Partial Purification, and Some Properties and Reactivities of Cetraxate[1] Benzyl Ester Hydrochloride-Hydrolyzing Enzyme[2]

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Debenzylating enzyme from *Aspergillus niger* enzyme (commercial crude cellulase) catalyzes the hydrolysis of cetraxate benzyl ester hydrochloride (2), a precursor of the antiulcer agent (1). The enzyme was highly purified by three kinds of chromatographies (hydrophobic, ion exchange, gel filtration) with a recovery of 36%. The content of the debenzylating enzyme was about 0.1% in the crude cellulase, but the enzyme showed no cellulase activity. The purified enzyme was inactivated by Hg^{2+}, and diisopropyl phosphorofluoridate (DFP). It was a monomer with a molecular weight of about 35000, and its isoelectric point was estimated to be 5.3. It showed a debenzylating activity for the phenylpropionic acid benzyl ester moiety of various benzyl ester derivatives, and the benzyl ester of phenylalanine or that of tyrosine was also well hydrolyzed.

Keywords cetraxate hydrochloride; enzymatic debenzylation; *Aspergillus niger* enzyme; purification; debenzylating enzyme; enzyme property; substrate specificity

Cetraxate hydrochloride (**1**), a widely used antiulcer agent, has been industrially produced by a route involving chemical deprotection of *p*-hydroxypropionic acid derivatives.[3-5] We investigated various kinds of enzymes in the hope of achieving the reaction under in mild conditions (*e.g.*, room temperature, ordinary pressure and in water), and it was found that *Aspergillus* sp. enzyme(s) had the required specific esterase activity. In the previous paper,[6] we demonstrated the selective hydrolysis of the terminal benzyl ester of cetraxate benzyl ester hydrochloride (**2**)[3,4] (Chart 1).

We found that some commercial cellulase enzyme preparations catalyzed the hydrolysis of benzyl groups.[6] Cellulase itself hydralyzes glycosyl bonds,[7] but not ester bonds. Consequently, it was suggested that the enzyme (esterase) selectively hydrolyzing the benzyl ester in **2** is a contaminant in the commercial cellulases.

In this paper, we report the purification of the debenzylating enzyme from cellulase(s) and describe some properties and the substrate specificity of this purified enzyme.

Materials and Methods

Materials H_2O: Water distilled in our laboratory was used. Commercial preparation of cellulase: Sumizyme AC (*Aspergillus niger*) was obtained from Shin-Nippon Chemical Industries Co., Ltd. (cellulase activity was 2—5 unit/mg.) Diisopropyl phosphorofluoridate (DFP) and eserine were purchased from Sigma Chemical Co., Ltd., ethylenediaminetetraacetic acid (EDTA), *o*-phenanthroline monohydrate and monoiodoacetic acid (MIA) wer purchased from Nacalai Tesque Inc. Butyl-Toyopearl 650-S, DEAE-Toyopearl 650-S and TSKgel G-3000 SW were purchased from Toso Co., Ltd. Ultrafilter membranes (Millipore PT M.W. < 10000) were purchased from Millipore Co. Superose-12 and standard proteins for electrophoresis were purchased from Pharmacia Fine Chemicals. Dialysis membrane was obtained from Viskase Sales Co.

Carboxylic Acid Derivatives for Examination of Substrate Specificity Commercial products and synthetic ones were used. Protecting groups (alkyl, phenyl, benzyl, *etc.*) were introduced into commercial carboxylic acids according to the conventional method in our laboratory.

Determination of 1 by High Performance Liquid Chromatography (HPLC) Column, YMC-gel ODS(AM) i.d. 6 × 100 mm; eluting solution, H_2O–CH_3OH–0.5 M CH_3COONH_4 (65 : 35 : 8, v/v); flow rate, 1.5 ml/min; temperature, 40 °C; detector, ultraviolet (UV) 230 nm.

Determination of Benzyl Alcohol by Gas Chromatography (GC) Column, 10% PEG-20M 200 cm; temperature, 200 °C (oven), 230 °C (injection); flow rate, 40 ml/min (N_2); detector, hydrogen flame ionization detector.

