PURIFICATION AND PROPERTIES OF D-GLUCOSAMINATE DEHYDRATASE FROM *AGROBACTERIUM RADIOBACTER*

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1. Introduction

D-Glucosaminate dehydratase (EC 4.2.1.26)* catalyzes the conversion of D-glucosaminate (GlcNA) to 2-keto-3-deoxy-D-gluconate and ammonia; it requires pyridoxal-5'-phosphate (pyridoxal-P) as a cofactor and is analogous to serine dehydratase and threonine dehydratase. The enzyme was discovered independently by Merrick and Roseman [1] and by Imanaga [2] in the cells of unidentified bacteria in their studies of metabolism of D-glucosamine. In the former case, the enzyme was formed inducibly by GlcNA, which was used as a sole source of carbon, and partially purified from the cell free extract [3]. It is the exclusive pyridoxal-P enzyme which acts specifically on an amino-sugar derivative. However, since the early works [1–3], little attention has been given to the enzyme. We here describe the purification of the GlcNA dehydratase from the glucosamine-adapted cells of *Agrobacterium radiobacter* to homogenity, and some of its properties.

2. Materials and methods

GlcNA was prepared by oxidation of glucosamine with the cells of *Pseudomonas fluorescens* as described previously except that phosphate buffer in the reaction system was replaced by water [4]. Pyridoxal-P was obtained from Nakarai Chemicals, Kyoto, Japan; DEAE-cellulose from Brown Co. USA; DEAE-Sephadex A-50 and Sephadex G-150 from Pharmacia Fine Chemicals, Uppsala; Sweden and hydroxyapatite from Seikagaku Kogyo, Tokyo, Japan. The other chemicals were analytical grade reagents. Disc-gel electrophoresis was carried out according to the method of Davis [5]. The enzyme was assayed as follows. The standard reaction mixture consisted of 20 μmol of GlcNA, 0.2 μmol of pyridoxal-P, 120 μmol of potassium phosphate buffer (pH 8.0), and enzyme in a final volume of 1.0 ml. Enzyme was replaced by a boiled enzyme in a blank. After incubation was carried out at 37°C for 15 min, the reaction was stopped by heating the mixture for 3 min in a boiling water bath and 2-keto-3-deoxy-gluconate formed was determined with 0.1 ml aliquots of the mixture by the semicarbazide method of Macgee and Doudoroff [6]. One unit of enzyme was defined as the amount of the enzyme that catalyzes the formation of 1 μmol of 2-keto-3-deoxy-gluconate per min. Specific activity was expressed as units per mg of protein. Protein was determined by the method of Lowry et al. [7] with crystalline bovine serum albumin as a standard.

* This enzyme is erroneously classified also as EC 4.3.1.9

3. Results and discussion

3.1. *Purification of the enzyme*

Agrobacterium radiobacter (IAM 1526) was grown in a medium composed of 0.1% glycerol, 0.05% urea, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4 \cdot 7\ H_2O$, 0.01% yeast extract, and 0.2% glucosamine (pH 7.2). The cultures were grown at 28°C for 22 h under aeration. The yield of cells was approximately 3.3 g (wet wt.) per 1 medium. The harvested cells were washed twice with 0.15 M KCl. All operations were carried out at 0–5°C unless otherwise specified. Potassium phosphate buffer (pH 7.2) containing 20 μM pyridoxal-P and 0.01% 2-mercaptoethanol was used throughout the experiments.

3.1.1. Preparation of cell-free extract

The washed cells (about 1.6 kg, wet wt.) were suspended in 2.0 l of the buffer and sonicated in 100 ml portions with a Kaijo denki oscillator for 10 min. The intact cells and cell debris were removed by centrifugation.

3.1.2. Ammonium sulfate fractionation

The supernatant solution (4.0 l) was brought to 35% saturation with ammonium sulfate, and the resultant precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant solution up to 70% saturation. The precipitate was collected by centrifugation, dissolved in 1.5 l of 0.01 M buffer, and dialyzed against the same buffer. The insoluble materials formed during the dialysis were removed by centrifugation.

