Horse Liver Aldehyde Dehydrogenase

PURIFICATION AND CHARACTERIZATION OF TWO ISOZYMES

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Two isozymes of horse liver aldehyde dehydrogenase (aldehyde, NAD oxidoreductase (EC 1.2.1.3)), F1 and F2, have been purified to homogeneity using salt fractionation followed by ion exchange and gel filtration chromatography. The specific activities of the two isozymes in a pH 9.0 system with propionaldehyde as substrate were approximately 0.35 and 1.0 pmol of NADH/min/mg of protein for the F1 and F2 isozymes, respectively.

The multiporosity polyacrylamide gel electrophoresis molecular weights of the F1 and F2 isozymes were approximately 230,000 and 240,000, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gave subunit molecular weight estimates of 52,000 and 53,000 for the F1 and F2 isozymes, respectively. The amino acid compositions of the two isozymes were found to be similar; the ionizable amino acid contents being consistent with the electrophoretic and chromatographic behavior of the two isozymes.

Both isozymes exhibited a broad aldehyde specificity, oxidizing a wide variety of aliphatic and aromatic aldehydes and utilized NAD as coenzyme, but at approximately 300-fold higher coenzyme concentrations could use NADP. The F1 isozyme exhibited a very low $K_m$ for NAD (3 μM) and a higher $K_m$ for acetaldehyde (70 μM), while the F2 isozyme was found to have a higher $K_m$ for NAD (30 μM) and a low $K_m$ for acetaldehyde (0.2 μM). The two isozymes showed similar chloral hydrate and p-chloromercuribenzoate inhibition characteristics, but the F1 isozyme was found to be several orders of magnitude more sensitive to disulfiram, a physiological inhibitor of acetaldehyde oxidation. Based on its disulfiram inhibition characteristics, it has been suggested that the F1 isozyme may be the primary enzyme for oxidizing the acetaldehyde produced during ethanol oxidation in vivo.

Biological oxidation of ethanol to acetate requires two NAD-linked dehydrogenases: alcohol dehydrogenase and aldehyde dehydrogenase. As a result of extensive studies over the past 25 years, it is now possible to discuss the structure-function correlation of aldehyde dehydrogenase in detail. Studies on aldehyde dehydrogenase, however, are far less advanced. Since 1949 when Racker (1) showed that the dismutation of acetaldehyde into ethanol and acetate catalyzed by animal tissues was due to two separable enzymes, many NAD linked aldehyde dehydrogenase enzymes from a number of sources have been studied (2, 3). Jakoby (4) first purified several isozymes of yeast to homogeneity but later showed the multiple isozymes to be due to in vitro proteolytic degradation of the native enzyme (5). Because liver is the primary site of mammalian ethanol oxidation in vivo, liver aldehyde dehydrogenase is considered to be of major importance in the regulation and control of ethanol metabolism. The presence of kinetically and physically distinct isozymes of aldehyde dehydrogenase in various subcellular fractions of rat liver is now well documented (3, 6-8). In order to understand the effects of ethanol on cellular metabolism, localization and characterization of each individual isozyme are important. We have developed a large scale method for purifying both major isozymes of horse liver to homogeneity and have investigated their physical and catalytic properties. This paper describes the purification and characterization of two isozymes, F1 and F2, of aldehyde dehydrogenase from horse liver, the latter of which appears to be identical with that studied by Okuda et al. (9) and purified to homogeneity by Feldman and Weiner (10, 11).

EXPERIMENTAL PROCEDURE

Materials—Reagent grade or purified chemicals were used throughout. Coenzymes were purchased from Sigma. Purified grade aldehydes were used as received; others were distilled under nitrogen. Ion exchange celluloses, Whatman CM52 and DE52, were obtained from Reeve Angel and gel filtration resin, Bio-Gel A-1.5m, was obtained from Bio-Rad.

Activity Assay—To facilitate comparison to previous work, the activity assay of Feldman and Weiner (10) was used during the isolation procedure. One unit of aldehyde dehydrogenase activity has been defined as that amount of enzyme producing 1 pmol of NADH/min under these conditions (10). Further work on characterization of the purified isozymes was done at 25° in sodium phosphate buffer, pH
Horse Liver Aldehyde Dehydrogenase 237

Purification Procedure

Fresh horse livers were obtained periodically from a local slaughter house. Within a few hours after removal, the liver was perfused with approximately 8 liters of 0.15 m NaCl to remove excess blood. The liver capsule and large blood vessels were removed, and the liver was then divided into approximately 800-g lots and frozen for later use.

