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Separation and Properties of Two Novel NADP+-dependent Alcohol Dehydrogenases from *Euglena gracilis* Z

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Abstract: We report here for the first time about the occurrence of a new NADP+-dependent ADH-I (EC1.1.1.2) in *Euglena gracilis* Z, which was separated from the already reported one (ADH-II) by hydrophobic chromatography on Butyl Toyo-pearl 650s and partially characterized. Both the enzymes were named as ADH-I and ADH-II based on their elution profile. ADH-1 seems to be constitutive with broader substrate specificity and reacted almost equally with all types of alcohols tested, while the inducible ADH-2 was active towards the middle and long-chain fatty alcohols and less active towards the primary short-chain and secondary alcohols. The ADH-1 was misophillic in nature with an optimal temperature of 55°C, while the other one was thermophilic. For ADH-1, the optimal pH was at the range of 8-9.0, while ADH-2 gave its maximal activity at pH range of 7.8 to 9.5 at 55°C. Accordingly, the two isozymes were proved to be different from each other in their properties.

Key words: *Euglena gracilis* Z, fatty alcohol assimilation, 1-Hexanol, NADP+-alcohol dehydrogenases, isozymes

INTRODUCTION

Many alcohol dehydrogenases have been reported, purified and characterized; the vast majority of the enzymes studied have been from animals-mostly mammals-or from plants and microorganisms (Jornvall *et al.*, 1987). Mammalian and plants ADHs have been purified from many sources and their properties, structures, functions and physiological significance have been studied in detail (Branden *et al.*, 1975). Most of alcohol dehydrogenases are NAD+-dependent (EC1.1.1.1) or NADP+-dependent (EC1.1.1.2) (Danielsson *et al.*, 1994) and exhibit a wide variety of substrate specificity (Jones *et al.*, 1976).

A NAD+-specific alcohol dehydrogenase (alcohol: NAD+ oxidoreductase, EC 1.1.1.1) activity was detected in cell-free extracts of *Euglena gracilis* var. *bacillaris* by Hurlbert and Rittenberg (1962), where as they did not report any NADP+-linked ADH activity. On the other hand a NADP+-dependent ethanol oxidation was reported by Danforth and Hunter (1966) without the occurrence of NAD+-dependent activity in the same organism. Similarly, both NADP+- and NAD+-specific alcohol dehydrogenases

have been reported to occur in the particle associated and soluble fractions of *Astasia longa* (Begin-Heick and Heick, 1970), but the detailed properties of these enzymes have not been elucidated.

Here we describe for the first time about the occurrence of a new enzyme (ADH-I) and its separation from our previously reported novel NADP+-linked alcohol dehydrogenase, ADH-2 (Munir *et al.*, 2002) in 1-hexanol grown *Euglena gracilis* Z and compare some of their properties. The characterization of enzymes is a prerequisite for their industrial application. Therefore, these enzymes were partially characterized for pH, temperature and substrate specificity.

MATERIALS AND METHODS

This experiment is part of the research conducted at the Department of Applied Biological Chemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University during 2000-2004. The work is now being continued at the Institute of Biotechnology and Genetic Engineering, NWFP Agricultural University Peshawar, Pakistan.

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Materials: Standard proteins were from Sigma Chemical (St. Louis, USA), NAD^+, $NADP^+$, DEAE cellulose, butyl toyo-pearl 650s and all other chemicals were from Nacalai Tesque, Kyoto, Japan and were of the highest purity and analytical grade.

Cells and culture: *Euglena gracilis* Z was cultured under illumination (2,000 lux) with shaking (90 strokes/min) at 27°C in CM (Cramer and Myers) medium (Cramer and Myers, 1952), containing 1-hexanol (0.025% v/v) as a sole carbon source.

Protein estimation: Protein was estimated using CBB dye-binding method of Bradford (1976) with BSA as a standard.

Preparation of the crude enzyme: Cells were harvested by centrifugation (1500×g, 10 min) at 4°C, suspended in 100 mL of 50 mM Tris-HCl buffer, pH 8.0, containing 20% ethylene glycol, 1 mM $MgCl_2$ and 1 mM DTT and subjected to sonic disruption (10 kHz, 1 min×6). The resulting lysate was centrifuged at 12,000×g for 15 min at 4°C. The supernatant fraction was used as a crude enzyme. All operations were conducted below 4°C.

