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Purification and characterization of *N*-acyl-D-glutamate deacylase from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6.

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The purification and properties of *N*-acyl-D-glutamate deacylase from the cell extracts of *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6 were studied. The two active fractions (peaks I and II) were obtained by a Mono Q column chromatography. The predominant enzyme (peak I) has been purified, 1960-fold to homogeneity and characterized. The enzyme was a monomer with a molecular weight of 59 000. The optimum pH and the isoelectric point were 8.0 and 5.5, respectively. The enzyme catalyzed the hydrolysis of *N*-acyl derivatives of D-glutamate. The K_ms for *N*-acetyl, *N*-butyryl and *N*-propionyl derivatives of D-glutamate were 0.129, 0.066 and 0.01 mM, respectively.

N-Acyl-D-glutamate deacylase; *Alcaligenes;* D-Glutamate

1. INTRODUCTION

N-Acyl-D-glutamate deacylase (*N*-acyl-D-glutamate amidohydrolase EC 3.5.1) (D-AGase) catalyzes the hydrolysis of *N*-acyl-D-glutamate (Glu). The enzyme was first reported by us in 1990 [1]. Though its physiological role in *Alcaligenes* is not quite clear, it can be utilized for the resolution of racemic Glu. It has been reported that *N*-acyl-L-glutamate deacetylase (L-AGase) from *Pseudomonas aeruginosa* is involved in the arginine biosynthetic pathway [2], and that *N*-formyl-L-glutamate amidohydrolase (L-FGase) from *P. putida* participates in histidine metabolism [3].

In this paper, we describe the purification and characterization of D-AGase from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6.

2. MATERIALS AND METHODS

A. xylosoxydans subsp. *xylosoxydans* A-6 was cultured in a medium containing 0.2% *N*-acetyl-D-Glu, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.01% yeast extract and 0.01% $MgSo_4{\cdot}7H_2O$ (pH 7.0) for 40 h at 30°C.

D-AGase activity was assayed at 30°C in 50 mM HEPES buffer, pH 7.8, containing 10 mM *N*-acyl-D-Glu and enzyme. D-Glu formed was measured by high-performance liquid chromatography or by 2,4,6-trinitrobenzene sulfonic acid [4]. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of D-Glu per min. Protein was measured as described previously [5]. Purification of D-AGase was performed as follows: the buffer used in this purification was 10 mM potassium phosphate buffer (pH 7.0). The dialyzed cell extract (84 g of cells) was applied onto a column (6.1 × 35 cm) of DEAE-Toyopearl pre-equilibrated with buffer. The fraction between 0.04 M and 0.1 M NaCl was pooled. To the eluate, glycerol and 2-mercaptoethanol were added at a final concentration of 10% and 0.1%, respectively. The eluate was subjected to ammonium sulfate fractionation from 20 to 70% saturation. The pellet was dissolved in buffer containing 20% ammonium sulfate, 10% glycerol and 0.1% 2-mercaptoethanol, and applied to a Butyl-Toyopearl column (2.4 × 15 cm) pre-equilibrated with the same buffer. The enzyme was eluted with 600 ml of a linear gradient of 20 to 10% ammonium sulfate in the same buffer. The active fractions were applied to a Sephadex G-100 column (2.4 × 130 cm) equilibrated with the buffer. The column was eluted with the buffer. Active enzyme was loaded on a hydroxylapatite FPLC column (0.8 × 10 cm, Mitsui Toatsu Chemicals, Inc.) equilibrated with 1 mM of the buffer. The active enzyme was eluted as non-binding protein with 1 mM of buffer. Active fractions were applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer, washed with buffer containing 0.08 M NaCl, and eluted with 25 ml of a 0.08 to 0.14 M gradient of NaCl in the buffer. The active enzyme was separated into two peaks (I and II). We denoted the activity in peak I as D-AGase I and the activity in peak II as D-AGase II (Fig. 1). Both peaks were concentrated by ultrafiltration.

A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% polyacrylamide gel was performed as described by

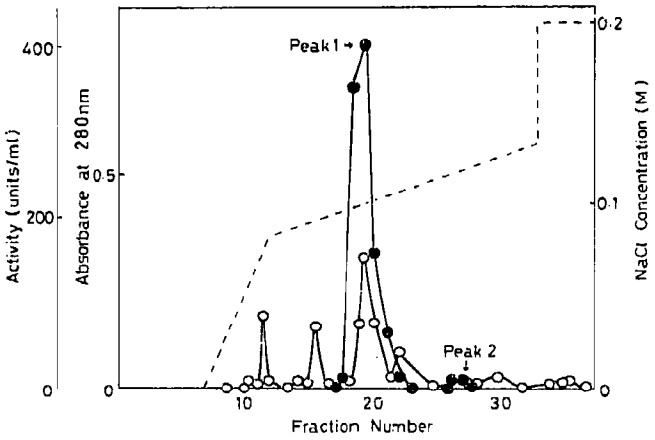

Fig. 1. Mono Q chromatography. 1-ml fractions were collected. Symbols: (○), protein: (●), D-AGase activity; (· · ·), NaCl.