3.1.3. 1st DEAE-cellulose chromatography

The enzyme solution (2.0 l) was placed on a DEAE-cellulose column (6.4 × 30 cm) equilibrated with 0.01 M buffer. After the column was washed thoroughly with the same buffer and then with the buffer containing 0.10 M KCl, the enzyme was eluted with the buffer supplemented with 0.15 M KCl. The active fractions were pooled and concentrated by addition of ammonium sulfate (80% saturation). The precipitate collected was dissolved in 0.01 M buffer and dialyzed against 50 volumes of the same buffer. The insoluble materials formed during the dialysis were removed by centrifugation.

3.1.4. DEAE-Sephadex A-50 chromatography

The enzyme solution (38 ml) was applied to a DEAE-Sephadex A-50 column (2 × 30 cm) equilibrated with 0.01 M buffer. After the column was washed with the same buffer containing 0.1 M KCl, the enzyme was eluted with the same buffer supplemented with 0.15 M KCl. The active fractions were collected and concentrated by addition of ammonium sulfate (80% saturation). The precipitate was dissolved in 0.01 M buffer and dialyzed against 200 volumes of 1 mM buffer.

3.1.5. Hydroxyapatite chromatography

The enzyme solution (15.6 ml) was applied to a hydroxyapatite column (2 × 12 cm) equilibrated with 1 mM buffer. The enzyme was eluted with 5 mM buffer. The active fractions were collected and concentrated by addition of ammonium sulfate (40–50% saturation). The precipitate was dissolved in 0.01 M buffer and dialyzed against the same buffer.

3.1.6. Sephadex G-150 chromatography

The enzyme (3.7 ml) was applied to a Sephadex G-150 column (3.5 × 80 cm) equilibrated with 0.01 M buffer and eluted with the same buffer. The active fractions were pooled.

3.1.7. 2nd DEAE-cellulose chromatography

The enzyme solution (49 ml) was placed on a DEAE-cellulose column (1.2 × 12 cm) equilibrated with 0.01 M buffer. After the column was washed with the same buffer, the enzyme was eluted with the same buffer supplemented with 0.1 M KCl. The active fractions were collected and concentrated. A summary of the purification is presented in table 1.

3.2. *Properties of the enzyme*

The purified enzyme was shown to be homogeneous upon disc-gel electrophoresis (fig.1A). The molecular weight was determined to be approximately 62 000 by Sephadex G-150 chromatography [8], with bovine serum albumin (68 000), egg albumin (45 000), chymotrypsinogen (25 000) and cytochrome *c* (12 500) as standard proteins. The subunit structure of the enzyme was examined by SDS-disc gel electrophoresis [9]. There was a single band of stained protein (fig.1B). To determine the molecular weight of the subunit, we ran a series of marker proteins in the

Table 1
Purification of D-glucosaminate dehydratase

Step		Protein (mg)	Total act. (units)	Specific activity	Yield (%)
(1)	Crude extract	63 100	30 900	0.490	100
(2)	Ammonium sulfate fractionation (35–70%)	29 200	28 300	0.969	91.6
(3)	DEAE-cellulose chromatography (1st)	1540	19 200	12.5	62.1
(4)	DEAE-Sephadex A-50 chromatography	293	17 500	59.7	56.6
(5)	Hydroxyapatite chromatography	25	6300	252.6	20.4
(6)	Sephadex G-150 chromatography	14	4100	292.9	13.3
(7)	DEAE-cellulose chromatography (2nd)	7.8	2900	371.8	9.4

same manner; bovine serum albumin, egg albumin and chymotrypsinogen. The molecular weight was calculated to be about 32 000 from a semilogarithmic plot of molecular weight against mobility. This suggests that the enzyme consists of two subunits with identical molecular weight.

