NEW SIMPLE METHOD FOR PURIFICATION OF CLASS I ALCOHOL DEHYDROGENASE

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Abstract — Aims: The purpose of this study was to develop a new simple method for purification of rat class I alcohol dehydrogenase (ADH, EC 1.1.1.1). Methods and Results: Immobilized p-hydroxyacetophenone was used as a ligand for affinity chromatography for the initial purification step after ammonium sulfate precipitation of the cytosolic fraction of rat liver. Then the eluant was separated by using ion-exchange chromatography, and homogenous class I ADH, as judged by the results of SDS-PAGE and confirmed by the results of the amino-acid sequence of peptides degraded from a 39 kDa protein, was obtained with a high yield (57%). The purified ADH showed kinetic constants of 1.3 mmol/l for Km and 62.4 per min for Kcat with ethanol as a substrate. ADH was also successfully purified from yeast by a similar method using p-hydroxyacetophenone affinity chromatography. Conclusions: This simple method involving only two chromatographic procedures may be very useful for purification of ADH.

INTRODUCTION

The major pathway for ethanol disposition involves alcohol dehydrogenase (ADH, EC 1.1.1.1), an enzyme that catalyses conversion of ethanol to acetaldehyde. In addition to metabolism of exogenous ethanol, ADH is known to eliminate small amounts of endogenous alcohol produced by fermentation in the gut (Baraona et al., 1986). ADH has a broad substrate specificity, including dehydrogenation of steroids (Knutson and Ungar, 1982), oxidation of intermediary alcohols of the shunt pathway of mevalonate metabolism (Keung, 1991), and ω-oxidation of fatty acids (Björkhem, 1972). ADH of the rat liver is an enzyme with five classes (I, II, III, IV and VI) of different molecular forms based on homology of amino-acid sequences, catalytic properties and expression patterns. Class II ADH cDNA was cloned from screening of a rat liver cDNA library using human class II cDNA fragment as a probe (Höög, 1995). However, protein expression of class II ADH has not been reported in any organs of the rat (Höög and Brandt, 1995). Class VI ADH was isolated from a rat cDNA library using a human class V ADH cDNA as a probe (Höög and Brandt, 1995). Two new mouse Adh genes (Adh5a and Adh5b) have recently been identified and are thought to be closely related to the human ADH5 gene (Szalai et al., 2002). Km values of rat recombinant class VI ADH have been reported to be 20 nmol/l for ethanol and 10 nmol/l for octanol at pH 10 (Höög and Brandt, 1995). Classes I, III and IV ADH have been purified and characterized. Class III ADH has been detected in all organs of rats and has a low specificity constant for ethanol (Julia et al., 1988). Class IV ADH is distributed in the skin, cornea and mucosal tissues of the digestive, respiratory and sexual tracts (Parés et al., 1994). Class I and IV ADH have also been shown to have high levels of activity for oxidation of retinol and for reduction of retinal (Boleda et al., 1993). Class IV ADH in the retina and pigmented epithelium is thought to play a role in the interconversion system of retinoid in visual cycle reactions (Parés et al., 1985).

Class I ADH is mainly located in the liver and is also present in the intestine, testis and kidney (Julia et al., 1988). Among the above-mentioned classes, class I ADH has a much lower Km (1.4 mmol/l) for ethanol than that of class IV ADH (5000 mmol/l) (Julia et al., 1987). Class I ADH in the liver has been investigated as a major enzyme for understanding alcohol toxicity and its metabolism (Crabb et al., 1983; Forsander and Sinclair, 1992). Thus, purified class I ADH is needed for various investigations, for example X-ray diffraction of class I ADH using its protein crystals and in-vivo interaction of administered drugs with ethanol or retinoids. However, the yields of ADH obtained by methods used for its purification so far have not necessarily been high. This may be due to a decrease in the level of enzymatic activity during several ion-exchange and/or gel filtration chromatography procedures after extraction of the cytosolic fraction.

In the present study, we developed a new method for purification of class I ADH using p-hydroxyacetophenone (p-HAP)-Sepharose affinity chromatography.