Extraction—When beginning a preparation, an 800-g lot of liver was allowed to thaw partially and was then minced in a meat grinder. All subsequent procedures were performed below 5º, using nitrogen-saturated buffers and a nitrogen atmosphere where possible. To the minced liver, 1 liter of 0.25% mercaptoethanol and 1 mM EDTA was added, and the mixture was homogenized for 3 min using a Torax Tissumizer. The insoluble debris was removed by centrifugation at 15,000 × g for 60 min.

Ammonium Sulfate Fractionation—To this extract, 2 ml of mercaptoethanol and 209 g/liter of solid ammonium sulfate were added. After dissolution of the ammonium sulfate, the slurry was allowed to stand for 60 min before centrifugation at 24,000 × g for 60 min. The precipitate was discarded. To the supernatant, 129 g/liter of solid ammonium sulfate and 1 ml/liter of mercaptoethanol were added. After dissolution of the ammonium sulfate, the slurry was allowed to stand for 90 min followed by centrifugation at 94,000 × g for 30 min. The supernatant was discarded, and the pellet suspended in approximately 400 ml of 10 mM sodium phosphate buffer, pH 6.3, containing 0.25% mercaptoethanol and 1 mM EDTA. This material was then dialyzed overnight against several 10-liter changes of the suspending buffer.

CM-cellulose Chromatography—The dialyzed material was centrifuged at 24,000 × g for 30 min to remove insoluble material. The supernatant was then applied to a CM-cellulose column (4 × 15 cm) equilibrated with 10 mM sodium phosphate, pH 6.3, containing 0.25% mercaptoethanol and 1 mM EDTA. The material was then dialyzed overnight against several 10-liter changes of the suspending buffer.

DEAE-cellulose Chromatography—After dialysis, the sample was added to a DEAE-cellulose column (5 × 20 cm) equilibrated with the same imidazole buffer. All of the aldehyde dehydrogenase activity is firmly bound under these conditions. First, several extraneous proteins were removed by washing the column with 250 ml of the equilibration buffer. The aldehyde dehydrogenase activity was then removed using a linear salt gradient prepared from 1 liter of 5 mM imidazole hydrochloride, pH 7.2, containing 0.25% mercaptoethanol and 1 liter of 5 mM imidazole hydrochloride, pH 7.2, containing 0.25% mercaptoethanol and 500 mM sodium chloride. This gradient resolved the two isozymes.

Final Purification of F1 Isozyme—Prior to gel filtration chromatography, the fractions containing the F1 isozyme from the above column were combined and were concentrated on DEAE-cellulose using the above imidazole buffer. The sample was then applied to a Bio-Gel A-1.5m column, this time equilibrated with 10 mM sodium phosphate buffer, pH 5.8, containing 0.25% mercaptoethanol and 1 mM EDTA. The column was then eluted with the same buffer. The fractions which contained the F1 isozyme activity were combined and stored under nitrogen at 4º for further use.

Final Purification of F2 Isozyme—Fractions from the DEAE-cellulose column containing the F2 isozyme were then combined and concentrated using a DEAE-cellulose column. The sample was then applied to the same Bio-Gel A-1.5m column, this time equilibrated with 10 mM sodium phosphate buffer, pH 5.8, and 300 ml of 200 mM sodium phosphate buffer, pH 5.8, both containing 0.25% mercaptoethanol and 1 mM EDTA. The fractions containing the F2 isozyme were combined and stored at 4º under nitrogen for further use.

RESULTS

Isolation—Results of a typical preparation are summarized in Table I. Approximately 100 to 200 mg of each isozyme can be obtained in a homogeneous state from 800 g of liver, representing approximately a 10% yield. The elution profile of the first DEAE-column on which the F1 and F2 isozymes are separated is shown in Fig. 1. The F1 and F2 isozymes were then combined as shown and purified further as described above. The final specific activities of the homogeneous F1 and F2 isozymes were generally 0.35 to 0.40 and 0.8 to 1.0 units/mg, respectively. Both purified isozymes appeared on acrylamide gel electrophoresis as single bands with less than 5% extraneous proteins. The F1 isozyme is particularly unstable when exposed to air even when EDTA and excess reducing agent (mercaptoethanol or bovine serum albumin) are employed. All staining was done with Coomassie brilliant blue.

