Purification of Mitochondrial Matrix Aldehyde Dehydrogenase from Pig Brain

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Abstract: The activity of aldehyde dehydrogenase (ALDH) in the cerebrum, cerebellum, striatum, and medulla oblongata was examined and mitochondrial matrix ALDH was purified prior to immunohistochemical study on the localization of ALDH isozymes in pig brain. Relatively high enzyme activity was found in the striatum and medulla oblongata when using indole-3-acetaldehyde as substrate, and in the striatum when using 3,4-dihydroxyphenylacetaldehyde (DOPAL). The main part of mitochondrial ALDH activities with both acetaldehyde and DOPAL existed in the matrix fraction. The ratio of activity of the matrix to the membrane fraction in the cerebrum was higher than in the cerebellum, suggesting that the distribution pattern of ALDH isozymes was different according to the brain regions. The 276-fold purified mitochondrial matrix ALDH from pig brain was identified to be homologous tetramers with 53 KD subunits. The enzyme showed maximal activity at pH 9.0 and was stable in the temperature range from 25℃ to 37℃. The mitochondrial matrix ALDH activity was considerably inhibited by acetaldehyde *in vitro*. The K_m values of the enzyme for acetaldehyde and propionaldehyde were 5.8 mM and 4.9 mM, respectively, whereas K_m values for indole-3-acetaldehyde and DOPAL were 44 μM and 1.6 μM, respectively. The V_{max}/K_m ratio was the highest with DOPAL as compared with other substrates. These results suggested that mitochondrial matrix ALDH in the present work might be a low K_m isozyme involved in biogenic aldehyde oxidation in pig brain.

Key words: aldehyde dehydrogenase, brain, mitochondrial matrix.

The primary role of brain aldehyde dehydrogenase (ALDH; EC 1.2.1.3.) must be closely associated with the degradation pathway of aminergic neurotransmitters. Biogenic amines, such as dopamine, epinephrine, norephinephrine, and serotonin are converted into their respective biogenic aldehydes by monoamine oxidase (MAO; EC 1.4.3.4.). They are further oxidized to acids by NAD^+-dependent ALDH in brain tissue.

The first investigation of the brain aldehyde dehydrogenase was conducted by Erwin and Deitrich (1966) on rat, monkey, and bovine brain. They found the enzyme activity present in all subcellular compartments. Duncan and Tipton (1971) investigated the kinetic properties of the pig brain enzyme and concluded that brain aldehyde dehydrogenase was not a single enzyme.

Nonphysiological aldehydes, such as acetaldehyde derived from exogenous ethanol, appear to be transported into the brain tissue via blood (Westcott *et al.*, 1980). In the presence of acetaldehyde the competitive inhibition of biogenic aldehyde oxidation could shift the amine metabolism from the oxidative to the reduction pathway (Yoshimoto *et al.*, 1992). Based on these observations the main interest in ALDH has been focused on the relation between biogenic amines and exogenous ethanol metabolism. It has been suggested that biogenic aldehydes and acetaldehyde are oxidized in the brain tissue by an ALDH which has a low K_m for acetaldehyde (Pettersson and Kiessling, 1977; Maring *et al.*, 1985). A number of isozymes have been identified and their physical and kinetic properties have been partially characterized (Ryzlak *et al.*, 1987; Joo *et al.*, 1991a; Song *et al.*, 1991). Recently one of the isozymes was identified as γ-aminobutylaldehyde dehyrogenase in human liver (Kurys *et al.*, 1989). However, detailed biological function and the regional distribution of each ALDH isozyme in the brain have not yet been fully understood.