MATERIALS AND METHODS

Materials

All chemicals were of reagent grade. Epoxy-activated Sepharose 6B, Mono S HR 5/5 and µRPC C2/C18 SC2.1/10 columns were obtained from Amersham Pharmacia Biotechnology. Poros HQ column was obtained from PerSeptive Biosystems. p-Hydroxyacetophenone was from Wako Chemical. Molecular weight markers for SDS-PAGE were purchased from Bio-Rad Laboratories and Owl Scientific. NAD and ADH from yeast was purchased from Boehringer Mannheim and Oriental Yeast, respectively. Ultrafiltration materials (Amicon Centripurus YM50) were from Millipore.

Sample preparation from rat liver

The study protocols regarding treatment of animals were in accordance with the ‘Guidelines for Experiments Using Laboratory Animals in Yamagata University School of Medicine’. Male Wistar rats were killed by decapitation under anaesthesia using sodium pentobarbital (50 mg/kg). Livers were...
buffers were filtrated and degassed. Buffer 4: 0.1 mol/l Tris–HCl (pH 8.5) and 0.5 mmol/l DTT. All
Buffer 3: 50 mmol/l phosphate (pH 8.0) containing 1 mol/l NaCl.

...with a one-tenth volume of 400 mmol/l DTT at 37°C. Then, the excized gel was rinsed three times with 0.05 mol/l Tris–HCl (pH 9.0, 37°C). The digest was separated by reverse-phase high-performance liquid chromatography (HPLC) (SMART SYSTEM; Amersham Pharmacia Biotechnology), applying a gradient of acetonitrile in 0.060–0.055% aqueous trifluoroacetic acid (TFA), using a μRPC C2/C18 SC 2.1/10 column (3 × 250 × 4 mm). The column was developed with a linear gradient of an acetonitrile solution (0.055% TFA in 84% acetonitrile). The flow rate was 0.1 ml/min, and UV absorption was recorded at 214 nm.

**Determination of 39-kDa protein sequence**

After mapping of degraded peptides from a 39-kDa protein, two fractions were selected for determination of amino-acid sequence of the protein. Sequence degradations were carried out using an ABI 447A sequencer (Applied Biosystems). The results of sequence alignment were compared with the protein sequence from the data bank using a genome net (http://fasta.genome.ad.jp).

**Purification of ADH from yeast**

Yeast (*Saccharomyces cerevisiae*) extract was prepared by toluene autolysis as described previously (Rutter and Hunsley, 1966). The post-ammonium sulfate (50–70%) precipitate from the toluene autolysate was dissolved in a minimal volume of buffer 1 and applied to a p-HAP-Sepharose column that was pre-equilibrated with buffer 1. The column was washed with a four-column volume of buffer 1, and the bound proteins were eluted with a two-column volume of the same buffer containing 10 mmol/l p-HAP. Aliquots of the affinity chromatography sample were pooled and concentrated to 0.5 ml in an ultrafiltration cell with an Amicon Centriplus. The concentrated sample was then dialysed three times against buffer 2 and loaded onto a Mono S column equilibrated with buffer 2 and eluted by a linear NaCl gradient of buffer 3. The flow rate was 1 ml/min, and UV absorption was recorded at 280 nm. The eluted fraction from the first single peak was collected and dialysed against buffer 4. The purified class I ADH was stored at 4°C.

Prior to the following kinetic analysis, DTT was removed by dialysis.

**In-gel digestion and reverse-phase chromatography**

A concentrated sample of fractions eluted from the p-HAP-Sepharose column was dissolved in SDS sample buffer [0.625 mol/l Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.01% bromophenol blue] and reduced with a one-tenth volume of 400 mmol/l DTT at 37°C for 2 h. Then carboxymethylation was carried out by addition of 800 mmol/l iodoacetoamide with the same volume of DTT at 37°C for 30 min. After the pH of the solution had been adjusted with NaOH to 6.8, the samples were subjected to SDS–PAGE. The gel was stained with Coomassie brilliant blue. After destaining, the part of the 39-kDa protein stained in the gel was excised and transferred to an Eppendorf tube. Then the excized gel was rinsed three times with 0.05 mol/l Tris–HCl (pH 9.0) / acetonitrile (1:1) and dried. Next, the gel was treated with a Lys-specific protease from Achmobactor for 12 h in 0.05 mol/l Tris–HCl (pH 9.0, 37°C). The digest was separated by reverse-phase high-performance liquid chromatography (HPLC) (SMART SYSTEM; Amersham Pharmacia Biotechnology), applying a gradient of acetonitrile in 0.060–0.055% aqueous trifluoroacetic acid (TFA), using a μRPC C2/C18 SC 2.1/10 column (3 × 250 × 4 mm). The column was developed with a linear gradient of an acetonitrile solution (0.055% TFA in 84% acetonitrile). The flow rate was 0.1 ml/min, and UV absorption was recorded at 214 nm.