TABLE I

Purification of two aldehyde dehydrogenase isozymes from horse liver

<table>
<thead>
<tr>
<th>F1/F2 mixture</th>
<th>Total protein*</th>
<th>Total activity*</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>g</td>
<td>μmol NADH/min</td>
<td>μmol NADH/min</td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>61</td>
<td>1400</td>
<td>23</td>
</tr>
<tr>
<td>35% Ammonium sulfate</td>
<td>38</td>
<td>1100</td>
<td>29</td>
</tr>
<tr>
<td>50% Ammonium sulfate</td>
<td>24</td>
<td>800</td>
<td>33</td>
</tr>
<tr>
<td>CM-cellulose chromatography</td>
<td>12</td>
<td>500</td>
<td>42</td>
</tr>
<tr>
<td>F1 Isozyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>0.61</td>
<td>100</td>
<td>160</td>
</tr>
<tr>
<td>Gel filtration chromatography</td>
<td>0.28</td>
<td>74</td>
<td>370</td>
</tr>
<tr>
<td>F2 Isozyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>1.53</td>
<td>200</td>
<td>150</td>
</tr>
<tr>
<td>Gel filtration chromatography</td>
<td>0.23</td>
<td>131</td>
<td>570</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>0.10</td>
<td>80</td>
<td>800</td>
</tr>
</tbody>
</table>

* By Lowry method (12) except as noted below.

In the pH 9 sodium pyrophosphate buffer system with propionaldehyde substrate.

Using E 1%1 = 0.95 mg/ml/cm for the F1 isoenzyme.

Using E 1%1 = 1.08 mg/ml/cm for the F2 isoenzyme.
dithiothreitol) are present. In fact, the yield of protein was approximately three times the yield when the preparation was done under nitrogen. We feel the loss of protein is probably due to oxidation of the sulfur-hydryl groups on the native protein. As long as oxygen was carefully excluded, the final solutions of both isozymes were quite stable, losing less than 5% activity per month.

**Native Protein and Subunit Molecular Weights**—As shown in Fig. 2, multiporosity polyacrylamide gel electrophoresis gave molecular weight estimates for the F1 and F2 isozymes of 230,000 and 240,000. The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gave subunit molecular weight estimates of 52,000 and 53,000.

**Absorption Spectra**—The absorption spectra of both isozymes from 250 to 600 nm showed only a single peak at 280 nm with no absorption in the visible region. The extinction coefficients at 280 nm in sodium phosphate, pH 7.0, were calculated to be 0.95 and 1.05 cm$^{-1}$ mg/ml$^{-1}$ for the F1 and F2 isozymes, respectively. The $A_{280}/A_{260}$ was 1.90 to 1.95 for both the isozymes. The lack of visible spectra and the high $A_{280}/A_{260}$ indicates the isozymes are not flavoproteins nor do they contain bound nucleotide as does glyceraldehyde-3-phosphate dehydrogenase.

**Amino Acid Composition**—As seen in Table II, the two isozymes have somewhat similar amino acid compositions. Differences in ionizable amino acids may agree with the electrophoretic and chromatographic behavior. The cysteine contents as measured by the mercury orange method of Sakai (13) were calculated to be 1.1 and 0.8 free sulfhydryl groups/10$^4$ g of protein for the F1 and F2 isozymes, respectively.

Because recoveries on amino acid analysis were slightly lower than expected (81% for F1 and 82% for F2), tests for the presence of lipids and carbohydrate were performed. An approximately 10% higher 280-nm extinction in 6 M guanidine hydrochloride was observed after chloroform-methanol extraction of the distilled water-dialyzed, lyophilized proteins compared to the unextracted isozymes. These results suggest some lipid may be present in the purified samples, although possible changes in water content caused by the extraction process must be considered. No carbohydrate was detected in the purified samples by the phenolsulfuric acid method of Hirs (19).

