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IN VITRO OXIDATION OF PYRAZINAMIDE AND ALLOPURINOL BY RAT LIVER ALDEHYDE OXIDASE

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Abstract—Aldehyde oxidase was purified about 120-fold from rat liver cytosol by sequential column chromatography using diethylaminoethyl (DEAE) cellulose, Benzamidine-Sepharose 6B and gel filtration. The purified enzyme was shown as a single band with M_r of 2.7×10^5 on polyacrylamide gel electrophoresis (PAGE) and M_r of 1.35×10^5 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Using this purified enzyme, *in vitro* conversion of allopurinol, pyrazinamide and pyrazinoic acid was investigated. Allopurinol and pyrazinamide were oxidized to oxypurinol and 5-hydroxypyrazinamide, respectively, while pyrazinoic acid, the microsomal deamidation product of pyrazinamide, was not oxidized to 5-hydroxypyrazinoic acid. The apparent K_m value of the enzyme for pyrazinamide was 160 μM and that for allopurinol was 1.1 mM. On PAGE, allopurinol- or pyrazinamide-stained band was coincident with Coomassie Brilliant Blue R 250-stained band, respectively. These results suggest that aldehyde oxidase may play a role in the oxidation of allopurinol to oxypurinol and that of pyrazinamide to 5-hydroxypyrazinamide with xanthine dehydrogenase which can oxidize both allopurinol and pyrazinamide *in vivo*. The aldehyde oxidase may also play a major role in the oxidation of allopurinol and pyrazinamide in the subgroup of xanthinuria patients (xanthine oxidase deficiency) who can oxidize both allopurinol and pyrazinamide.

The metabolic conversion of allopurinol and pyrazinamide is generally attributed to the enzyme xanthine oxidase (EC 1.2.3.2) [1, 2]. However, it has been suggested that aldehyde oxidase (EC 1.2.3.1), one of the molybdoflavoproteins, also oxidizes these compounds [3, 4]. However, so far, there have been few detailed studies on the metabolism of allopurinol and pyrazinamide by purified aldehyde oxidase. Therefore, we conducted an *in vitro* incubation study to examine whether or not aldehyde oxidase is able to oxidize allopurinol and pyrazinamide, using purified rat liver aldehyde oxidase.

MATERIALS AND METHODS

Chemicals and animals

P-Dimethylaminocynnamaldehyde (DMAC†), xanthine, hypoxanthine, ammonium sulfate, hydroxyapatite, benzamidine, acrylamide, bis-acrylamide, ammonium persulfate, *N,N,N′,N′*-tetramethylethylendiamine, sodium dodecyl sulfate (SDS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT) and 2-hydroxypyrimidine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Diethylaminoethyl (DEAE) cellulose (DE 52 microgranular) was from Whatman Paper Ltd (Maidstone, U.K.) and Benzamidine-Sepharose 6B from Pharmacia LKB (Uppsala, Sweden). Phenazine-methosulfate (PMSF) and *N′*-methylnicotinamide (NMN) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Allopurinol and oxypurinol were kindly provided by Tanabe Pharmaceutical Co. (Osaka, Japan). Pyrazinamide and pyrazinoic acid were kindly provided by Sankyo Pharmaceutical Co. (Tokyo, Japan). BOF-4272 [5], a newly developed xanthine oxidase inhibitor, was kindly provided by Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). Other chemicals were of analytical grade.

Male Wistar rats weighing 250–300 g were used in the following enzyme preparation.

Methods

Enzyme preparation. Unless otherwise stated, the enzyme preparation was performed at 0–4°. Male Wistar rats were anesthetized using diethylether. Then the livers were carefully removed, rinsed, minced and homogenized with 3 vol. of 100 mM phosphate buffer, pH 7.4 in a polytron tissue homogenizer. The homogenate was then rapidly warmed to 55° in a water bath. After 10 min at 55°, the homogenate was cooled on ice and centrifuged at 15,000 *g* for 45 min in a Beckman refrigerated ultracentrifuge.