**Activity assay and measurement of kinetic parameters of class I ADH**

The enzymatic activity of the purified class I ADH was measured with 0.1 mol/l glycine/NaOH (pH 10) buffer, 2.4 mmol/l NAD and 33 mmol/l ethanol in a Beckmann DU7000 spectrophotometer. One unit of activity (U) equals 1 μmol NAD(H) produced/min at 25°C, based on an absorption coefficient of 6220 mol/l/cm for NADH at 340 nm. The same procedure as that described above was also used for kinetic analysis. The range of ethanol concentrations used for the activity assay was 0.25–25 mmol/l. The data were fitted using Lineweaver–Burk kinetic plots.

**Protein determination**

Protein concentration was measured by the Bradford method with bovine serum albumin as a standard.

**RESULTS**

**SDS–PAGE of eluant from p-HAP affinity chromatography**

p-HAP was effectively coupled to epoxy-activated Sepharose to generate an affinity matrix, and p-HAP affinity chromatography...
was successively conducted using the fraction of liver cytosol after ammonium sulfate precipitation as a material loaded to the affinity column. Four polypeptides, which had molecular weights of 54, 43, 39 and 25 kDa as estimated by SDS–PAGE, were simultaneously eluted with p-HAP (Fig. 1, lane 1). The yields of the 54- and 39-kDa polypeptides were higher than those of the other polypeptides as judged by the SDS–PAGE pattern.

**Internal amino-acid sequence of the 39-kDa polypeptide**

We conducted a peptide mapping of the 39-kDa polypeptide by reverse-phase HPLC after cleavage with a Lys-specific protease. Two fractions, were selected for analysis of amino-acid sequence. The nine amino-acid residues of one peak were determined to be Ile, Asp, Ala, Ala, Pro, Leu, Asp and Lys, and the seven amino-acid residues of the other peak were determined to be Phe, Pro, Leu, Glu, Pro, Leu and Ile. A computer data-bank search showed that the sequences of the nine amino-acid residues of the former peak and seven amino-acid residues of the latter peak were identical to the partial amino-acid sequences (161–169 and 341–347, respectively) of rat class I ADH.

**Purification of class I ADH from rat liver cytosol**

We further purified the 39-kDa polypeptide from the eluant of p-HAP affinity chromatography by using ion-exchange chromatography. A high and sharp peak, discriminated from other contaminating proteins as shown by the arrow in Fig. 2, was obtained, by Mono S ion-exchange chromatography and was detected as a single band with 39 kDa on SDS–PAGE (Fig. 1, lane 2). Table 1 summarizes the data on purification of class I ADH. The enzyme was purified 74-fold at 57% yield, compared with the initial cytosolic fraction. The values of $K_m$ and $K_{cat}$ for ethanol are 1.3 mmol/l and 62.4 per min, respectively.

![Fig. 1. SDS–PAGE analysis of purified rat liver class I ADH. The polyacrylamide gel (12.5%) was stained with Coomassie blue. Lane 1, eluant from a p-hydroxyacetophenone-Sepharose affinity column preparation. Lane 2, eluant from a Mono S column. Molecular weight standards are shown on the left. The 39-kDa ADH subunit mass is indicated by an arrow.](image1)

![Fig. 2. Mono S ion-exchange chromatography of the eluant fraction from a p-hydroxyacetophenone-Sepharose column. Conditions of chromatography are described in 'Materials and methods'. The arrow indicates a peak fraction that was collected and then subjected to SDS–PAGE.](image2)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (µl)</th>
<th>Total protein (µg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>5000</td>
<td>19850</td>
<td>0.024</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>50–70% (NH₄)₂SO₄</td>
<td>600</td>
<td>11160</td>
<td>0.038</td>
<td>1.6</td>
<td>89</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>500</td>
<td>512</td>
<td>0.56</td>
<td>23</td>
<td>61</td>
</tr>
<tr>
<td>Mono S chromatography</td>
<td>500</td>
<td>152</td>
<td>1.78</td>
<td>74</td>
<td>57</td>
</tr>
</tbody>
</table>
Purification of ADH from yeast

As shown in Fig. 3, the 39-kDa protein was also successfully purified from yeast using two column procedures (affinity chromatography using p-HAT and ion-exchange chromatography using a Poros HQ column following post-ammonium sulfate (50–70%) precipitation.