In view of the biological significance of brain ALDH and the biochemical effects of ethanol on the central nervous system, the regional distribution of ALDH is important in understanding the relation of ALDH to the functional and structural systems of the brain. Until recently, studies on the regional distribution of brain ALDH activity have been performed in human (Pietru-

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szko *et al.*, 1984; Hafer *et al.*, 1987) and in rat (Weiner and Ardelt, 1984; Lee and Joo, 1993, 1994). However the regional distribution of ALDH activity in pig brain has not been reported. Prior to the study on the localization of ALDH isozymes in brain using the immunohistochemical method, this study was undertaken primarily to determine the ALDH activity in different brain region and to purify and characterize the mitochondrial matrix ALDH from pig brain.

Materials and Methods

Materials

Fresh pig brains (not later than 3 h after killing) were obtained from a slaughterhouse. Acetaldehyde, indole-3-acetaldehyde, pyrazole, NAD^+, and Coomassie Brilliant Blue R-250 were purchased from Sigma Chemical Co. (St. Louis, USA). Propionaldehyde was obtained from Fluka (Buchs, Swiss). DEAE-Sephacel and 5'-AMP Sepharose 4B were purchased from Pharmacia LKB Chemimicals (Uppsala, Sweden). 3,4-dihydroxyphenylacetaldehyde (DOPAL) was prepared enzymatically from dopamine in our laboratory.

Enzyme activity assay

The ALDH activity was determined spectrophotometrically by the increase in absorbance at 340 nm for NADH produced at 25℃ in 1 ml total volume. The assay mixture contained (final concentration) 50 mM sodium pyrophosphate buffer (pH 8.4), 0.5 mM pyrazole, 1 mM NAD^+, and an appropriate concentration of substrate. One unit of enzyme activity was defined as the amount of enzyme which produced 1 nmol of NADH per min under the standard assay conditions. For calculations the extinction coefficient for NADH, 6.22 $mM^{-1}cm^{-1}$ was used. The K_m and V_{max} values were determined from a straight line fitted to Lineweaver-Burk plots by least squares analysis.

Preparation of 3,4-dihydroxyphenylacetaldehyde (DOPAL)

DOPAL was prepared enzymatically by the methods of Nilsson and Tottmar (1987). The incubation mixtures contained (final concentration) 8 mM potassium phosphate buffer (pH 7.5), 4 mM sodium bisulfite, 1 mM dopamine, and mitochondrial membrane (2 mg protein/ml) from rat liver as monoamine oxidase source. The incubation was performed for 8 h in nonsealed bottles in a shaking water bath at 30℃. After reaction the incubation mixture was centrifuged at 105,000×*g* for 30 min and the supernatant was used as substrate without further purification.

Preparation of tissue and subcellular fractionation

Four brain regions, cerebral cortex, cerebellum, striatum, and medulla oblongata were dissected on an ice-cooled glass plate and kept at −70℃ until used. A 20% (w/v) homogenate of each brain region was prepared using a Potter-Elvehjem homogenizer with a homogenizing medium containing 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, and 0.5 mM EDTA.

For subcellular fractionation two hundred grams of pig brains was chopped with a scissor and homogenized with 800 ml of homogenizing medium. The homogenate was centrifuged at 800×*g* for 10 min to remove the nuclei and cell debris. The supernatant was then centrifuged at 10,000×*g* for 15 min. The pellet was resuspended in homogenizing medium and centrifuged at the same speed for 15 min. The resulting mitochondrial pellet was resuspended in 10 mM sodium phosphate buffer containing 1 mM EDTA and 2 mM β-mercaptoethanol and disrupted using a sonicator (Heat Systems Co. USA). The sonicated suspension was centrifuged at 10,000×*g* for 15 min to remove the undisrupted mitochondria fraction. The supernatant was then centrifuged at 105,000×*g* for 60 min to obtain mitochondrial membrane and soluble matrix fraction. All procedures of subcellular fractionation was performed at 4℃.

