Note

Monoclonal Antibody Recognizing the Quinoprotein Subunit of Alcohol Dehydrogenase Complex from *Gluconobacter* Species

Emiko SHINAGAWA, Kazunobu MATSUSHITA,
Takeshi INOUE,* Osao ADACHI
and Minoru AMEYAMA

Department of Agricultural Chemistry and
*Veterinary Medicine, Faculty of Agriculture,
Yamaguchi University, Yamaguchi 753, Japan

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Quinoprotein alcohol dehydrogenase (ADH), a key enzyme in vinegar production, is located on the cytoplasmic membrane of acetic acid bacteria and linked to the respiratory chain. We purified ADH from the membranes of two representative acetic acid bacteria, *Gluconobacter suboxydans* IFO 12528^{1,2)} and *Acetobacter aceti* IFO 3284.³⁾ The purified ADHs from *G. suboxydans* and *A. aceti* have three and four subunits, respectively. It is important to study the role of each subunit and the relationship between ADH activity and each peptide. In this report, to investigate the participation of the dehydrogenase peptide in ADH activity, we prepared a monoclonal antibody against the dehydrogenase peptide which is one of the three subunits of ADH from *G. suboxydans*, dehydrogenase (mol. wt. 85 kDa), cytochrome *c* (49 kDa) and an unknown peptide (14.4 kDa).

Gluconobacter spp. listed in Table I, were cultivated as described in our previous report.¹⁾ The protein and ADH activity were measured⁴⁾ and immunoblotting was done⁵⁾ under the same conditions as reported previously. ADH was purified from the membrane of *G. suboxydans* to homogeneous state as was also described previously.⁶⁾

The preparation of monoclonal antibodies were done under essentially the same conditions as described by Galfre and Milstein.⁷⁾ Male BALB/c mice (6 weeks old) were immunized intraperitoneally with 50 µg of the purified ADH emulsified in complete Freund's adjuvant and after 2 weeks, the mice were further immunized with 100 µg of the purified ADH emulsified in incomplete Freund's adjuvant. After 1 week more, the mice were boosted with 100 µg of the antigen and fusion was done after 3 days. NS-1 myeloma cells were cultured in RPMI-1640 (Nissui) supplemented with 15% fetal calf serum (FCS) and antibiotics (50 IU/ml of penicillin G and 50 µg/ml of streptomycin). Spleen cells from the immunized mice were fused at a 5:1 ratio with myeloma cells

using 50% polyethylene glycol 4000 in RPMI-1640. The cells were plated into 96-well tissue culture plates at a density of 5×10^5 spleen cells per well (100 µl) and selected by adding selective medium containing RPMI-1640, 15% FCS, and HAT (100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine). After 10 days, the culture fluid was assayed for anti-ADH activity by enzyme-linked immunosorbent assay (ELISA) as described later. Hybridomas producing antibodies were cloned by the limiting dilution method. All monoclonal antibodies from several hybridomas obtained were shown to react with the dehydrogenase peptide with immunoblotting. We selected a hybridoma having the highest anti-ADH activity and obtained the monoclonal antibody from the hybridoma supernatant as a 50% ammonium sulfate precipitate.

ELISA was done as follows. Fifty microliters of the purified ADH (10 µg/ml of protein) in 0.1 M carbonate buffer, pH 9.5, was dispensed in each well of the microplate (Greiner). After overnight incubation at 4°C, the solution was aspirated off. After blocking with 0.2% gelatin for 1 hr at room temperature, wells were washed four times with phosphate buffered saline containing 0.1% Tween 20 (PBS-Tween) and 50 µl of hybridoma supernatant or conventional antibodies were added to each well of the plates, which were incubated for 2 hr at 37°C. After the wells were washed, 50 µl of the peroxidase-labelled goat anti-mouse immunoglobulins (Cappel) were added to each well. After incubation for 2 hr at 37°C, wells were washed four times with PBS-Tween and then were filled with 100 µl of substrate solution (McIlvaine buffer, pH 5.0, containing 2 mM *o*-phenylenediamine and 0.02% H₂O₂). After incubation for 30 min at room temperature the enzyme reaction was stopped by the addition of 50 µl of 4 M H₂SO₄. The absorbance of the solution was read at

Fig. 1. Immunoblotting of Membranes from *Gluconobacter* spp. with Monoclonal Antibody against the Dehydrogenase Peptide of Alcohol Dehydrogenase.

Each membrane (100 µg of protein) was electrophoresed by SDS-PAGE and immunoblotted as described in the text. 1, *G. nonoxygluconicus* IFO 3275; 2, *G. gluconicus* IFO 3286; 3, *G. cerinus* IFO 3265; 4, *G. suboxydans* var. α IFO 3257; 5, *G. suboxydans* var. α IFO 3256; 6, *G. suboxydans* var. α IFO 3255; 7, *G. suboxydans* var. α IFO 3254; 8, *G. suboxydans* IFO 3130; 9, *G. suboxydans* IFO 3172; 10, *G. suboxydans* IFO 12528.

Table I. ALCOHOL DEHYDROGENASE ACTIVITY
IN *Gluconobacter* SPECIES

Enzyme activity was assayed with membrane fraction using phenazine methosulfate—2,6-dichlorophenolindophenol as an electron acceptor, and expressed by specific activity (units/mg).

Strain	Enzyme activity
<i>G. oxydans</i> IFO 3189	0.11
<i>G. oxydans</i> IFO 3287	1.55
<i>G. dioxyacetonicus</i> IFO 3272	0.86
<i>G. cerinus</i> IFO 3262	1.53
<i>G. cerinus</i> IFO 3265	0.51
<i>G. cerinus</i> IFO 3268	0.92
<i>G. cerinus</i> IFO 3270	0.69
<i>G. gluconicus</i> IFO 3285	0.34
<i>G. gluconicus</i> IFO 3286	0.17
<i>G. albidus</i> IFO 3251	0.94
<i>G. albidus</i> IFO 3253	1.73
<i>G. suboxydans</i> IFO 3130	0.21
<i>G. suboxydans</i> IFO 3172	0.34
<i>G. suboxydans</i> IFO 12528	3.70
<i>G. suboxydans</i> var. α IFO 3254	0.02
<i>G. suboxydans</i> var. α IFO 3255	tr.*
<i>G. suboxydans</i> var. α IFO 3256	0.01
<i>G. suboxydans</i> var. α IFO 3257	tr.*
<i>G. suboxydans</i> var. IFO 3258	tr.*
<i>G. nonoxygluconicus</i> IFO 3275	0.77
<i>G. nonoxygluconicus</i> IFO 3276	3.62
<i>G. rubiginosus</i> IFO 3244	1.63

* Enzyme activity was less than 0.01 units/mg.

450 nm.

Immunocross-reactivity of the membranes of twenty-two *Gluconobacter* strains was examined by an immunoblotting using the monoclonal antibody against the dehydrogenase peptide and all strains examined here revealed

this peptide is present in variable amounts in the membranes as shown in Fig. 1 which is a part of an immunoblotting sheet. The results could not show the correlation of the amount of the dehydrogenase peptide with ADH activity. Although all strains of *G. suboxydans* var. α scarcely showed ADH activity as described in Table I, the 85-kDa peptide could be observed in these strains. These strains have been shown to contain little amount of the cytochrome *c* peptide, which is different from most of *Gluconobacter* sp. having high concentrations of cytochrome *c* (unpublished results). Thus, ADH activity seems to be dependent on not only the dehydrogenase peptide but also the cytochrome *c* peptide.

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