Note

Subunit Structure of Chondroitinase ABC from *Proteus vulgaris*

Nobuyuki SATO, Kousaku MURATA* and Akira KIMURA*

Institute for Research and Development,
Taiyo Fisheries Co., Ltd., 3-2-9 Tsukishima,
Chuo-ku, Tokyo 106, Japan*Research Institute for Food Science,
Kyoto University, Uji,
Kyoto 611, Japan

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Chondroitinases that catalyze the depolymerization of chondroitin sulfate have been found in bacterial strains such as *Flavobacterium heparinum*,^{1,2)} *Bacteroides thetaiotaomicron*,³⁾ *Aeromonas* sp.,⁴⁾ and *Proteus vulgaris*.⁵⁾ Among these bacteria, *P. vulgaris* has been shown to be a potent producer of chondroitinase ABC [EC 4.2.2.4]. The enzyme has been purified from *P. vulgaris* and its properties have been characterized in detail,⁶⁾ except for the molecular structure of the enzyme. The chondroitinase ABC is an inducible enzyme and its activity remarkably increases on incubation of *P. vulgaris* cells in a medium containing chondroitin sulfate as an inducer.⁷⁾ To clarify the nature of the chondroitinase ABC induced, we first purified the enzyme from *P. vulgaris* cells and determined the subunit structure of the enzyme.

Cells of *P. vulgaris* NCTC4636 grown on a nutrient medium (1.0% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.2) at 30°C for 14 hr with shaking were harvested and then washed once with chilled 0.85% saline solution. The cells (5.0 g wet wt.) were resuspended in 1000 ml of induction medium (0.7% K_2HPO_4 , 0.3% KH_2PO_4 , 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, 0.1 mg/ml nicotinic acid, 0.1% yeast extract, 0.3% chondroitin sulfate, pH 7.0)⁷⁾ and then incubated at 30°C for 9 hr with shaking. The cells (90 g wet wt.) harvested from 10 liter induction medium were resuspended in 300 ml of 10 mM potassium phosphate buffer (pH 7.0) and then disrupted with a Dyno-Mill for 3 min at 0°C. The cell debris was removed by centrifugation at $25,000 \times g$ for 15 min and the resultant supernatant was directly applied to a DEAE-cellulose column (5 $\times$ 56 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The column was washed with the same buffer and the eluate was collected at 20 ml/tube/3 min. The active fractions were collected, concentrated by Amicon ultrafiltration with a PM10 membrane and then loaded onto a hydroxylapatite column (2 $\times$ 15 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The enzyme was eluted with a

linear gradient of potassium phosphate buffer (pH 7.0) of increasing concentration, from 10 to 500 mM (total volume, 200 ml). The active fractions, which were eluted at approximately 0.3 M buffer, were combined and concentrated as before. The enzyme solution was dialyzed against 10 mM Tris-HCl buffer (pH 7.0) for 4 hr at 0°C, and then the dialysate was applied to Zn^{2+} -iminodiacetate-agarose column (1 $\times$ 5 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0). The Zn^{2+} -iminodiacetate-agarose column was prepared by passing a 0.1 M $ZnCl_2$ solution through a column packed with iminodiacetate agarose (Sigma Chemical Co., St. Louis, MO.). The column was washed with 50 ml of 10 mM Tris-HCl buffer (pH 7.0) and then the enzyme was eluted with 50 ml of 0.5% chondroitin sulfate. The fraction eluted with chondroitin sulfate was concentrated as before and then applied to a Sephadex G-200 column (1.6 $\times$ 50 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer and then stored at 4°C after concentration as before. The assay mixture for chondroitinase ABC activity consisted of 0.5% chondroitin sulfate, 10 mM potassium phosphate buffer (pH 7.0) and enzyme (volume, 3.0 ml). After incubation at 37°C for 20 min, the *N*-acetylgalactosamine end group produced in the mixture was determined by the method of Reissig *et al.*⁸⁾ The activity was expressed as mg of *N*-acetylgalactosamine-containing polymers formed per hr per mg of protein. Protein was determined by the method of Lowry *et al.*⁹⁾ The molecular weight of chondroitinase ABC was determined on a calibrated column of Sephadex G-200 (1.6 $\times$ 50 cm) by the method of Andrews.¹⁰⁾ Proteins were eluted with 10 mM potassium phosphate buffer (pH 7.0). Polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate (SDS) was performed according to the method of Laemmli.¹¹⁾ The gels were stained for proteins with Coomassie brilliant blue.¹²⁾

