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ORIGINAL PAPER

Alcohol Dehydrogenase Activity in *Carassius carassius* White Skeletal Muscle.

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Abstract

A form of alcohol dehydrogenase was prepared from white skeletal muscle of Carassius carassius by acetone precipitation, Sephacryl S-200 gel filtration and DEAE-Fractogel anion-exchange chromatography. We have determined the molecular weight and the kinetic properties of this enzyme. The molecular weight value, 81,400 Da, was very similar to mammalian alcohol dehydrogenase. The high Km value for ethanol (555 x 10^{-1} M) suggested that this enzyme might be a class III alcohol dehydrogenase.

Keywords: *alcohol dehydrogenase, Carassius carassius, enzyme purification, Km values.*

Introduction

Alcohol dehydrogenase (ADH) activity is amply distributed in numerous phyla that include organisms belonging to each of the different realms in which living beings are classified. Three protein families, which seem to have arisen independently throughout evolution, carry out this activity [1]. The first family is comprised of "short chain" alcohol dehydrogenases, which do not require a metallic ion as cofactor and which are found characteristically among insects [2]. The second family is made up of the "medium and long chain" alcohol dehydrogenases and requires zinc atoms as cofactor. The third and last family corresponds to the Fe-dependent alcohol dehydrogenases, which are characteristic of unicellular organism [3].

When it comes to vertebrates, just the two first protein families are present, but only the "medium and long chain" family exhibits a significant alcohol dehydrogenase activity in these organisms. Different forms of this family were characterized from prevertebrates as anurochordate and cephalocordate [4].

Among vertebrates, alcohol dehydrogenases were specially studied in rats. In these mammals, the alcohol dehydrogenase family is divided into six distinct classes, which make up, at least, twenty different isozymes codified by more than seven distinct genes [5]. Class I was the most studied class of this enzyme family and comprises the "classic" hepatic enzyme [6]. Indeed, in mammals the liver is the main site of ethanol metabolism, but other tissues may contribute to ethanol metabolism as well [7].

Few investigations on the alcohol dehydrogenase system in fishes were reported. One class of alcohol dehydrogenase has been described in fish, and has been found to be

structurally similar to mammalian class III alcohol dehydrogenase (gluthatione-dependent formaldehyde dehydrogenase, EC 1.2.1.1) but functionally similar to class I (primarily responsible foe ethanol dehydrogenase, EC 1.1.1.1) [8]. In grass carp (*Ctenopharyngodon idellus*) and common carp (*Cyprinus carpio*), alcohol dehydrogenase activity was detected in the liver [9,10], while in crucian carp (*Carassius carassius*) alcohol dehydrogenase was found only in red and white skeletal muscle [11].

The current study was undertaken to investigate the multiplicity and the enzymatic properties of alcohol dehydrogenase from the white skeletal muscle of crucian carp.

Materials and Methods

Enzyme's extraction

After the fish were killed, the white skeletal muscle was quickly dissected and rinsed with cold saline solution to remove blood. The tissue was immediately submerged in demineralised water and homogenized with a motor-driven Potter-Elvejhem homogenizer at 0-4^{o}C until a uniform suspension was obtained. The homogenates were centrifuged at 15,000g for 15 minutes and the clear supernatants were stored at –80^{0}C.

Assay of enzyme activity and protein concentration

Alcohol dehydrogenase (ADH) was determined following the decrease of absorbency at 340 nm [12]. One unit of activity (1U) is the amount of enzyme that oxidizes 1 μmole NADH in a minute.

Protein concentration was determined using the Lowry method [13] and by measuring the absorbency at 280 nm.

The specific activity of alcohol dehydrogenase was expressed as U/mg protein.

Results and Discussions

Purification procedure

A three-step procedure was used to purify the alcohol dehydrogenase from the homogenates of white skeletal muscle.

In the first step, two volume of acetone (at – 70^{0}C) were added to each volume of the clear supernatant with stirring of this mixture in an ice bath. The precipitated protein was collected by centrifugation for 30 min at 14,000 g and the residual acetone was eliminated under a stream of air. The pellet was resuspended in a minimal volume of 0.1 M TRIS-HCl buffer, pH 8.0, and again centrifuged for 10 min at 10,000 g to remove any insoluble material. Typically, 82.6% of the starting activity is recovered after this step (**Table 1**).