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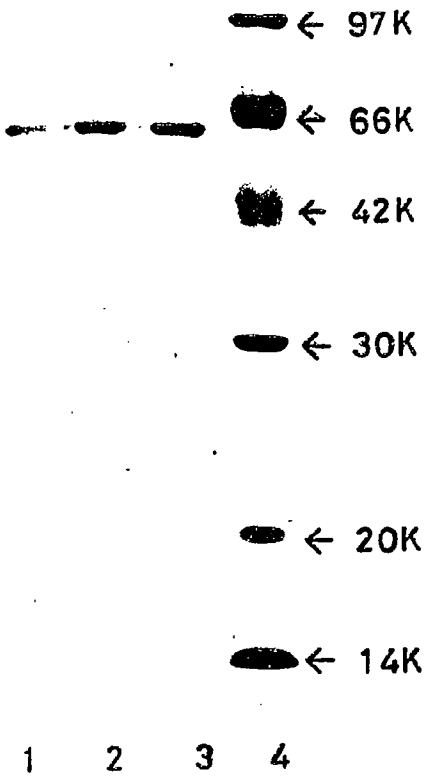

Fig. 2. SDS-PAGE of purified D-AGases I and II. Lane 1, D-AGase I. Lane 2, D-AGase I plus II. Lane 3, D-AGase II. Lane 4, molecular weight markers: phosphorylase (94K), bovine serum albumin (66K), aldolase (42K), carbonic anhydrase (30K), trypsin inhibitor (20K) and lysozyme (14K).

Laemmli [6]. Protein was blotted from SDS-PAGE onto an Immobilon polyvinylidene difluoride membrane, and used for N-terminal amino acid sequence analysis (Applied Biosystems). The molecular weights of the native enzyme and the subunit were determined by gel filtration on a Superose 12 column (FPLC) (1.0 × 30 cm, Pharmacia) and SDS-PAGE, respectively. Standard proteins from commercially available molecular weight marker kits were used as references.

3. RESULTS AND DISCUSSION

3.1. *Purification of D-AGase*

Activity profiles from the Mono Q column showed two peaks (I and II) (Fig. 1). Two D-AGases have been purified to homogeneity. On SDS-PAGE, both had one band, respectively (Fig. 2). Table I summarizes the purification of D-AGase I. The enzyme was purified about 1960-fold, with a 19.1% yield.

Table II

Substrate specificity

Substrate	Relative activity (%)
N-Formyl-D-Glu	449
N-Acetyl-D-Glu	100
N-Propionyl-D-Glu	23
N-Butyryl-D-Glu	7.9
N-Glycyl-D-Glu	45.0
N-Chloroacetyl-D-Glu	411
N-Carbobenzoxy-D-Glu	0
N-Acetyl-L-Glu	0
N-Acetyl-D-Asp	0

Table III

Amino acid composition

Amino acid	Number of residues	Amino acid	Number of residues
Cys	7	Ile	21
Asx	52	Leu	35
Thr	21	Tyr	7
Ser	30	Phe	11
Glx	55	Lys	12
Gly	60	His	26
Ala	85	Arg	40
Val	34	Pro	27
Met	10	Trp	19

Asx and Glx represent the sum total of Asp and Asn, Glu and Gln, respectively. The number of residue was calculated from a molecular weight of 59 000.

3.2. *Properties of D-AGase I*

Molecular weight was estimated to be 49 000 by gel filtration and 59 000 by SDS-PAGE, indicating that the enzyme is a monomeric protein. The optimum pH was 8.0, and the isoelectric point was determined to be 5.5. The enzyme was D-specific, active with *N*-acyl derivatives of D-Glu, but not with *N*-acetyl-D-aspartate (Table II). *N*-Formyl-D-Glu was the most preferred substrate. The activity decreased with increasing *N*-acyl chain length of D-Glu. L-AGase from *P. aeruginosa* hydrolyzed various *N*-acyl derivatives of L-Glu and *N*-

Table I

Summary of purification of D-AGase I from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6

Step	Total protein (mg)	Specific activity (U/mg)	Total units (U)	Purification (fold)	Recovery (%)
Cell extract	9990	0.53	5360	1	100
DEAE-Toyopearl	1120	4.32	4840	8.1	90.2
20–70% $(NH_4)_2SO_4$	672	5.21	3510	9.7	72.0
Butyl-Toyopearl	38	36.2	1380	67.5	25.6
Sephadex G-100	10.3	131	1360	246	25.3
Hydroxylapatite-HPLC	3.04	408	1240	762	23.1
Mono Q	0.925	1100	1017	1960	19.1

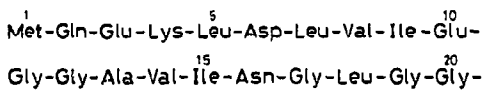

Fig. 3. N-terminal amino acid sequence.

acetyl derivatives of L-amino acids [2], but *Alcaligenes* D- AGase did not hydrolyze *N*-acetyl derivatives of neutral D-amino acids [1]. The K_ms of D-AGase I for *N*-acetyl- D-Glu, *N*-butyryl-D-Glu and *N*-propionyl-D-Glu were 0.129, 0.066 and 0.01 mM, respectively. Co^{2+} has been reported to activate L-AGase and L-FGase [2,3]. D- AGase I activity was not stimulated by divalent cations. The N-terminal sequence (residues 1–20) was determined (Fig. 3). The amino acid analysis showed alanine as the most abundant amino acid (Table III).

We have purified and characterized D-AGase I from *A. xylosoxydans* subsp. *xylosoxydans* A-6, and found a possibility for the existence of an isoenzyme, but the experimental data available are not sufficient to characterize the second enzyme, D-AGase II.

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