The overall purification procedure for chondroitinase ABC is summarized in Table I. Zn^{2+} has been shown to be a potent inhibitor of the enzyme.^{5,7)} So, affinity chromatography on iminodiacetate-agarose with Zn^{2+} as a ligand was attempted. Almost all the contaminating proteins in a sample after hydroxylapatite column chromatography (Fig. 1A, lane 4) were eliminated by the Zn^{2+} -agarose column (Fig. 1A, lane 5). However, another protein with a molecular weight of 94,000 was co-purified with chondroitinase ABC on Zn^{2+} -agarose affinity chromatography (Fig. 1A, lane 5). Though the nature of the enriched protein (MW 94,000) having affinity for both Zn^{2+} and chondroitin sulfate is unclear, the introduction of the Zn^{2+} -agarose affinity column seems to be useful for the purification of chondroitinase ABC. For further purification and to remove chondroitin sulfate, the enzyme solution after Zn^{2+} -agarose column chromatography was loaded on a Sephadex G-200 column. The final enzyme preparation thus obtained showed a single protein band on polyacrylamide gel electrophoresis (Fig. 1B, lane 7) and determination of the molecular weight of the enzyme on a

TABLE I. PURIFICATION OF CHONDROITINASE ABC FROM *P. vulgaris*

Step	Preparation	Protein (mg)	Specific activity (mg/hr/mg-protein)	Total activity (mg/hr)	Yield (%)	Fold
1.	Cell extract	4100	2.30	9430	100	1.0
2.	DEAE-cellulose	144	40.8	5880	62.4	17.7
3.	Hydroxylapatite	23.8	108	2570	27.3	47.0
4.	Zn ²⁺ -iminodiacetate-agarose	3.11	N.D.	N.D.	—	—
5.	Sephadex G-200	2.20	114	251	2.70	49.6

The purification procedures were as described in the text.

N.D., not determined because of the high concentration of chondroitin sulfate.

FIG. 1. Chondroitinase ABC at Various Purification Steps.

[A] SDS (0.1%) / polyacrylamide gel (12.5%) electrophoresis of samples at various purification steps. Lane 1, standard proteins: (from top) myosin (H-chain); phosphorylase b; bovine serum albumin; ovalbumin; α -chymotrypsinogen; β -lactoglobulin and lysozyme. Lane 2, cell extract (29 μ g); lane 3, after DEAE-cellulose column chromatography (20 μ g); lane 4, after hydroxylapatite column chromatography (920 μ g); lane 5, after Zn²⁺-iminodiacetate-agarose column chromatography (20 μ g). [B] Polyacrylamide gel (6.0%) electrophoresis. Lane 6, standard proteins (the same as those in lane 1 of [A]); lane 7, purified chondroitinase ABC (20 μ g).

calibrated column of Sephadex G-200 gave a value of 120,000 (Fig. 2A). The molecular weight of chondroitinase ABC has been estimated to be lower than that of yeast alcohol dehydrogenase (MW 150,000) on centrifugation in a sucrose density gradient.⁶⁾ On SDS/polyacrylamide gel electrophoresis, the enzyme was separated into two non-identical subunits with molecular weights of 86,000 and 32,000 (Fig. 2B, lane 1).

From the results reported above, we concluded that the chondroitinase ABC purified from *P. vulgaris* cells consists of two non-identical subunits [light (MW 32,000) and

FIG. 2. Determination of the Molecular Weight and Subunit Structure of Chondroitinase ABC.

[A] Gel filtration on a Sephadex G-200 column. The standard proteins used were: a, aldolase (MW 145,000); b, glyceraldehyde 3-phosphate dehydrogenase (MW 130,000); c, deoxyribonuclease I (MW 60,000). The position of chondroitinase ABC is shown by a closed circle. [B] SDS (0.1%) / polyacrylamide gel (12.5%) electrophoresis. Lane 1, purified chondroitinase ABC (20 μ g); lane 2, standard proteins (the same as those in lane 1 of Fig. 1A).

heavy (MW 86,000)]. The followings are now considered regarding the formation of such a unique enzyme structure. 1. Association of light and heavy subunits, the syntheses of which are governed by separate genes. 2. Processing of a mature enzyme (MW 120,000), the synthesis of which is governed by a single gene. To obtain information on the regulation of the synthesis and activity of chondroitinase ABC, we are at present attempting to isolate the genes responsible for the chondroitinase ABC of *P. vulgaris*.

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