Purification of mitochondrial matrix ALDH

The mitochondrial matrix fraction was treated with ammonium sulfate and the fraction precipitating between 30 and 65% saturated ammonium sulfate was obtained by centrifugation at 15,000×*g* for 30 min. The resulting pellet was resuspended in 10 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA and 2 mM β-mercaptoethanol and dialyzed overnight against 100 volumes of the same buffer. The dialysed preparation was applied on a DEAE-Sephacel column (20 cm×3 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA and 2 mM β-mercaptoethanol. The elution was performed with the same buffer until all nonbound proteins were eluted. The column was then eluted with a linear gradient of sodium chloride (0~1 M) in the same buffer. The fractions containing the ALDH activity were collected and dialyzed against 100 volumes of 100 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 2 mM β-mercaptoethanol. This dialysate was applied on an affinity column (1.5 cm×10 cm) of 5'-AMP Sepharose 4B equilibrated with 100 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 2 mM β-mercaptoethanol. The bound enzyme was eluted with the same buffer containing 0.25 mM NAD^+. All purification steps were performed at 4℃. In the purification procedure protein concentration was estimated using A_{280}. Other samples were assayed by the Bradford (1976) method. Bovine serum albumin was

used as the standard.

Molecular weight determination of native enzyme and subunits

The molecular weight of mitochondial matrix ALDH from pig brain was determined by 4~20% gradient polyacrylamide gel electrophoresis employing molecular weight standard proteins. Using the calibration curve prepared by measuring the Rf on the gel plate, the molecular weight of the native enzyme was estimated. The molecular weight of subunit was determined by SDS polyacrylamide gel electrophoresis. The purified enzyme (20 μg) was dissolved in 10 ml of 62.5 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue and heated in a boiling water bath for 2 min. The denatured enzyme and standard proteins were loaded on SDS polyacrylamide gel electrophoresis according to the Laemmli (1970) method.

Effects of pH and temperature on ALDH activity

The enzyme activity with 2 mM indole-3-acetaldehyde as a substrate was measured in the range from pH 6.0 to 11.0. Instead of standard assay buffer, 50 mM sodium phosphate buffer (pH 6.0 to 8.0) and 50 mM glycine-NaOH buffer (pH 8.0 to 11.0) were used. To estimate the heat stability the enzyme was placed in the heat block at 25℃, 37℃, and 55℃ for a measured length of time (from 5 to 20 min). Aliquots of enzyme solution were taken and the enzyme activity was measured with 2 mM indole-3-acetaldehyde as substrate.

Effect of acetaldehyde on ALDH activity

15, 30, and 50 mM (final concentration) of acetaldehyde was added to the enzyme reaction mixture without substrate and incubated for 1 min at 25℃. The enzyme activity was assayed with 2 mM indole-3-acetaldehyde as substrate.

Results and Discussion

Regional distribution of ALDH activity in brain

The distribution of aldehyde dehydrogenase activities with various substrates in four brain regions, cerebrum, cerebellum, striatum, and medulla oblongata were determined and the results were as given in Table 1. Although a lower concentration of indole-3-acetaldehyde (2 mM) than that of acetaldehyde or propionaldehyde (10 mM) was used in total activity measurement, The ALDH activity with indole-3-acetaldehyde was higher than with acetaldehyde and propioaldehyde as substrate in all brain regions examined. These results coincided with the view that aromatic aldehydes are a more feasible substrate for brain ALDH in respect that ALDH involved in biogenic amine catabolism.

Table 1. The distribution of ALDH activity with various substrates in the different regions of pig brain[a].

	Cerebrum	Cerebellum	Striatum	Medulla oblongata
Acetaldehyde	0.49	0.77	0.45	0.79
Propionaldehyde	1.46	1.03	1.70	0.99
Indole-3-acetaldehyde	3.80	2.58	4.61	5.35
3,4-dihydroxyphenyl acetaldehyde(DOPAL)	0.22	0.17	0.34	0.15

[a] ALDH activity was expressed as nmol NADH produced/min/mg protein. Activity was assayed with 10 mM acetaldehyde and propionaldehyde, 2 mM indole-3-acetaldehyde and 100 μM DOPAL as substrate.