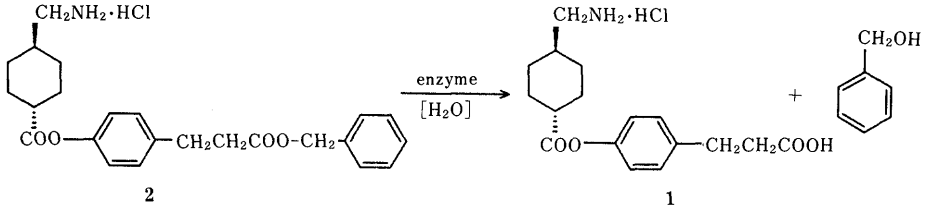
Measurement of Debenzylating Activity by HPLC A mixture of 1 M sodium acetate buffer (pH 4.5, 1 ml), **2** (15 mg) and H_2O (8 ml) was heated to 30 °C, enzyme corresponding to 0.1 unit[8] was added to it, and the mixture was kept at 30 °C for 30 min. Acetone–ethanol (1 : 2, 8 ml) and 1 drop of 1 N HCl were immediately added after 30 min, followed by addition of H_2O to make a total volume of 20 ml, and then **1** formed was assayed by HPLC.

Measurement of Kinetic Parameters The K_m and V_{max} values were calculated from Hanes–Woolf plots, using 1 ml of a reaction mixture composed of 1.25—40 mM substrate (**2**) in 0.1 M sodium acetate buffer (pH 4.5) and 0.1 unit of enzyme. Reaction conditions: 30 °C for 30 min.

Measurement of Protease Activity and Lipase Activity Protease activity was measured by the conventional method (Anson-hemoglobin-280 nm) and lipase activity was measured with olive oil as the substrate.

Measurement of Debenzylating and Deesterifying Activities for Evaluation of a Substrate Specificity Substrate (50 mM; 5—15 mg) was added to a mixed solution (1 ml) of the purified enzyme (0.1 unit; 50—100 μl), 10% CH_3OH (0.7—0.75 ml) and 0.5 M sodium acetate buffer (pH 4.5, 0.2 ml) or 0.5 M sodium phosphate buffer (pH 7.5, 0.2 ml). This solution was kept at 30 °C for 1 h, and then CH_3OH (1 ml) was rapidly added to it. The resulting benzyl alcohol was determined by GC, and the resulting carboxylic acid (Phe or **1**) by HPLC.

Electrophoresis Sodium dodecyl sulfate (SDS) slab gel electrophoresis was performed in 12.5% polyacrylamide according to the method of Laemmli[9] (60 mA for 1 h, staining with CBB). Apparatus and reagents: Daiichi Chemical Pharmaceutical Co., Ltd. Transferrin (M.W. 80000), bovine serum albumin (M.W. 67000), ovalbumin (M.W. 43000), and carbonic anhydrase (M.W. 31000) were used as references to determine the



2 1

Chart 1

molecular weight.

Isoelectric focusing was carried out using carrier ampholite with a pH range from 3.5—10 (LKB-Produkter AB) at 200 V for 30 min and 700 V for 90 min.

Results

Purification of the Debenzylating Enzyme Preliminary experiments with several kinds of chromatographic columns suggested that the desired enzyme is a strongly hydrophobic acidic protein. So, three kinds of column chromatographies (hydrophobic—ion exchange—gel filtration) were chosen to isolate the intended enzyme. The debenzylating enzyme activity in the chromatographic fractions was assayed by measuring the formation rate of **1** from the substrate (**2**) (HPLC method).

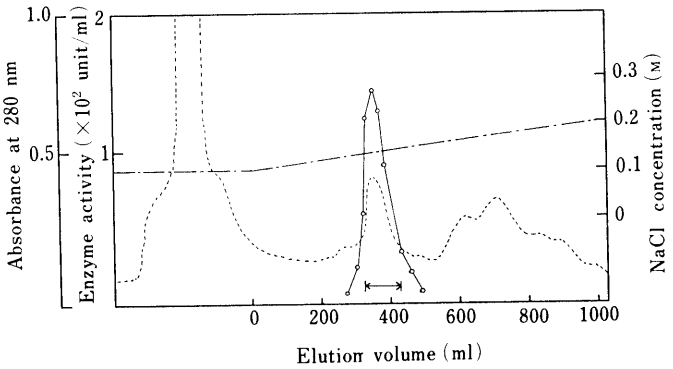


Fig. 1. Ion Exchange Column Chromatography of the Fraction from Hydrophobic Chromatography

Column, DEAE-Toyopearl 650-S (2.2 × 20 cm); eluting solution, a linear gradient of 0.1—0.2 M NaCl in 0.1 M Tris–HCl buffer (pH 7.5); flow rate, 180 ml/h. ------, A_{280} (absorbance at 280 nm); ——, debenzylating activity; — - —, linear gradient of NaCl.