DISCUSSION

Judging from the molecular weight information on the eluant from the column of p-HAP, the 54-kDa protein may be aldehyde dehydrogenase (ALDH). We also found the 43-, 39- and 25-kDa proteins in the liver cytosolic fraction that have affinity to p-HAP. The 39-kDa protein was demonstrated to be rat class I ADH by peptide mapping after reverse-phase HPLC. This protein was further purified in high yield by using ion-exchange chromatography.

Affinity chromatography using 4-[3-(N-6-aminocaproyl) aminopropyl]pyrazole-Sepharose (CapGapp–Sepharose) is often used for purification of class I ADH from human and rat livers (Lange and Vallee, 1976). However, in this method, the presence of NAD at high concentrations (a millimolar range) is needed for the enzyme to bind to the resign of the affinity chromatography, and a high concentration of ethanol (0.5 mol/l) is required to elute the enzyme from the affinity resign. On the other hand, p-HAP-Sepharose is used for purification of two enzymes, mitochondrial ALDH from the rat liver and chloramphenicol acetyl transferase in E. coli (Ghenbot and Weiner, 1992). In the present study, we demonstrated that p-HAP-Sepharose also has an affinity to class I ADH from the rat liver cytosolic fraction, and we developed a simple and effective method for its purification using immobilized p-HAP resign. Moreover, p-HAP affinity chromatography was also shown to be useful for purification of ADH from yeast.

Table 2. Comparison of columns used during purification step as well as the specific activities and yields of the final product between this study and previous studies

<table>
<thead>
<tr>
<th>Reference (material)</th>
<th>Columns used during purification steps</th>
<th>Specific activity (µmol/min/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markovic et al. (rat liver)</td>
<td>1. DEAE-Sephadex 2. Sephadex G100 3. Sephadex G100</td>
<td>1.732 (23.5°C)</td>
<td>14.3</td>
</tr>
<tr>
<td>Lad and Leffert (rat liver)</td>
<td>1. Sephadex G100 2. DEAE- Affi- Gel Blue 3. Affi-Gel Blue 4. AMP- agarose 5. HPLC I-125</td>
<td>2.2 (37°C)</td>
<td>3.8</td>
</tr>
<tr>
<td>Lange and Vallee (human liver)</td>
<td>1. DEAE-Sephrose 2. CapGapp-Sepharse</td>
<td>5.7 (25°C)</td>
<td>65</td>
</tr>
<tr>
<td>Julià et al. (rat liver)</td>
<td>1. DEAE-Sephrose 2. DEAE-Sephrose 4. CapGapp-Sepharse 4. Sephadex G-25</td>
<td>1.3 (25°C)</td>
<td>22</td>
</tr>
<tr>
<td>Negoro and Wakabayashi (rat liver)</td>
<td>1. p-HAP-Sephrose 2. Mono S</td>
<td>1.78 (25°C)</td>
<td>57</td>
</tr>
</tbody>
</table>
Table 2 shows the column used for purification of class I ADH and the specific activities and yields of finally isolated products obtained by using our method and methods in other studies. Although the specific activity in the present study of the final product of ADH purification obtained by Markovic et al. (1971) is similar to that in the present study, the Km value (2.13 mmol/l) obtained by using their method is higher than that obtained by using our method (1.3 mmol/l). It is difficult to simply compare the properties of the final product of ADH purification obtained by Lad and Leffert (1983) with the results of other studies, because the temperature at which the enzyme activity of ADH was measured in the study of Lad and Leffert (1983) is different from those in other studies. Our method using p-HAP was comparable to the CapGapp method by Lange and Vallee (1976) from the viewpoint of high yield. However, Julià et al. (1987), using a CapGapp–Sepharose affinity chromatography, reported a much lower yield of class I ADH purification, which is possibly due to the preceding column of DEAE-Sepharose chromatography, while the Km value (1.4 mmol/l) reported by Julià et al. was similar to that of our experiment. In addition, our method has the advantage that no special chemical, such as NAD in the CapGapp method, is needed for binding of ADH to the resin.

In conclusion, using p-HAP-Sepharose affinity chromatography, we have developed a new method for purification of ADH that may be useful for various alcohol-related studies.

REFERENCES