Solid ammonium sulfate was slowly added with gentle stirring to the clear supernatant to obtain a final concentration of 30%. The suspension was allowed to stand for 30 min, and then fractionated by centrifugation at 5000 *g* for 30 min. The pellet was discarded and additional ammonium sulfate was

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† Abbreviations: DMAC, *P*-dimethylaminocynnamaldehyde; SDS, sodium dodecyl sulfate; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide; DEAE, diethylaminoethyl; PMSF, phenazine-methosulfate; NMN, *N′*-methylnicotinamide; PAGE, polyacrylamide disc gel electrophoresis; BPB, bromophenol blue.

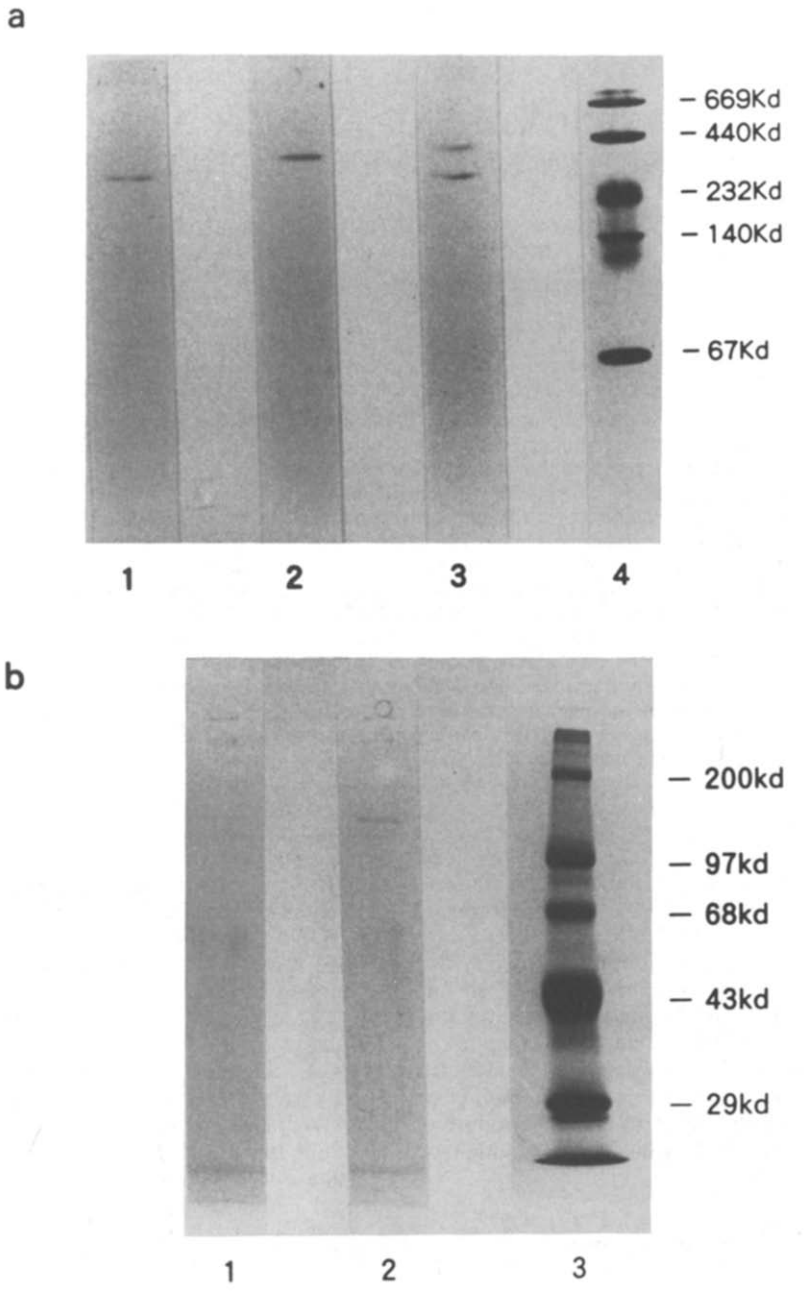

Fig. 1. PAGE of the purified rat liver aldehyde oxidase in the absence (a) and presence (b) of SDS. (a) Lane 1, aldehyde oxidase, lane 2, xanthine oxidase, lane 3, aldehyde oxidase and xanthine oxidase were simultaneously applied to the gel, showing distinct separation of these two enzymes. Lane 4, marker proteins. (b) Lane 1, aldehyde oxidase 2 μg, lane 2, aldehyde oxidase 5 μg, lane 3, marker proteins.