Table 1. Purification of alcohol dehydrogenase from white skeletal muscle of *Carassius carassius.*

Step	Volume (ml)	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Purification factor	Yield (%)
Crude extract	100	30 260.3	340.0	89.0	1.0	100.0
Acetone precipitation (66% saturation)	20	24 994.8	163.4	152.8	1.7	82.6
Gel filtration (S-200)	24	15 952.0	43.2	369.3	4.2	52.7
Anion-exchange (DEAE-Fractogel)	50	9560.4	11.3	846.1	9.5	31.6

The supernatant from the above step was subjected to gel filtration on Sephacryl S-200 (60.0 x 1 cm) preequilibrated with 0.1 M TRIS-HCl buffer, pH 8.0. The column was eluted with the same buffer at a constant flow rate of 25 ml h^{-1}. Fractions (3 ml) were collected and assayed for enzyme activity and protein content. Alcohol dehydrogenase activity was eluted as a single symmetrical peak (**Figure 1**). Fractions eluted with volumes between 72 and 96 ml, were precipitates by addition of ammonium sulphate to 100% (w/v) saturation. The pellet was recovered by centrifugation at 14,000 g for 30 min and dissolved in a minimal volume of demineralized water.

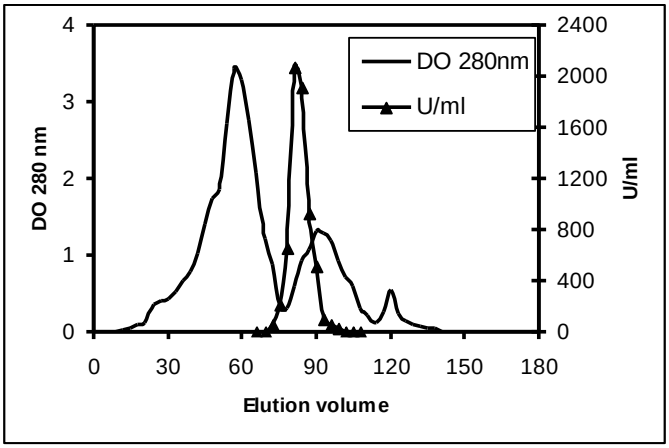


Figure 1. Gel-filtration on Sephacryl S-200

The concentrated solution was dialyzed against 100 volumes of 0.02 M TRIS-HCl buffer, pH 7.8, and subjected to anion-exchange chromatography on DEAE- Fractogel TSK 650 (column size 12 x 2 cm) preequilibrated with the above buffer. Unbound protein was removed by washing with 100 ml volumes of started buffer at a flow rate of 25 ml h^{-1}. For bounded proteins a stepwise elution, with increasing ionic strength in NaCl, was applied. Fractions of 19 ml were collected. Alcohol dehydrogenase activity eluted at 0.1 M NaCl (**Figure2**), with a 9.5-fold purification factor and 31.6% recovery with respect to the starting material (**Table 1**).

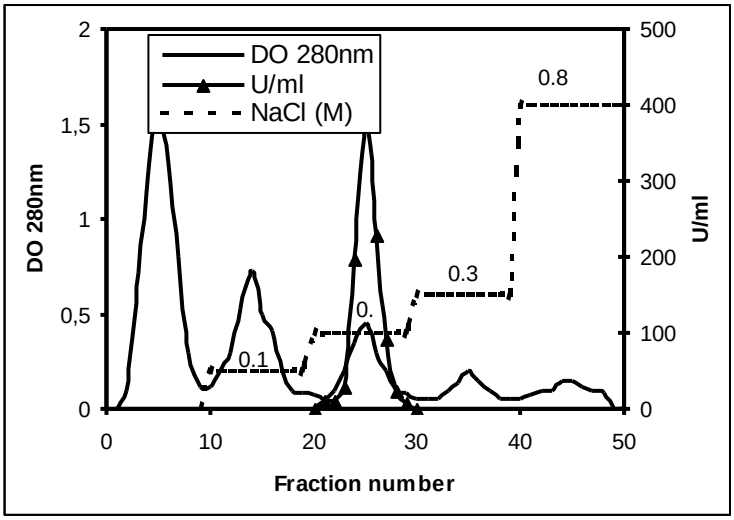


Figure 2. ADH separation on DEAE - Fractogel

The anion-exchange chromatography showed that, at pH 7.8, the alcohol dehydrogenase from white skeletal muscle of *Carassius carassius* was negatively charged; therefore it has an isoelectric point under 7.8.

A summary of the purification of alcohol dehydrogenase is shown in **Table 1**. With respect to the crude extract, it can be seen that alcohol dehydrogenase was obtained in 31.6% yield and represented 9.5-fold purification.

Molecular weight value

The molecular weight of alcohol dehydrogenase purified from white skeletal muscle of *Carassius carassius* was estimated by gel filtration on Sephacryl S-200, using a Sigma kit for molecular weights 12,000-200,000 Da. By comparison with standard proteins, the relative molecular weight (Mr) value was calculated to be 81,400 Da (**Figure 3**). This value is very similar to that reported for mammalian alcohol dehydrogenases, which are dimeric proteins with subunits of approximately 40,000 Da [7,14].