Table 2. The distribution of ALDH activity in the mitochondria of pig brain .

	ALDH activity[a] with					
	Acetaldehyde			3,4-dihydroxyphenyl acetaldehyde(DOPAL)		
	Matrix	Membrane	Amat/Amem	Matrix	Membrane	Amat/Amem
Cerebrum	3.22	0.51	6.3	1.61	0.28	5.8
Cerebellum	3.30	1.39	2.4	1.65	0.40	4.1

[a] ALDH activity was expressed as nmol NADH produced/min/mg protein. Activity was assayed with 10 mM acetaldehyde and 100 μM DOPAL as substrate.

Relatively high enzyme activity was found in the striatum and medulla oblongata when using indole-3-acetaldehyde as substrate and in the striatum when using DOPAL. In a previous study with rats as the experimental animal a similar distribution pattern of the activity has been reported (Lee and Joo, 1993). High ALDH activity in the striatum compared to other brain regions investigated could be attributed to the abundance of dopaminergic neurons and to a higher tendency of DOPAL to the oxidation rather than the reduction pathway. These results present plausible evidence for alteration of dopamine metabolism in the liver (Tank *et al.*, 1976) and in the basal ganglia including structurally the corpus striatum (Hunt, 1981) after ethanol uptake.

Distribution of ALDH activity in mitochondria

It is well documented that ALDH exists in the cytosol, mitochondrial and microsomal fractions. The ALDH activity with propionaldehyde as substrate in rat brain was found to be 12% in cytosol, 40% in mitochondria, and 28% in microsomal fraction (Weiner and Ardelt; 1984). In addition Joo *et al.* (1991b) reported that

Table 3. Purification of the mitochondrial matrix ALDh from pig brain.

Step	Total activity[a] (U)	Total protein (mg)	Specific activity[a] (U/mg protein)	Purification (fold)
Crude mitochondrial matrix	1414.6	1386	1.0	1
Ammonium sulfate (30~65%)	837	55.8	15	14.7
DEAE-Sephacel	257.2	3.7	70.1	69.1
5'-AMP-Sepharose 4B	56.3	0.2	281.5	276

[a]One enzyme unit was defined as 1 nmol of NADH produced per min.

enzyme activity with *p*-nitrobenzaldehyde was found to be 22% in cytosol, 58% in mitochondria, and 20% in the microsomal fraction from rat brain. However, Agarwal *et al.* (1982) have found that the major portion (41%) of total activity with DOPAL was localized in the cytosolic fraction in the case of the striatum. The broad subcellular distribution of ALDH activity suggests that ALDH isozymes having different properties could be present in each subcelullar component of brain. There seems to be a general agreement that mitochondrial low K_m ALDH plays an important role in biogenic aldehyde oxidation in brain (Pettersson and Kiessling, 1977). As shown in Table 2, the main part of mitochondrial ALDH activities in the presence of either acetaldehyde or DOPAL was localized in the matrix fraction, suggesting that the major site of acetaldehyde and biogenic aldehydes oxidation might be the mitochondrial matrix. With both substrates, the ratio of activity of the matrix to the membrane fraction in the cerebrum was higher than in the cerebellum. These results led to the postulation that the mitochondrial ALDH activity is closely related to the difference in biogenic aldehyde metabolism in brain regions, which further indicates that there must be regional difference in the distribution pattern of isozymes, i.e. matrix soluble and membrane-bound enzyme.

Purification of mitochondrial matrix ALDH

A number of previous studies with liver and brain preparations from various animal sources indicated the presence of multiple forms of the enzyme, which could be divided into two classes of ALDH with low and high K_m (Tipton *et al.*, 1989). The establishment of the regional distribution of ALDH isozymes in the central nervous system is important for understanding the functional implications of brain ALDH and the biochemical effects of ethanol on neurotransmitter metabolism. As an experiment preparatory to a study on the localization of ALDH isozymes in the brain using the immunohistochemical method, primarily mitochondrial matrix ALDH was purified from the pig brain.