added to the supernatant to 50% saturation. After a second centrifugation at 5000 *g* for 30 min, the precipitate was collected and dissolved in 10 mM phosphate buffer, pH 7.4 using 1 mL of buffer solution for every 10 g of liver and dialysed overnight against 200 vol. of the same buffer. Dialysed ammonium sulfate precipitate was applied onto a DEAE-cellulose column (2.5 × 15 cm), pre-equilibrated with 10 mM phosphate buffer, pH 7.4, and the column was washed with a large volume of the same buffer until protein was not detected. The column was eluted with a linear gradient of 10–200 mM phosphate buffer, pH 7.4, and fractions of 10 mL were collected. To the fractions containing aldehyde oxidase activity were added 200 mg dry weight per mL of hydroxyapatite. The mixture was allowed to stand for 30 min with occasional stirring. The gel was centrifuged at 8000 *g* for 10 min and the

supernatant in which enzyme activity resided was pooled. Then hydroxyapatite was washed twice in succession with 100 mM phosphate buffer, pH 7.4. The gel was removed by centrifuge and the three washings were combined, concentrated in an NMWL 10,000 ultrafiltration membrane (Nihon Millipore Kogyo KK, Yonezawa, Japan) to a final volume of 10–15 mL and dialysed, with stirring, overnight against 200 vol. of 0.1 M glycine–NaCl buffer, pH 9.0. The dialysed enzyme preparation was loaded onto a Benzamidine-Sepharose 6B column (2 × 6 cm) pre-equilibrated with 0.1 M glycine–NaCl buffer, pH 9.0. After washing with ample column volumes of 0.1 M glycine–NaCl buffer, the enzyme adsorbed to the column was eluted with 0.1 M phosphate buffer, pH 6.5. Fractions (each of 3 mL) were collected and fractions containing enzyme activity were applied to a gel filtration column (TSK-gel G3000SW, TOSOH, Tokyo, Japan), then subjected to gel electrophoresis.

Enzyme assay. Aldehyde oxidase activity was assayed spectrophotometrically by measuring the rate of conversion of DMAC as previously described by Kurth and Kubiciel [6]. All enzyme assays were performed in a Hitachi spectrophotometer at 37°. The assay mixture contained DMAC at a final concentration of 40 μM; potassium phosphate buffer, pH 7.4; at 100 mM; and 25 μL of the enzyme sample in a final volume of 1.5 mL. The specific activity of aldehyde oxidase was expressed as units per mg of protein. One unit of activity was defined as the amount of enzyme that oxidizes 1 μmol DMAC per minute at 37°.

Gel electrophoresis. Polyacrylamide disc gel electrophoresis (PAGE) was performed on a 7.5% native gel under non-denaturing conditions to determine the purity of the final enzyme preparation. Enzyme solution (90 μL) and 30 μL of a 3:1 mixture of sample buffer [33% sucrose, 1% bromophenol blue (BPB)] were applied onto each tube (5 × 80 mm). The reservoir buffer of the electrophoresis system consisted of 0.05 M Tris and 0.38 M glycine (pH 8.9). Electrophoresis was performed at 2 mA per tube. The gels were stained for protein with Coomassie Brilliant Blue R-25. As for activity staining, 3.75% gel was used and the gels were stained by 75 μM DMAC, 400 μM 2-hydroxypyrimidine, 1.5 mM allopurinol, 65 mM pyrazinamide and 0.7 mM hypoxanthine in 0.1 M Tris–HCl, pH 7.4 containing 0.04% MTT, 0.01% PMSF. Standards for the molecular mass determination included bovine serum albumin (67 kDa), lactic dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa). The molecular mass of purified aldehyde oxidase was estimated from a best-fit line obtained with the R_f values of the standards described above. SDS–PAGE was performed according to Laemmli [7] after boiling the samples using 9.0% acrylamide. Standards for the molecular mass determination were carbonyl anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorylase B (97 kDa) and myosin (H-chain) (200 kDa).

Inhibition of aldehyde oxidase by benzamidine and BOF-4272. The inhibition studies of partially purified aldehyde oxidase by benzamidine or BOF-4272 were performed by adding appropriate amounts of these inhibitors to the reaction mixture described above.