Enzyme assays: All enzyme assays were done at 55°C in a total volume of 1 mL.

Alcohol dehydrogenase activity for the oxidation reaction was assayed by the method of Munir *et al.* (2002). The reaction mixture for contained 100 mM Tris-HCl buffer, pH 8.8, 10 mM $MgCl_2$, 2 mM $NADP^+$ and 20 mM 1-hexanol. The reaction was initiated by the addition of 1-hexanol and the formation of NADPH during the reaction was monitored by measuring the increase in absorbance at 340 nm.

Separation of $NADP^+$-linked alcohol dehydrogenases: *Euglena gracilis* Z grown on 1-hexanol for 24 h was used as the starting material. The harvested cells were washed and suspended in 100 mL of 50 mM Tris-HCl buffer, pH 8.0, containing 20% ethylene glycol, 1 mM $MgCl_2$ and 1 mM DTT. All operations were conducted below 4°C. The crude enzyme was obtained by sonic disruption (10 kHz, 1 min×6 with 1 min interval) and sonicate was centrifuged at 12,000×g for 15 min. After optimisation, $(NH_4)_2SO_4$ was added at 30% saturation to the supernatant of centrifuged homogenate at 0°C, incubated for 4 h and centrifuged at 35,000×g for 30 min at 4°C. The precipitate was discarded and further $(NH_4)_2SO_4$ was added to the supernatant at 0°C to attain 70% saturation. The precipitate was collected by centrifugation 35,000×g for 30 min at 4°C and dissolved in 50 mM Tris-HCL buffer,

pH 8.0, containing 20% ethylene glycol, 1 mM $MgCl_2$ and 1 mM DTT. After desalting with the same buffer for 24 h with 3-4 changes, the supernatant was applied at 0.5 mL min^{-1} onto a column of DEAE cellulose (2.5×4.0 cm) pre-equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 20% ethylene glycol, 1 mM $MgCl_2$ and 1 mM DTT (buffer A). Once loaded and after allowing 15 min for protein binding, the column was washed with at least five column volumes of the same buffer at 1 mL min^{-1} until the A_{280} of the effluent was < 0.05. The retained proteins were eluted at 0.5 mL min^{-1} with a linear 0-1 M KCl gradient in buffer A. Fractions containing higher enzyme activity were pooled, dialyzed against buffer A for 24 h with 4 changes. Ammonium sulfate was added to the dialyzed enzyme solution at a final concentration of 1 M and put on a Butyl Toyo-pearl 650s column (3.5 × 10 cm) that was pre-equilibrated with buffer A containing 1 M ammonium sulfate. After allowing 15 min for the protein to bind, column was washed with the same buffer and elution was performed with a linear gradient containing decreasing ammonium sulfate concentration (1-0 M). Active fractions were pooled, dialyzed against buffer A for 24 h with 4 changes, concentrated and stored in -80°C until use.

Temperature optimum: $NADP^+$-linked alcohol dehydrogenases were assayed at different temperature ranging from 25-60°C as described before. The enzyme assay was carried out up to 60°C due to limited temperature range of the spectrophotometer.

pH optimum: $NADP^+$-linked alcohol dehydrogenases were assayed at different pH ranging from 5.0-10.5 at 55°C. The buffers used were: pH 5-5.5 (100 mM sodium acetate/acetic acid), pH 6-6.5 (100 mM K_2HPO_4 buffer) pH 7.0 (100 mM MOPS-KOH buffer), pH 7.5 (100 mM HEPES-KOH buffer), pH 8.0-8.8 (100 mM Tris-HCl buffer), pH 9.0-10.0 (100 mM glycine-NaOH buffer) and pH 9.0-10.5 (100 mM $(NH_4)_2CO_3$-NH_3 buffer) (Stoll and Blanchard, 1990, Tipton and Dixon, 1979).