**Aldehyde Dehydrogenase Activity**—As shown in Table III, both isozymes show rather broad aldehyde specificity. The $K_m$ values reported in Table III were derived from Lineweaver-Burk plots with the aldehyde concentration in the solutions being based either on the volume of pure aldehyde added or for the low $K_m$ aldehydes, on NADH production after allowing the
reaction to progress to completion. It is well known, however, that in aqueous solutions aldehydes exist as a mixture of the free aldehyde, RCHO, and the hydrate, RCH(OH), the exact ratio being determined by the gem-diol equilibrium constant (20). It is likely that only one form is the true substrate for the dehydrogenase (21-23), and the large range in \( K_m \) values for different aldehydes may in part be due to the widely varying fractions existing as free aldehyde.

Both isozymes show a relative, but not absolute specificity for NAD over NADP as coenzyme. In sodium phosphate, pH 7.0, with 1 mM acetaldehyde as substrate, the \( K_m \) values are about the same.

### Inhibition Studies

Chloral hydrate, \( p \)-chloromercuribenzoate, and disulfiram (tetrathialthiuram disulfide, Antabuse) are all found to be inhibitors of both isozymes. Using approximately 10 \( \mu \)g/ml of protein in the pH 7 system with 1 \( mM \) NAD and 1 \( mM \) acetaldehyde, the \( K_m \) values were 3 \( \mu M \) and 700 \( \mu M \), respectively, for the Fl isozyme and 30 \( \mu M \) and approximately 10 \( \mu M \), respectively, for the F2 isozyme. For each isozyme, the \( V_{max} \) values with the two coenzymes were about the same.

Both isozymes exhibit broad aldehyde specificity. One isozyme (Fl) had a very low extinction coefficient and higher specific activity. Both isozymes show a relative, but not absolute specificity for NAD over NADP as coenzyme. In sodium phosphate, pH 7.0, with 1 mM acetaldehyde as substrate, the \( V_{max} \) and \( K_m \) values with the two coenzymes were about the same.

### DISCUSSION

We have described a practical method for purification of large quantities of the two major aldehyde dehydrogenase from horse liver. The principle molecular and catalytic properties have been ascertained. Both isozymes appear to be tetrameric with molecular weights about 200,000 to 250,000. There was no evidence on sodium dodecyl sulfate electrophoresis for nonidentical subunits in either isozyme. Both isozymes exhibit broad aldehyde specificity. One isozyme (Fl) had a very low \( K_m \) for NAD, and a higher \( K_m \) for acetaldehyde than the other isozyme (F2) had a very low \( K_m \) for acetaldehyde and a higher \( K_m \) for NAD. Based on the physical, catalytic, and chromatographic properties, we identify the F2 isozyme with the steroid aldehyde dehydrogenase (trihydroxyprogrenal dehydrogenase) which Okuda et al. (9) partially purified from horse liver and with the purified horse liver aldehyde dehydrogenase of Feldman and Weiner (10, 11). The only major differences between the Feldman and Weiner enzyme and our F2 isozyme is that we find it to have a lower extinction coefficient and higher specific activity.

![Fig. 3 (left). Inhibition of the Fl and F2 isozymes by disulfiram and \( p \)-chloromercuribenzoate (PCMB). Approximately 30 \( \mu \)g of Fl or 15 \( \mu \)g of F2 which had been extensively dialyzed to remove reducing agents were combined with \( p \)-chloromercuribenzoate (○) or disulfiram (●) in 1.5 ml of the standard pH 7.0 assay buffer. After 5-min incubation, 0.2 ml of 10 \( mM \) acetaldehyde/10 \( mM \) NAD was added to start the reaction. Inhibitor concentrations on the figure represent the final concentration in the 2.0-ml volume.](https://example.com/fig3)

[![Disulfiram inhibition of the Fl isozyme. Small samples (0.2 ml) of reducing agent free Fl isozyme (0.5 mg/ml) were prepared using sodium phosphate buffer, \( \mu = 0.1 \), pH 7.0, so as to contain increasing amounts of disulfiram (0 to 10 \( \mu M \)). After a 5-min incubation, 20-\( \mu l \) aliquots were removed for assay in 1 ml of 5 \( mM \) acetaldehyde/0.5 mM NAD in the same pH 7.0 buffer.](https://example.com/fig4)

#### Table III

Relative maximal velocities and \( K_m \) values of two isozymes for various aldehydes