In vitro *incubation of purified aldehyde oxidase with allopurinol, pyrazinamide and pyrazinoic acid.* The oxidization of allopurinol, pyrazinamide or pyrazinoic acid was determined by measuring the formation of oxypurinol, 5-hydroxypyrazinamide or 5-hydroxypyrazinoic acid, respectively, with or without benzamidine or BOF-4272. The assay mixture contained 50 mM potassium phosphate buffer, pH 9.0, 2.4 mM allopurinol and 25 μL of enzyme preparation in a final volume of 150 μL with or without 10 μM BOF-4272 or 10 mM benzamidine. When pyrazinamide or pyrazinoic acid was used as the substrate, the assay mixture contained 5.4 mM pyrazinamide or 5.4 mM pyrazinoic acid instead of allopurinol in the reaction mixture described above. The reaction was terminated after a 120 min incubation at 37° by the addition of 25 μL of 20% $HClO_4$, then neutralized with 25 μL of 1 M K_2CO_3. The precipitated protein was removed by centrifugation and filtered through Chromatodisc 4A (0.2 μm). The oxidation products of allopurinol, pyrazinamide and pyrazinoic acid, that is, oxypurinol, 5-hydroxypyrazinamide and 5-hydroxypyrazinoic acid, were measured by the modified method of Yamamoto *et al.* [8] using HPLC. The modification was as follows. The column used was Wakosil $5C_{18}$ 200 (i.d. 4.6 × 150 mm), the mobile phase was 0.02 M potassium phosphate buffer (pH 2.2), the flow rate was 1.0 mL/min and a 20 μL sample was applied.

Protein determination. Protein was assayed either by the calorimetric method of Lowry *et al.* [9] or the dye binding method [10] using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

RESULTS

Purification of aldehyde oxidase from rat liver

By the linear gradient (10–200 mM) elution with potassium phosphate from DE-52 column to which the dialysed ammonium sulfate fraction was applied, the aldehyde oxidase activity was eluted as a single peak at about 120 mM. Meanwhile, xanthine oxidase protein was eluted at about 70 mM, and part of the xanthine oxidase eluate overlapped the aldehyde oxidase eluate. To separate the protein possessing xanthine oxidase activity from that of aldehyde oxidase completely, xanthine oxidase protein present in the collected fraction containing aldehyde oxidase activity was adsorbed to hydroxyapatite and after centrifugation, the supernatant in which aldehyde oxidase resided was applied to an affinity column. All the aldehyde oxidase activity was eluted at a single peak. Table 1 shows a summary of the data from the purification procedure. The procedure resulted in a final purification of the enzyme about 120-fold.

Enzyme characteristics

The prepared sample was homogeneous as judged by PAGE. Aldehyde oxidase was distinctly separated from xanthine oxidase applied simultaneously and the estimated molecular mass of this protein was 270 kDa (Fig. 1a). When compared against molecular

Table 1. Purification of rat liver aldehyde oxidase

Step	Volume (mL)	Activity (U)	Protein (mg)	Specific activity (U/mg)
Cytosol	205	247,811	4370	567
30–50% $(NH_4)_2SO_4$	25	100,826	721	139.8
DEAE-cellulose	80	46,102	100	461.0
Hydroxyapatite	16	15,926	17.7	899.8
Benzamidine-Sepharose 6B and gel filtration	40	4233	0.6	7055.0

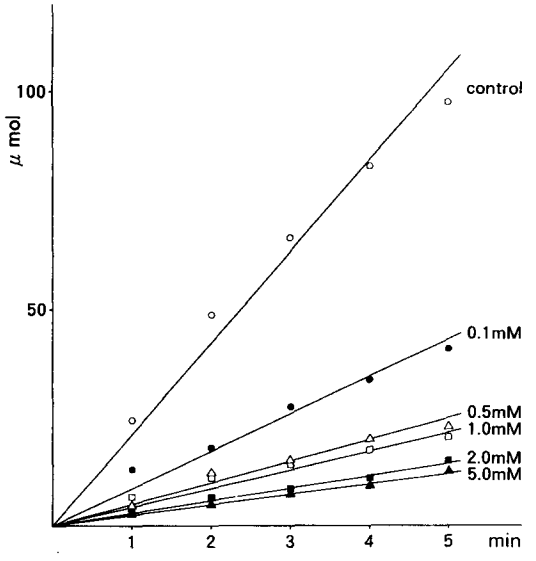

Fig. 2. Inhibition of aldehyde oxidase by various concentrations of benzamidine; 5 mM benzamidine inhibited aldehyde oxidase activity about 90%.