RESULTS AND DISCUSSION

Separation and partial purification of the $NADP^+$-dependent ADH-I and ADH-II: The enzymes solution when applied on to ion exchange and hydrophobic chromatographies following ammonium sulfate precipitation, yielded two different $NADP^+$-dependant activities, which were completely separated at 0.4 and 0.2 mM $(NH_4)_2SO_4$ and were designated as $NADP^+$-dependent ADH-1 and ADH-2, respectively, based on the elution pattern (Fig. 1). This confirmed
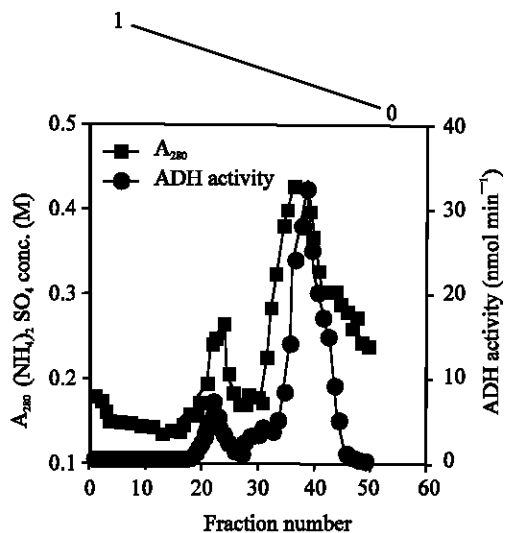
Fig. 1: Separation of two NADP⁺-dependent alcohol dehydrogenases activities by hydrophobic interaction chromatography on Butyl Toyo-pearl 650s using linear gradient method

the presence of two NADP⁺-dependent isoenzymes in *Euglena gracilis* Z. These findings are in continuation of our previous work reporting the separation of NAD⁺-dependent and NADP⁺-dependent ADHs on ion exchange chromatography (Munir *et al.*, 2002). This study suggest that the two proteins differ markedly in their amino acid composition that could be fractionated on the basis of hydrophobic interactions. As the ADH-1 was produced in low quantity, it could be used for partial characterization only, while ADH-2 was further purified to electrophoretic homogeneity (paper submitted). These results are in agreement to the previous work on the occurrence of a novel NADP⁺-dependent ADH in *Saccharomyces cerevisiae* (Tani *et al.*, 2000).

Substrate specificity: The two NADP⁺-dependent ADHs, separated at the Butyl Toyo-pearl 650s (Hydrophobic Interaction Chromatography) step of purification (Fig. 1), were evaluated for substrate specificity. ADH-1 showed broader substrate specificity and reacted almost equally with all types of alcohols tested, while the ADH-2 was more specific for the middle-and long-chain length of fatty alcohols, showing less activity in the presence of primary short-chain and secondary alcohols (Table 1). The low activity of the ADH-II with long-chain alcohols may be due to the poor solubility of the long-chain alcohols. Similar enzymes have been reported in some bacteria and yeast (Wales and Fewson, 1994; Tsigos *et al.*, 1998). In other microorganisms the presence of multiple isoforms have been indicated by substrate specificity (Wales and

Table 1: Substrate specificity of NADP⁺-dependent ADHs from *E. gracilis* Z

| Substrate	ADH-1 (nmole min⁻¹ mL⁻¹)	ADH-2 (nmole min⁻¹ mL⁻¹)
Ethanol (20 mM)	29.74 (0.74)	14.06 (0.12)
1-Propanol (20 mM)	30.06 (0.75)	18.87 (0.16)
2-Propanol (20 mM)	24.37 (0.61)	26.76 (0.23)
1-Butanol (20 mM)	37.59 (0.94)	37.38 (0.32)
2-Butanol (20 mM)	35.71 (0.89)	26.76 (0.15)
1-Pentanol (20 mM)	37.32 (0.93)	104.20 (0.89)
1-Hexanol (20 mM)	40.04 (1.00)	116.23 (1.00)
1-Octanol (20 mM)	34.06 (0.83)	57.98 (0.50)
1-Decanol (1 mM)	21.39 (0.53)	14.67 (0.12)
Lauryl alcohol (3 mM)	29.76 (0.74)	16.22 (0.14)
Myristyl alcohol (2 mM)	21.32 (0.53)	18.74 (0.16)
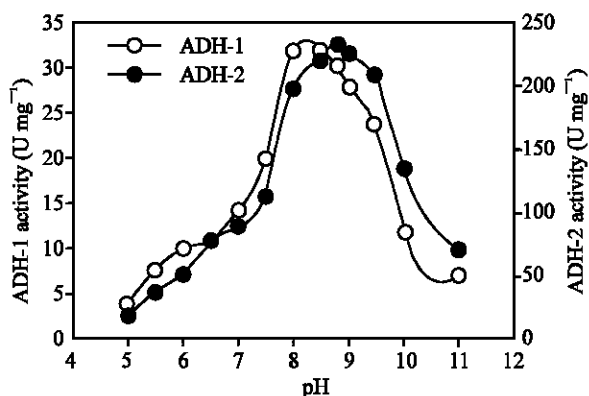
Fig. 2: Effect of pH on the activities of NADP⁺-dependent ADHs. (The ADHs activities with 1-hexanol were assayed at 55°C at different pHs as explained in the Materials and Methods)