TABLE I. Purification of the Debenzylating Enzyme

Fraction	Total protein[a] (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)
Crude enzyme	23800	21070	0.89	100
Butyl-Toyopearl	540	15600	28.9	74.0
DEAE-Toyopearl	17.4	10700	615	50.8
Superose-12	9.2	7280	791	36.4

a) Protein content was measured by the method of Lowry *et al.*[10]

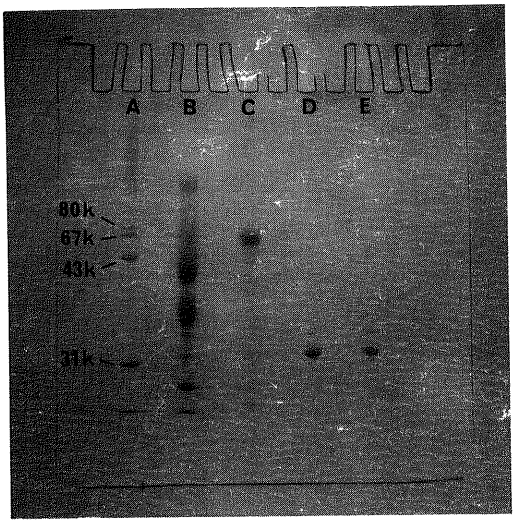


Fig. 2. SDS-Polyacrylamide Gel Electrophorogram of the Purified Enzyme

A, Reference proteins: B, crude enzyme (cellulase(s)); C, butyl-Toyopearl fraction; D, DEAE-Toyopearl fraction; E, Superose-12 fraction. Protein (about 10 μg) was stained with CBB.

Purification of the Enzyme by Column Chromatography All procedures were carried out at 4 °C. Cellulase(s) (23.8 g) was dissolved in 100 ml of 0.1 M sodium sulfate buffer (pH 7.5) containing 1 M ammonium sulfate. This solution was applied to a Butyl-Toyopearl column (5 × 52 cm) equilibrated with 0.1 M sodium phosphate buffer (pH 7.5) containing 1 M ammonium sulfate and 10% CH_3OH. The enzyme was eluted with a linear gradient of 1—0 M ammonium sulfate in 0.1 M sodium phosphate buffer (pH 7.5). The active fractions were collected.

The fraction eluted from the hydrophobic column was concentrated to 200 ml by ultrafiltration and dialyzed against 50 mM Tris–HCl buffer (pH 7.5). The enzyme solution was put on a DEAE-Toyopearl column (2.2 × 20 cm) previously equilibrated with 0.1 M Tris–HCl buffer (pH 7.5). The column was washed with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, and the enzyme was eluted with a linear gradient of 0.1—0.2 M NaCl in 0.1 M Tris–HCl buffer (pH 7.5) (Fig. 1). The active fractions were pooled.

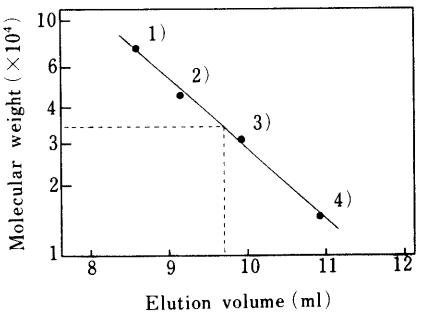


Fig. 3. Estimation of Molecular Weight of the Enzyme by Gel Filtration

Column, TSKgel G-3000 SW (7.5 × 30 cm); buffer, 50 mM Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl; flow rate, 1.0 ml/min. Standard proteins: 1) bovine serum albumin (M.W. 67000), 2) ovalbumin (M.W. 43000), 3) carbonic anhydrase (M.W. 31000), 4) ribonuclease A (M.W. 13700). Protein in the eluates was measured in terms of the absorption at 280 nm, and the enzyme was detected by following the debenzylating activity toward **2**.

TABLE II. Effect of Metal Ions and Enzyme Inhibitors

Metal ion and inhibitor	Relative residual activity (%)
None	100
Hg^{2+}	9
Mg^{2+}	64
Co^{2+}	68
Ca^{2+}	71
Fe^{2+}	81
Mn^{2+}	79
Cu^{2+}	72
Zn^{2+}	70
Ni^{2+}	67
EDTA	102
o-Phenanthroline	80
MIA	88
DFP	9
Eserine	101

The enzyme (0.1 unit) was preincubated at 30 °C for 30 min with 1 mM metal ion (the metal chloride was used) or 1 mM inhibitor in 0.1 M sodium acetate buffer (pH 4.5), and was incubated at 30 °C for 30 min with substrate (**2**) (15 mg) in the same buffer (pH 4.5). The residual activity was measured by the HPLC assay of **1** formed.