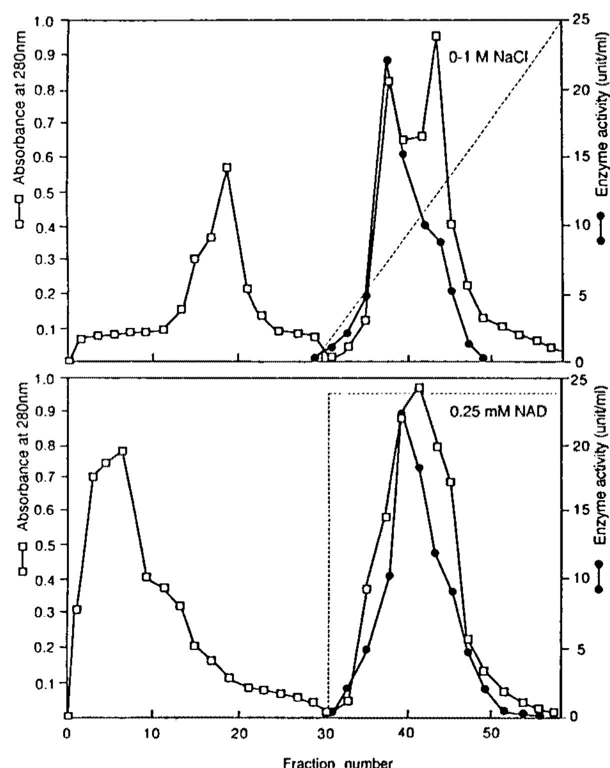

Fig. 1. (a) Fractionation of pig brain mitochondrial matrix ALDH by DEAE-Sephacel column chromatography. The column was equilibrated with 10 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol and eluted by 0~1 M NaCl gradient using the above buffer. (b) Fractionation of mitochondrial matrix ALDH by 5'-AMP Sepharose 4B column chromatography. The column was equilibrated with 100 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol and the enzyme was eluted with the same buffer containing 0.25 mM NAD$^+$. One enzyme unit of enzyme activity was defined as the amount of enzyme to produce 1 nmol of NADH per min.

The purification scheme for mitochondrial matrix ALDH is given in Table 3. The crude mitochondrial matrix preparation was subjected to 30~65% ammonium sulfate precipitation followed by DEAE-Sephacel column chromatography (Fig. 1a). The final enzyme sample, as a single peak eluted from a column of 5'-AMP Sepharose 4B (Fig. 1b), showed a 276-fold increase in specific activity (281.5 nmol NADH min^{-1}mg^{-1}) from the initial crude mitochondrial matrix fraction.

Polyacrylamide gel electrophoresis of the purified enzyme yielded a single protein band with 205 KD molecular weight, and a single protein band with molecular weight of 53 KD was detected in SDS-polyacrylamide gel electrophoresis (Fig. 2), suggesting that the enzyme might be homologous tetramers.

K_m and V_{max} values of mitochondrial matrix ALDH

The Michaelis constant and V_{max} of mitochondrial

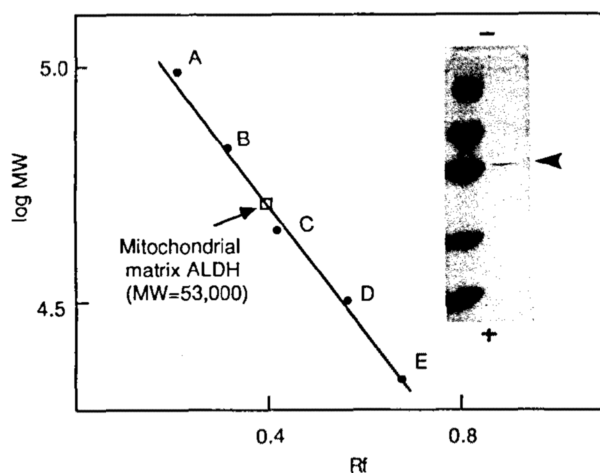

Fig. 2. Determination of subunit moelcular weight of mitochondrial matrix ALDH from pig brain by SDS-polyacrylamide gel electrophoresis. A: phosphorylase b (97,400), B: bovine serum albumin (66,200), C: ovalbumin (45,000), D: carbonic anhydrase (31,000) and E: trypsin inhibitor (21,500).