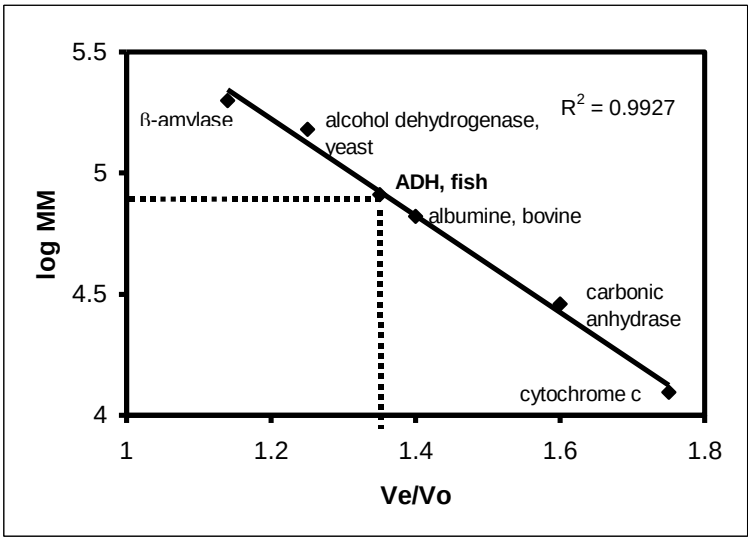


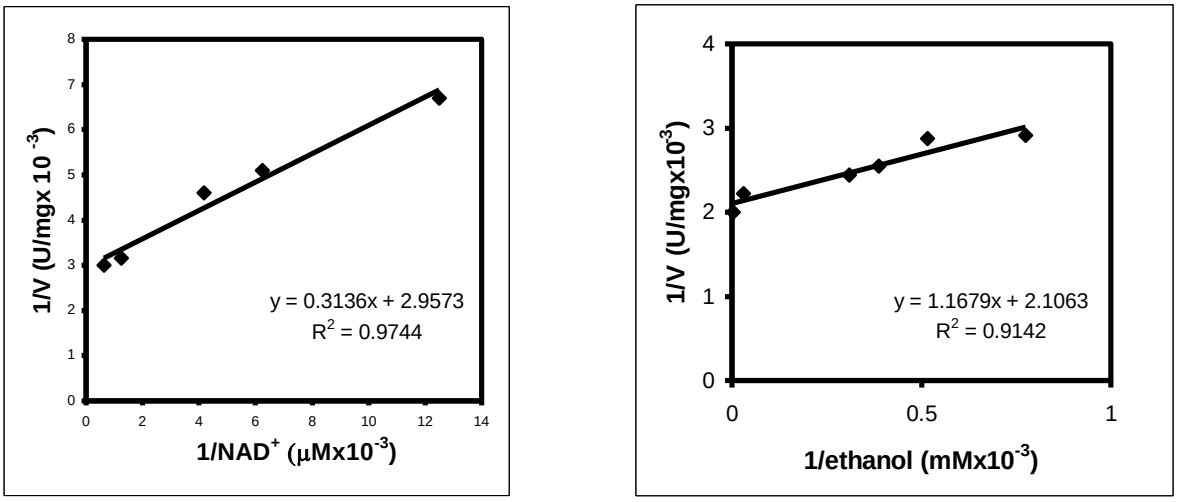
Figure 3. Molecular weight of ADH estimated by gel-filtration on Srphacryl S-200.

Kinetic properties

The kinetic properties of the purified enzyme were determined with ethanol as substrate. Lineweaver-Burk analysis of enzymatic activity as a function of NAD^+ concentration (**Figure 4a**) indicated the Km to be 1.06×10^{-4}M and the Vmax to be 338.18 U/mg. This Km value is higher that determinate for Class I mammalian enzymes, which comprises the "classic" hepatic alcohol dehydrogenases with a Km value in range 10^{-5} M [15].

The alcohol dehydrogenase attempted with difficulty the ethanol saturation. The Km value for ethanol was 555×10^{-1} M (**Figure 4b**), enzyme showing a less affinity for this substrate and a limited capacity to oxidize ethanol.

Our studies founded a single form of alcohol dehydrogenase from white skeletal muscle of *Carassius carassius*. The proprieties of this enzyme, pI less 7.0 and a higher Km from ethanol, suggested its affiliation to class III alcohol dehydrogenases. The class III alcohol dehydrogenases includes homodimer proteins which possess a great capacity to metabolize long chain alcohols, while its capacity to oxidize ethanol is very limited [4].



a b

Figure 4. Estimation of Km and Vmax with Lineweaver-Burk double reciprocal plot: a) for NAD^+; b) for ethanol

The number of alcohol dehydrogenases in fish is controversial. In zebrafish *(Danio rerio*) one class of alcohol dehydrogenase has been described and has been found to be structurally similar to mammalians class III ADH, but functionally similar to class I ADH [8]. The others studies showed that two active forms of alcohol dehydrogenase could be purified from grass carp (*Cteropharyngodon idellus*) liver [9,10,16]. One of these forms was derived from the ADH-1 through limited and specific proteolytic clevage [9].

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