraacetic acid. The presence of divalent cations (i.e., Co^{2+} or Zn^{2+}) is essential for its activity.

KEYWORDS — novel enzyme; Urethane Hydrolase II; hydrolytic enzyme; Z-Arg; *Lactobacillus fermenti* 36 ATCC 9338; characterization; purification

In the course of our studies on the deprotection reaction of N^{α}-protecting group of acylamino acids by microorganisms, a new enzyme "N^{α}-benzyloxycarbonyl amino acid urethane hydrolase" (Urethane Hydrolase) having the characteristic activity of catalyzing the hydrolysis of the urethane bond in Z-amino acid was discovered in microbes. Previously, the Uretane Hydrolase I, which catalyzes the hydrolysis of Z-Gly to give equimolar amounts of benzyl alcohol and Gly, was isolated from *Streptococcus faecalis* R ATCC 8043 and its properties were characterized.[2)] We have successfully used this enzyme for analytical purposes.[3)] The present report deals with the purification and characterization of Urethane Hydrolase II, which catalyzes the hydrolysis of Z-Arg, from *Lactobacillus fermenti* 36 ATCC 9338.

The enzyme assay was carried out in 1.0 ml of 0.05 M phosphate buffer, pH 6.5, containing 0.5 μmol of Z-Arg, 1 μmol of Co^{2+} and the enzyme, with about 4.35×10^{-8} units (5.75 μg). The reaction mixture was kept at 33°C for 30 min. One unit of the enzyme activity is defined as the amount of the enzyme which catalyzes hydrolysis of 1.0 mol of Z-Arg per min. *Lactobacillus fermenti* 36 ATCC 9338 was cultured in 100 liters of synthetic medium[4)] containing glucose, vitamins, purine bases, amino acids and inorganic compounds. The activity was induced when the strain was grown on medium containing Z-Arg (3 g/100 liters). The cultivation was carried out

5 times at 37°C for 10 h in a 30 liters jar fermentor under anaerobic conditions. The harvested cells were disrupted with a Vibrogen Cell-Mill (Edmund Buhler, Tubingen, West Germany) with 0.1 mm glass beads. The enzyme was purified from cell-free extracts by methods involving fractionation with ammonium sulfate, column chromatographies on DEAE-Sephadex A-50, DEAE-Sepharose CL-6B, isoelectric focusing and preparative disc electrophoresis. This procedure resulted in a 94-fold purification with an over-all yield of about 2.8%. The purified enzyme gave a single band on acrylamide gel electrophoresis. The enzyme had an optimum pH around 6.5 with Z-Arg as substrate. The optimum temperature was found at 33°C in 0.05 M phosphate buffer (pH 6.0). This enzyme was stable in the pH range from 5.5 to 6.0 below 20°C. The apparent molecular weight of the purified enzyme was estimated to be about 200,000 by gel filtration on Sepharose 6B. The isoelectric point of the purified enzyme was 5.0. The enzyme was completely inhibited by PCMB and EDTA at the concentration of 1 mM. The purified enzyme required divalent cations (Co^{2+} or Zn^{2+}) for its activity, and the optimum concentration of Co^{2+} was about 1 mM. The substrate specificity of the purified enzyme is summarized in Table I.

Table I. The Rate of Hydrolysis of Various Substrates

	Reaction rate ($nmol \cdot min^{-1} \cdot mg^{-1}$, a))	*Km* (mM)
Z-Arg	727	0.11
Z(*p*-OCH_3)-Arg	698	-
Bz-Arg	683	0.18
Ac-Arg	160	2.50
Bz-Gly-Arg	370	1.60

Inert substrates[b)]

Z-Gly, -Met, -Phe, -Ala, -Ser, -Tyr, -Val, -Pro, -Trp, -Leu, -Asp, -Asn, -Lys, -Ile, -Gln, -His, -Thr, -Glu, -Cys(Bzl), -ornithine, N^{δ}-Z-ornithine, N^{ε}-Z-Lys, Z-D-Arg, Z-Arg(Tos), Z-Arg(NO_2), Boc-Arg, Z(*p*-OCH_3)-Arg(Tos), Bz-Arg-OEt, Bz-Arg-NH_2, Bz-Arg-pNA, Bz-Arg-Lys, Arg-Arg-pNA, Gly-Gly-Tyr-Arg, Thr-Lys-Pro-Arg

Initial velocity was measured by the standard assay method.