Table 4. K_m and V_{max} values for various substrates of mitochondrial matrix ALDH from pig brain.

Aldehyde	K_m (μM)	V_{max}[a] (U/mg protein/min)	V_{max}/K_m
Acetaldehyde	5800	534	0.09
Propionaldehyde	4900	612	0.12
Indole-3-acetaldehyde	44	1071	24.3
3,4-Dihydroxyphenyl acetaldehyde (DOPAL)	1.6	138	86.2

[a]One enzyme unit was defined as 1 nmol of NADH produced per min.

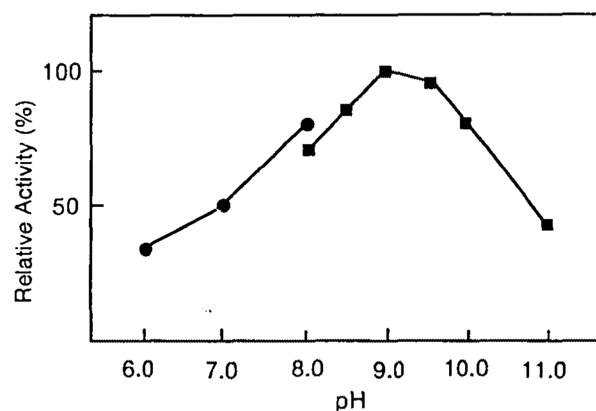

Fig. 3. The effect of pH on the activity of mitochondrial matrix ALDH from pig brain. ●-●: 50 mM sodium phosphate buffer, ■-■: 50 mM glycine-NaOH buffer.

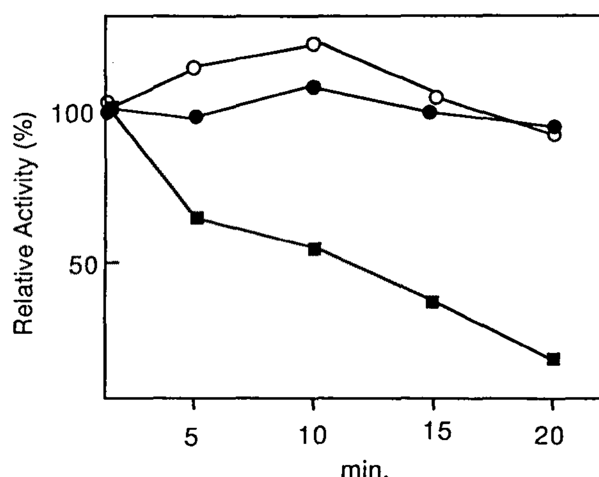

Fig. 4. Heat stability of mitochondrial matrix ALDH from pig brain. ●-●: 25℃, ○-○: 37℃, ■-■: 55℃.

matrix ALDH for acetaldehyde, propionaldehyde, indole-3-acetaldehyde, and DOPAL were determined by Lineweaver-Burk double reciprocal plot. As seen in Table 4, K_m values of mitochondrial matrix ALDH for acetaldehyde and propionaldehyde were 5.8 mM and 4.9 mM, respectively whereas K_m values obtained with indole-3-acetaldehyde and DOPAL as substrates were 44 μM and 1.6 μM, respectively. In addition the V_{max}/K_m ratio which means an index of the enzyme reaction activity for a substrate, was the highest with DOPAL as compared with other aldehydes as substrate. Thus this suggests that mitochondrial matrix aldehyde dehydrogenase might participate in the oxidation of biogenic aldehydes produced during biogenic amine metabolism. These results coincide with the data that DOPAL derived from dopamine, in contrast to norepinephrine, is metabolized by ALDH *via* an oxidative pathway in mitochondria (Tabakoff *et al.*, 1973). Zimatkin (1991) has also reported that brain ALDH activity was closely related to regions of dopaminergic terminals, high dopamine concentration and monoamine oxidase activity.