Table 2. The inhibitory effect of BOF-4272 on liver aldehyde oxidase activity

	% Activity
Control	100
BOF-4272 (μM)	
10	102
30	104
80	105
160	95
320	97

Assays were performed with DMAC as substrate at pH 9.0. The activity of aldehyde oxidase without benzamidine in the reaction mixture was taken as the reference standard. The activity shown represents a percentage of the aldehyde oxidase activity in the reference standard.

mass standards run simultaneously on thin layer SDS–PAGE, a molecular mass of approximately 135 kDa was estimated for this protein (Fig. 1b). No other bands were present in the TSK gel preparation.

Inhibition of aldehyde oxidase by benzamidine, allopurinol and BOF-4272

Benzamidine (5 mM) inhibited aldehyde oxidase activity by about 90% (Fig. 2). However, BOF-4272 had no effect on the activity of aldehyde oxidase (Table 2). Allopurinol did not inhibit the activity of aldehyde oxidase at a dose below 2.5×10^{-4} mol/L. The concentration of allopurinol required for 20–30% inhibition of aldehyde oxidase activity was 2.5–5.0×10^{-3} mol/L.

In vitro *incubation of aldehyde oxidase with various substrates*

After 120 min incubation of aldehyde oxidase with xanthine, uric acid in the reaction mixture was below the detection limit, indicating the present preparation free of xanthine oxidase (data not shown). During incubation aldehyde oxidase converted allopurinol to oxypurinol. This formation of oxypurinol was inhibited by the addition of benzamidine, while oxypurinol formation was not affected by the addition of BOF-4272 (Fig. 3). During incubation, aldehyde oxidase also converted pyrazinamide to 5-hydroxypyrazinamide and the formation of 5-hydroxypyrazinamide was inhibited by adding benzamidine, although addition of BOF-4272 had no effect on the formation of 5-hydroxypyrazinamide (Fig. 4). Pyrazinoic acid was not converted to 5-hydroxypyrazinoic acid by aldehyde oxidase during incubation.

Activity staining of aldehyde oxidase with various substrates

Activity staining with 2-hydroxypyrimidine and DMAC showed a strong positive band on 3.75% PAGE and those with allopurinol, pyrazinamide and hypoxanthine showed weak bands coincident with the band stained by Coomassie Brilliant Blue R-250 (Fig. 5).

K_m *values of aldehyde oxidase*

Purified preparations of aldehyde oxidase were assayed at different concentrations of either pyrazinamide or allopurinol. The apparent K_m values of the enzyme were 160 μM and 1.1 mM for pyrazinamide and allopurinol, respectively. The K_m value of xanthine oxidase for allopurinol was 0.5 mM,

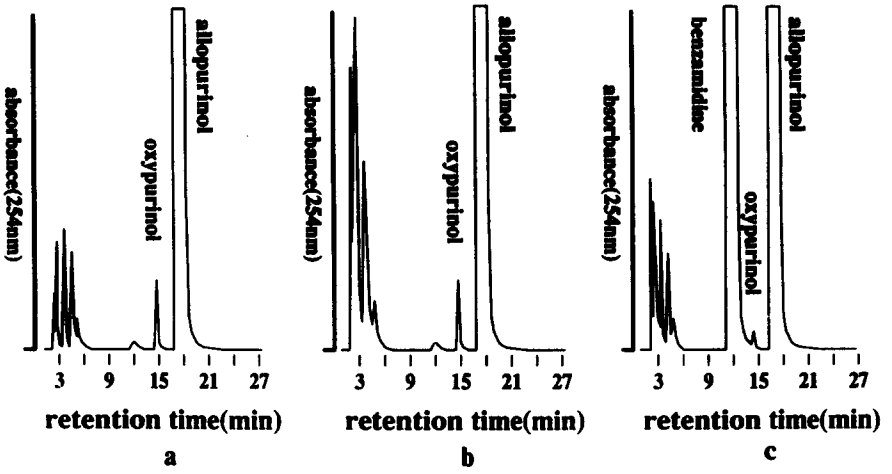

Fig. 3. *In vitro* incubation of aldehyde oxidase with allopurinol in the absence (a) and presence of BOF-4272 (b) and benzamidine (c). Benzamidine suppressed the formation of oxypurinol, while BOF-4272 had no effect on the formation of oxypurinol.