TABLE III. Substrate Specificity toward the Benzyl Esters of Aliphatic and Aromatic Carboxylic Acids and Amino Acids

Abbreviation	Substrate[a] Formula	Relative debenzylating activity (%) pH 4.5	pH 7.0
	$HCOOCH_2$–Ph	1	<1
	CH_3COOCH_2–Ph	3	<1
	$CH_3CH_2COOCH_2$–Ph	4	<1
	$CH_3CH_2CH_2COOCH_2$–Ph	11	<1
	$(CH_3)_2CHCOOCH_2$–Ph	4	<1
	$CH_3CH_2CH_2CH_2COOCH_2$–Ph	6	<1
	$(CH_3)_2CHCH_2COOCH_2$–Ph	3	<1
	$CH_3(CH_2)_{10}COOCH_2$–Ph	<1	<1
	Ph–$COOCH_2$–Ph	<1	<1
	Ph–CH_2COOCH_2–Ph	2	<1
	Ph–$CH_2CH_2COOCH_2$–Ph	10	<1
	Ph–$CH_2CH_2CH_2COOCH_2$–Ph	<1	<1
	HO–Ph–$COOCH_2$–Ph	<1	<1
	HO–Ph–CH_2COOCH_2–Ph	9	<1
	HO–Ph–$CH_2CH_2COOCH_2$–Ph	42	14
	CH_3COO–Ph–$CH_2CH_2COOCH_2$–Ph	24	<1
Gly–By	$H_2NCH_2COOCH_2$–Ph	3	4
Leu–By	$(CH_3)_2CHCH_2CH(NH_2)COOCH_2$–Ph	1	2
Pro–By	Pyrrolidine-2-carboxylate–$COOCH_2$–Ph	<1	<1
Trp–By	Trp–$CH_2CH(NH_2)COOCH_2$–Ph	2	8
Phe–By	Ph–$CH_2CH(NH_2)COOCH_2$–Ph	10	29
Tyr–By	HO–Ph–$CH_2CH(NH_2)COOCH_2$–Ph	12	34
TS–(O)–Tyr–By	$HCl \cdot H_2NCH_2$–Cy–COO–Ph–$CH_2CH(NH_2)COOCH_2$–Ph [b]	21	30
2	$HCl \cdot H_2NCH_2$–Cy–COO–Ph–$CH_2CH_2COOCH_2$–Ph [c]	100	5

a) Amino acid: L-form. *b*) A substrate in which tyrosine is coupled to tranexamic acid (TS).[11] *c*) Standard substrate. Debenzylating activity was measured by the method described under Materials and Methods.

The fraction eluted from the ion exchange column was concentrated to 2 ml by ultrafiltration, and applied to a Superose-12 column (1.6 × 50 cm) equilibrated with 0.1 M Tris–HCl buffer (pH 7.5). The enzyme was eluted with 0.1 M Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl. The purified enzyme was stored at −20 °C until use.

The purified enzyme, about 10 mg, was obtained from cellulase(s), about 20 g, by the three kinds of column chromatographies mentioned above. The debenzylating enzyme was purified about 800-fold in an overall yield of 36% (Table I).

The purified enzyme showed a single major band and a

trace amount of a minor band on SDS-polyacrylamide gel electrophoresis (Fig. 2).

Properties of the Debenzylating Enzyme The molecular weight of the enzyme was estimated to be 33000 and 35000, by SDS-polyacrylamide gel electrophoresis and gel filtration on TSKgel G-3000 (Fig. 3), respectively. The isoelectric point value was determined to be 5.3 by isoelectric focusing. The apparent K_m and V_{max} values were estimated from a Hanes-Woolf plot to be 4 mM and 1.1 μmol/min, respectively.

The enzyme was stable at pH 4—7 and at temperatures below 40 °C, and its optimal pH was at around 4.5 for debenzylating substrate (**2**). This result agreed very closely with that for the crude enzyme studied in the previous paper.[6] The debenzylating enzyme separated from cellulase(s) showed no cellulase activity. It also showed no protease or lipase activity.

The effects of 1 mM solutions of various metal ions and inhibitors were tested (Table II). The enzyme was inactivated strongly by Hg^{2+} and DFP, but not by EDTA and *o*-phenanthroline.