Three rat brain mitochondrial ALDH isozymes were identified and two of them were purified (Song and Joo, 1991). Whereas one matrix and a membrane bound ALDH have a high affinity for succinic semialdehyde (K_m value of 10^{-6} M level), another mitochondrial matrix ALDH isozyme did not react with succinic semialdehyde. This suggested that ALDH isozymes with different K_m values for aliphatic aldehyde and aromatic biogenic aldehyde might exist in brain mitochondria matrix. In the present work, although two or more fractions containing the enzyme activity with aromatic aldehydes could not be obtained in the mitochondrial matrix, isolated ALDH showed an extremely low K_m for DOPAL and a high affinity to DOPAL and therefore this enzyme is thought to be a low K_m isozyme participating in biogenic aldehyde oxidation in pig brain. The possibility of the presence of other isozymes with differ-

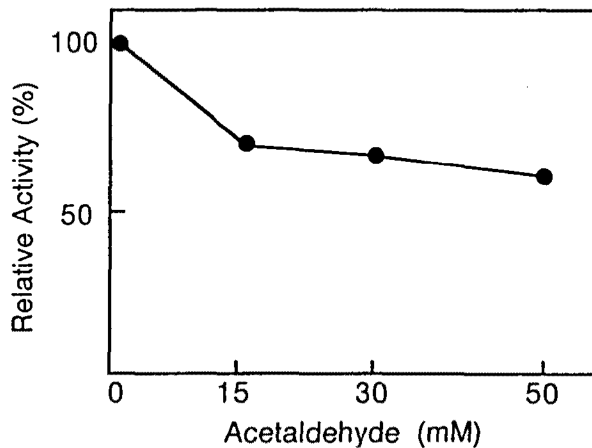

Fig. 5. The effect of acetaldehyde on the activity of mitochondrial matrix ALDh from pig brain.

ent properties in the mitochondrial matrix will be pursued later.

The effects of pH, temperature and acetaldehyde on ALDH activity

The purified mitochondrial matrix ALDH showed maximal activity with indole-3-acetaldehyde as substrate at pH 9.0 (Fig. 3). The purified enzyme activity was stable in the temperature range from 25℃ to 37℃, and a half of the activity remained for 10 min at 55℃ (Fig. 4).

The mitochondrial matrix ALDH was considerably inhibited by acetaldehyde *in vitro* (Fig. 5). The major portion of acetaldehyde derived from ethanol is oxidized to acetate in the liver and a low level of acetaldehyde is found in the blood and other tissues. Acetaldehyde in the brain must be oxidized by an aldehyde dehydrogenase which has a K_m for acetaldehyde in the micromolar range (Duncan and Tipton, 1971). In the presence of acetaldehyde biogenic aldehydes accumulate as a result of the competitive inhibition of biogenic aldehyde oxidation by acetaldehyde. This might lead to an increased reduction of the aldehydes to corresponding alcohols by aldehyde reductase. In contrast to ALDH, aldehyde reductase activity did not respond to acetaldehyde (Joo *et al.*, 1991b). However it is somewhat doubtful whether the enzyme purified in this work is responsible for acetaldehyde oxidation in the brain, considering the relatively high K_m value (5.8 mM) for acetaldehyde. In fact, a quite low level of acetaldehyde was found in the brain after ethanol administration for the reason that acetaldehyde entering the brain is metabolized so rapidly (Tabakoff *et al.*, 1976). In this respect acetaldehyde conceivably can saturate the ALDH sufficiently to have pharmacological effects in the brain.

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