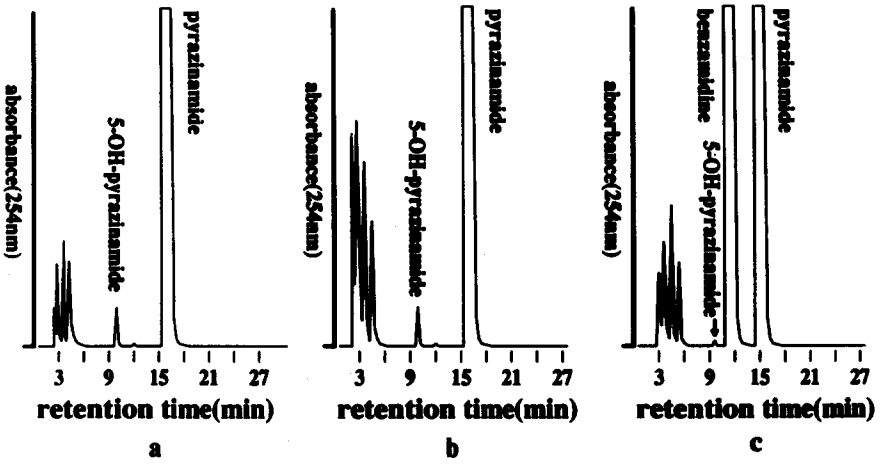

Fig. 4. *In vitro* incubation of aldehyde oxidase with pyrazinamide in the absence (a) and presence of BOF-4272 (b) and benzamidine (c). Benzamidine inhibited the formation of 5-hydroxypyrazinamide, while BOF-4272 had no effect on the formation of 5-hydroxypyrazinamide.

which was almost comparable to the value obtained by aldehyde oxidase. The K_m value of xanthine oxidase for pyrazinamide was 1.5 mM, about 10 times higher than that of aldehyde oxidase for pyrazinamide.

DISCUSSION

Allopurinol, a widely used antihyperuricemic agent, is generally believed to be oxidized to oxypurinol by the enzyme xanthine oxidase [1, 2]. Pyrazinamide (an antituberculous agent) and pyrazinoic acid (a metabolite of pyrazinamide) are also demonstrated to be metabolized to 5-hydroxypyrazinamide and 5-hydroxypyrazinoic acid, respectively, by xanthine oxidase [11].

Xanthinuria (xanthine oxidase deficiency) has been reported to consist of two subgroups. One is a subgroup that neither oxidizes allopurinol to oxypurinol [12] nor pyrazinamide to 5-hydroxypyrazinamide [13]. This subgroup is suggested to be ascribable to a combined deficiency of allopurinol–pyrazinamide oxidizing enzyme(s) or a variant form of xanthine oxidase which can not oxidize hypoxanthine, xanthine, allopurinol, pyrazinamide or pyrazinoic acid at all. The other is a subgroup that can oxidize both allopurinol and pyrazinamide to oxypurinol and 5-hydroxypyrazinamide, respectively, despite a lack of xanthine oxidase [14–16]. This indicates the existence of allopurinol–pyrazinamide oxidizing enzyme(s) other than xanthine oxidase or a variant form of xanthine oxidase