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>R group</th>
<th>Fl isozyme</th>
<th>F2 isozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( V_{max} )</td>
<td>( K_m )</td>
</tr>
<tr>
<td>Aliphatic aldehydes</td>
<td></td>
<td>( \mu M )</td>
<td>( \mu M )</td>
</tr>
<tr>
<td>Isovaleraldehyde</td>
<td>(CH(_3))CH(_2)CH(<em>2)</em></td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Valeraldehyde</td>
<td>CH(_2)CH(<em>2)</em></td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>CH(<em>3)</em></td>
<td>1.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>CH(<em>2)</em></td>
<td>1.0</td>
<td>70</td>
</tr>
<tr>
<td>Phenylacetaldehyde</td>
<td>(C(_6)H(_5))CH(<em>2)</em></td>
<td>1.9</td>
<td>4</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>H</td>
<td>0.8</td>
<td>940</td>
</tr>
<tr>
<td>Glycolaldehyde</td>
<td>HOCH(<em>2)</em></td>
<td>0.9</td>
<td>130</td>
</tr>
<tr>
<td>Chloroacetaldehyde</td>
<td>ClCH(<em>2)</em></td>
<td>0.7</td>
<td>30</td>
</tr>
<tr>
<td>Conjugated aldehydes</td>
<td></td>
<td>0.6</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>C(_6)H(<em>5)</em></td>
<td>0.5</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

\( ^* \) The \( V_{max} \) values are relative to acetaldehyde as substrate for each isozyme. With 1 \( mM \) NAD and 1 \( mM \) acetaldehyde in this pH 7.0 buffer, the specific activities of the purified Fl and F2 isozymes are approximately 0.13 and 0.35 \( \mu \)mol of NADH/min/mg, respectively.
Horse Liver Aldehyde Dehydrogenase

noted a minor activity peak eluting from a DEAE-column before their major activity. We identify this minor peak as the F1 isozyme. These previous investigators did not attempt to purify or characterize it and because of its instability in the presence of oxygen, probably underestimated its importance. Because several reports of multiple isozymes, especially in yeast, have subsequently been shown to be due to in vitro modification of a native species with partial retention of activity (5, 24, 25), this question was carefully considered. We feel, however, that the evidence is overwhelmingly against one of the two horse liver aldehyde dehydrogenase isozymes (F1 or F2) being a degradation artifact. First, the Fl and F2 isozymes represent a good model system.

In most cases of known proteolysis, the kinetic data from the “isozymes” are very similar (5, 24). Second, no interconversion of the purified F1 and F2 isozymes was ever detected, in contrast to the case of proteolytic modification where interconversion has been observed (5, 24). Third, subcellular localization studies provided kinetic evidence that both isozymes are present in the crude homogenate and that the F1 isozyme is cytosolic and the F2 isozyme mitochondrial in origin (96, 97). Finally, a fresh horse liver homogenate can be resolved into the two isozymes within 30 min of cellular disruption using DEAE-cellulose chromatography. In these latter experiments, the F1 isozyme accounts for approximately 90% of the total activity and hence is not a minor component in vivo.

From the physiological standpoint, the disulfiram inhibition characteristics may be important in understanding ethanol metabolism. Disulfiram (Antabuse) administration prior to alcohol ingestion is known to produce a complex of very sensitive aldehyde dehydrogenase activities have been found in rat liver homogenates (29). While horse liver aldehyde dehydrogenase enzymes are interesting in their own right from the standpoint of elucidating enzyme mechanisms, they are primarily of interest as models for the physiology of alcohol metabolism with the human enzyme system. While Kraemer and Deitrich (30) in a partial purification of human liver aldehyde dehydrogenase found evidence for only a single isozyme, Blair and Bodley (31) more recently observed two isozymes which were separable on DEAE-cellulose chromatography. Thus, the study of the human isozyme which was more firmly bound to the DEAE-cellulose shows surprisingly many similarities to the F2 isozyme of horse liver. Both show broad aldehyde specificity, sensitivity to sulphydryl reagents, molecular weight near 200,000, high pH optimum, micromolar range Ki for aliphatic aldehydes, and only moderate disulfiram inhibition (35% at 40 μM). While more work on the human system is obviously needed, we are encouraged that the readily available horse isozymes may represent a good model system.

REFERENCES

Horse liver aldehyde dehydrogenase. Purification and characterization of two isozymes.