Fewson, 1994; Tsigos *et al.*, 1998). We, therefore, suggest that *Euglena gracilis* ADH-1 and ADH-II are distinct enzymes in showing major differences in their preferences for different alcohols.

Effect of pH: The ionization of the active site residues mainly depends upon the concentration of H⁺ ions as well as the temperature. The two NADP⁺-dependent ADHs from 1-hexanol-grown *Euglena gracilis* Z were assayed at different pH. ADH-1 showed its ideal ionization of active site residues between pH range of 8 and 9.0, while ADH-2 gave its maximum activity at pH range of 7.8 to 9.5 at 55°C (Fig. 2), similar to the results reported in some bacteria, *E, coli* and yeast by Adachi *et al.* (2001), Tani *et al.* (2000) and Wales and Fewson (1994).

Effect of temperature: The NADP⁺-linked ADH isozymes were further precisely evaluated for optimal temperature. The ADH-1 showed a declining trend in activity at 60°C (Fig. 3), indicating it as its optimal temperature. The optimum temperature of ADH-2 was more than 60°C as it showed a linear increase at this temperature. The enzyme
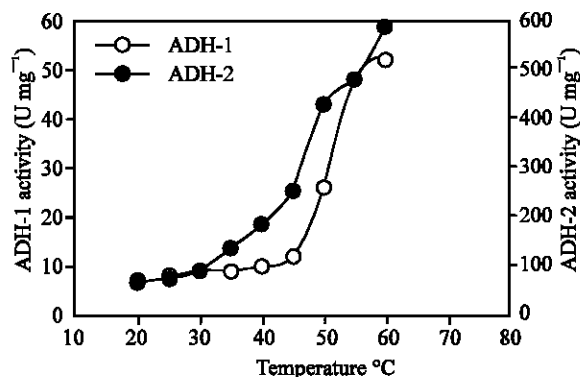
Fig. 3: Dependence of catalytic activity of the NADP⁺-dependent alcohol dehydrogenases on temperature. (Rates were measured at saturation concentration of substrates (for other details see the materials and methods)

assay was stopped beyond this temperature due to the limitation of spectrophotometer for higher temperature. This value was higher than those reported for mesophilic microorganisms ADHs (25-40°C) (Niehaus *et al.*, 1978; Neale *et al.*, 1986; Hensgens *et al.*, 1993; Tsigos *et al.*, 1998). The enzyme was therefore suggested to be thermophilic in nature. Similar results have also been reported for ADHs from *B. stearothermophilis* (65°C) (Guagliardi *et al.*, 1996), *Sulfolobus solfataricus* (95°C) (Rella *et al.*, 1987), *Thermoanaerobacter ethanolicus* (70°C) (Pham and Phillips, 1990) and *Thermococcus hydrothermalis* (80°C) (Antoine *et al.*, 1999).

In conclusion, we are reporting that NADP⁺-dependent ADH in *Euglena gracilis* is not a single enzyme. Our findings of the presence of more than one NADP⁺-dependent ADHs will not only facilitate proper characterization of ADHs in *Euglena gracilis* per se, but also suggest that the fatty alcohol metabolism in this organism should be scrutinized accordingly.

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