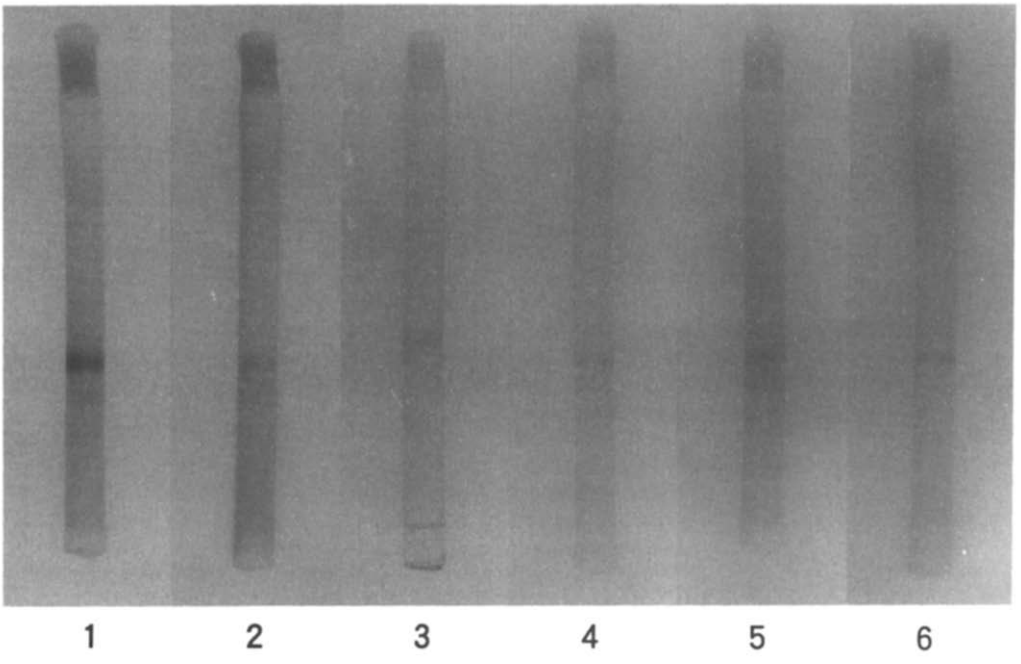

Fig. 5. Activity staining of aldehyde oxidase with various substrates; lane 1, protein staining, lane 2, DMAC, lane 3, 2-hydroxypyrimidine, lane 4, allopurinol, lane 5, pyrazinamide, lane 6, hypoxanthine.

which can not oxidize hypoxanthine, xanthine and pyrazinoic acid, but can oxidize allopurinol and pyrazinamide. In previous studies, it has been suggested that aldehyde oxidase (a molybdoflavoprotein) may oxidize allopurinol to oxypurinol [3, 4]. However, these experiments have been performed using partially purified aldehyde oxidase. Therefore, in the present study, we purified aldehyde oxidase and investigated the oxidation of allopurinol by aldehyde oxidase in detail, using purified aldehyde oxidase. In addition, it was investigated whether or not pyrazinamide and pyrazinoic acid are oxidized by aldehyde oxidase since the enzyme may be allopurinol–pyrazinamide oxidizing enzyme described previously [13]. On PAGE, the purified aldehyde oxidase was demonstrated to be a single band of molecular mass of approximately 270 kDa and on SDS–PAGE, it was demonstrated to consist of two identical subunits with a molecular mass of 135 kDa. Although the molecular mass of the purified enzyme (270 kDa) is smaller than that in the previous study [17], the difference of the molecular mass seems to be ascribed to the difference of species. To confirm that the purified aldehyde oxidase protein did not contain xanthine oxidase, we performed an inhibition study on xanthine oxidase activity, using the purified aldehyde oxidase protein, xanthine and BOF-4272 (xanthine oxidase inhibitor but not aldehyde oxidase inhibitor), and demonstrated that the purified aldehyde oxidase protein did not contain xanthine oxidase at all. Then, to examine the oxidation of allopurinol, pyrazinamide and pyrazinoic acid by the purified aldehyde oxidase, we conducted an *in vitro* study. The *in vitro* study demonstrated that aldehyde oxidase oxidized both allopurinol and pyrazinamide but did not oxidize pyrazinoic acid. These results suggested that aldehyde oxidase may play a role in the oxidation of allopurinol to oxypurinol and that of pyrazinamide to 5-hydroxypyrazinamide with xanthine dehydrogenase which can oxidize both allopurinol and pyrazinamide in humans, and it was also suggested that aldehyde oxidase may play a major role in the oxidation of allopurinol and pyrazinamide in the subgroup of xanthinuria (xanthine oxidase deficiency) that can oxidize both allopurinol and pyrazinamide as suggested by a previous study [18]. Further study must be made using human liver aldehyde oxidase to clarify the problem concerning subgroup of xanthinuria, especially in light of the relationship between xanthine oxidase deficiency and aldehyde oxidase.

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