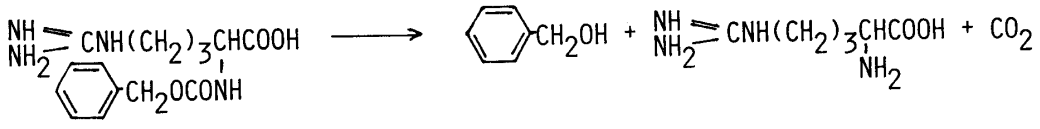
a) Protein was determined spectrophotometrically by measuring the absorbance at 280 nm, assuming that the absorbance at the concentration of 1 mg/ml is 1.0.

b) Reaction period was 24 h, and other conditions were the same as in the standard assay method.

The hydrolytic activity of this enzyme was restricted to Z-Arg, Bz-Arg, Ac-Arg, Z(p-OCH_3)-Arg and Bz-Gly-Arg. Z-Arg is the most favorable substrate for the enzyme. It is interesting that the enzyme was able to hydrolyze Ac-Arg at one-fifth of the reaction rate of Z-Arg, considering that Urethane Hydrolase I might require the benzene ring of the N^{α}-protecting group of a susceptible substrate. The COOH-blocked Bz-Arg derivatives (e.g., Bz-Arg-OEt, Bz-Arg-NH_2) were all inert to the enzyme. Further, $\overset{NH}{NH_2}\!\!>$CNH-blocked Z-Arg derivatives (e.g., Z-Arg(Tos), Z-Arg(NO_2)) were all resistant to the enzyme. This suggests that the free carboxyl and free guanidyl groups in the Arg residue are required for the catalytic activity of the enzyme. The stereospecificity of the enzyme was also confirmed by its failure to hydrolyze Z-D-Arg. The enzyme acts on Bz-Gly-Arg to release Arg, but its *Km* value was 15-fold larger than that for Z-Arg. The acyl group containing Gly (Bz-Gly-) is next in poorness of affinity to the Ac moiety as a substrate.

The acylases, which operate on N^{α}-acylamino acids, are widely distributed in animal tissues, fungi and bacteria and have been extensively studied. But Z-protected amino acids were all resistant to these acylases, although certain acylases were reported to show a trace effect on Z-Glu[5] and ε-Z-Lys.[6] Details were discussed in a previous paper.[2] Enzymes that release the basic amino acids, Lys and Arg, from the COOH-terminal position of peptides and proteins have been known since the first report by Waldschmidt-Leiz et al.[7] The enzyme, namely, carboxypeptidase B (EC 3.4.17.2), has been reported to be distributed in animal pancreas, spiny pacific dogfish and body fluids.[8] Subsequently, they were purified and characterized from human pancreas,[9] porcine pancreas,[10] bovine pancreas[11] and rabbit lung lysosome.[12] The other enzyme, carboxypeptidase N (EC 3.4.17.3), was discovered by Erdos and sloane as a kininase that inactivated bradikinin and kallidin.[13] Human carboxypeptidase N has been purified and some of its properties have been reported.[14] Further, L.J. Jeanneret et al. purified and characterized the enzyme in pig serum.[15] These enzymes act on synthetic peptide derivatives (e.g., Bz-Gly-Lys, Bz-Gly-Arg, Bz-Gly-ornithine, Bz-Lys-Lys),[10,15] but fail to hydrolyze Z-Arg according to our studies of porcine pancreas carboxypeptidase B (Boehringer) and human plasma (used as crude carboxypeptidase N). In addition, Bz-Gly-Lys, which is a favorable substrate for carboxypeptidase B and N, was completely resistant to Urethane Hydrolase II. These results confirm that Urethane Hydrolase II from *Lactobacillus fermenti* 36 ATCC 9338 is quite distinct from carboxypeptidase B or N, so the present enzyme is a novel one.

The reaction products were identified as benzyl alcohol and Arg on HPLC (Toyo Soda 803A) and TLC. The fluorometric assay, measuring the amount of the released amino acids, was used. Equimolar amounts of benzyl alcohol and Arg were detected stoichiometrically as shown in the following scheme:



A more detailed description of this work will be reported later.

REFERENCES AND NOTES

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Abbreviated designations of amino acids, peptides and their derivatives obey the tentative rules recommended by IUPAC-IUB Commission on Biochemical Nomenclature (1984). Except when specified, constituent amino acids were all in the L-configuration. The following abbreviations are used: EDTA = ethylenediaminetetraacetic acid, PCMB = *p*-chloromercuribenzoic acid.

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