Substrate Specificity of the Debenzylating Enzyme The enzyme showed a debenzylating activity for the structure of –(O)–⬡–$CH_2CH_2COOCH_2$–⬡ in the benzyl esters of aliphatic and aromatic carboxylic acids (Table III). In addition, the benzyl esters of phenylalanine (Phe–By) and tyrosine (Tyr–By) were hydrolyzed. The enzyme did not hydrolyze other ester groups such as those of Tyr–Me, Tyr–*tert*-Bu, **1**-Me, **1**-iso-Pro, and **1**-Ph.

Discussion

In the previous paper, it was found that *Aspergillus niger* cellulase catalyzed the selective hydrolysis of the benzyl esters of carboxylic acids without cleaving another ester bond in the molecule. A novel and potentially industrially useful enzymatic synthesis of cetraxate hydrochloride (**1**) was established by using cetraxate benzyl ester hydrochloride (**2**) as a substrate and a cellulase(s) preparation which showed debenzylating activity.

The authors undertook to isolate the debenzylating enzyme from the crude cellulase(s) and to elucidate its properties and reactivities. The debenzylating enzyme was initially found to be a strongly hydrophobic acidic protein. Through three successive column chromatographies (hydrophobic—ion exchange—gel filtration), the desired enzyme was obtained with about 800—fold purification in 70% yield at each step. The debenzylating enzyme content is very low (0.1%) in the cellulase(s) preparation, but the enzyme has a strong activity which is characteristic of a fungal enzyme.

The purified enzyme exhibited no cellulase activity, but the optimum conditions of debenzylation were 40 °C-pH 4.5, similar to those of the crude enzyme. We estimated the molecular weight by SDS-polyacrylamide gel electrophoresis (on the denatured protein) and gel filtration (on the native protein). Since the values were similar, the enzyme seems to be a monomer. The effect of enzyme inhibitors suggested that no metal ion is present and a serine residue exists in the active site of the enzyme.

Though the enzyme was separated from a crude cellulase, it is a novel debenzylating enzyme and has no protease or lipase activity. Its specificity was examined toward several synthetic substrates (Table III). The enzyme is specific for the –(O)–⬡–$CH_2CH_2COOCH_2$–⬡ structure of benzyl ester. The benzyl esters of phenylalanine (Phe–By) and tyrosine (Tyr–By) were also well hydrolyzed. Therefore, it is supposed that the enzyme is a kind of amino acid esterase which acts on Phe and Tyr.

From a practical point of view, the debenzylating reaction of **2** to give **1** may be better carried out by using the crude enzyme, cellulase(s), since the purified debenzylating enzyme is rather labile. The authors are planning to study the distribution of debenzylating enzyme activity in various sorts of microorganisms.

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References and Notes

1) Compound **1**: 4-(2-Carboxyethyl)phenyl *trans*-4-aminomethylcyclohexanecarboxylate hydrochloride, an antiulcer agent (Daiichi Seiyaku Co., Ltd.).
2) Enzymatic debenzylation of cetraxate benzyl ester hydrochloride into cetraxate hydrochloride. Part II. (Part I, see reference 6.)
3) Y. Yamamura, S. Fujii, A. Okano, M. Hirata, Y. Abiko, M. Inaoka, and S. Funabashi, Japan. Patent 46-19950 (1971) [*Chem. Abstr.*, **73**, 45139*a* (1970)]; S. Kitahara, Japan. Patent 52-48978 (1977) [*Chem. Abstr.*, **86**, 155266*r* (1977)].
4) M. Kamada, M. Mimura and M. Iwase, Japan. Patent 59-13498 (1984) [*Chem. Abstr.*, **87**, 84691*h* (1977)]; M. Kamada and M. Mimura, Japan. Patent 60-6352 (1985) [*Chem. Abstr.*, **89**, 197185*f* (1978)].
5) T. Hirayama, H. Kuroda and M. Iwase, Japan. Patent 63-28062 (1988) [*Chem. Abstr.*, **96**, 217482*a*] (1982)].
6) H. Kuroda, A. Miyadera, T. Kaneuchi and A. Imura, *Yakugaku Zasshi*, **109**, 157 (1989).
7) Report of the Commission on Enzymes of the International Union of Biochemistry, Enzyme No. 3214 (1961), Pergamon Press.
8) One unit is defined as the amount of enzyme that converted 1 μmol of substrate (**2**) into **1** per a minute. One unit of cellulase(s) corresponds to 1—2 mg of the preparations used.
9) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
10) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) *trans*-4-Aminomethylcyclohexanecarbocylic acid, an anti-inflammatory agent (Daiichi Seiyaku Co., Ltd.).