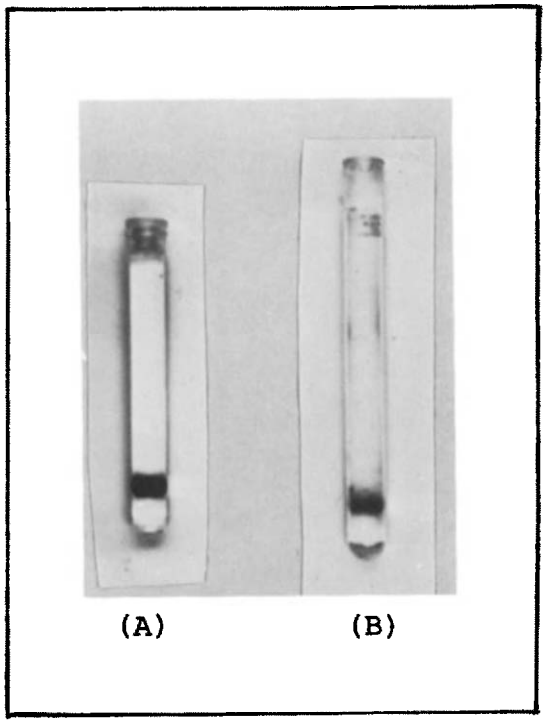

Fig.1. Polyacrylamide disc-gel electrophoresis of D-glucosaminate dehydratase. (A) The purified enzyme (36 μg of protein) was subjected to electrophoresis in Tris–HCl buffer (pH 8.3) according to the procedure of Davis [5]. The section of unstained gel corresponding to the protein band was cut out and crushed in 0.2 M potassium phosphate buffer (pH 8.0) to extract the enzyme. The protein solution contained the activity. (B) The 1% SDS-treated enzyme (11.5 μg of protein) was subjected to SDS-disc gel electrophoresis according to the method of Weber and Osborn [9]. The direction of migration was from the cathode (top) to the anode.

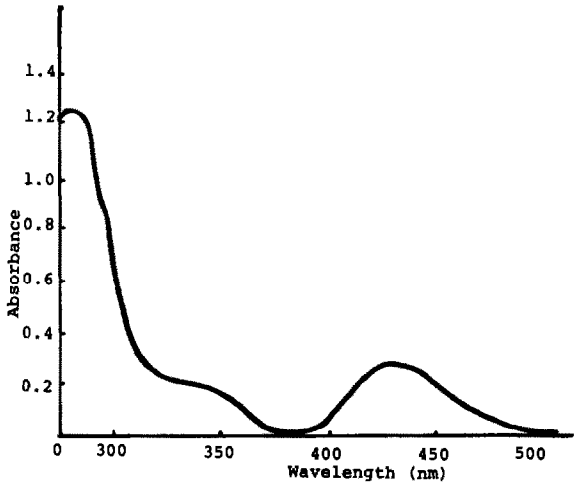

Fig.2. Absorption spectrum of D-glucosaminate dehydratase. Holoenzyme in 0.01 M potassium phosphate buffer, pH 7.0.

The purified enzyme was obtained substantially in apo-form and showed an absorption maximum at 280 nm and a shoulder at 330 nm. Pyridoxal-P was required for the maximum reactivity and the holo-enzyme showed another absorption maximum at 430 nm (fig.2). No spectral change occurred by varying the pH (6.5–8.8).

The enzyme activity was strongly inhibited by NH_2OH, L-penicillamine, PCMB and Hg^{2+}. The enzyme had a maximum activity in the pH range of 8.2–9.0. The K_m values were calculated to be 12 μM for pyridoxal-P and 2.77 mM for GlcNA. The enzyme catalyzes dehydration of D-threonine (2*R*, 3*S*), DL-allothreonine (2*R*, 3*R* and 2*S*, 3*S*), D-serine (2*R*) in addition of D-GlcNA (2*R*, 3*S*, 4*R*, 5*R*), which is the preferred substate, and L-serine (2*S*) also serves as a very poor substrate. L-Threonine (2*S*, 3*R*) is not a substrate. This suggested that the enzyme most predominantly acts on the amino carboxylic acids with (2*R*)-configuration, although not exclusively.

The reaction mechanism and cofactor content of the enzyme are currently under investigation.

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