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PURIFICATION AND CHARACTERIZATION OF 3-MERCAPTOLACTIC ACID *S*-CONJUGATE OXIDASES

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Abstract—Two enzymes catalysing the oxidative formation of 3-mercaptopyruvic acid *S*-conjugates from L-3-mercaptolactic acid *S*-conjugates were purified to apparent homogeneity from rat liver cytosol. The two enzymes, tentatively designated MLO-I and MLO-II, showed a molecular mass of 160 and 250 kDa and were composed of four and six subunits of 41 and 39 kDa, respectively. Both enzymes possessed flavin mononucleotide as prosthetic group and oxidized several aromatic and aliphatic *S*-substituted L-3-mercaptolactic acids as well as α-hydroxy acids such as L-3-phenyllactic acid and L-2-hydroxyisocaproic acid. Glycolic acid and 3-(4-hydroxyphenyl)-lactic acid were the specific substrates for MLO-I and MLO-II, respectively. Neither of the enzymes oxidized β- and γ-hydroxy acids such as 3- and 4-hydroxybutyric acid. 2-Hydroxyisobutyric acid, ethyl-2-hydroxybutyrate, malic acid, 1-butanol, benzyl alcohol and L-leucine did not act as substrates for the enzymes. MLO-I and MLO-II exerted their maximum activities around pH 5.5 with K_m of 0.5 and 0.25 mM and V_{max} of 0.9 and 0.2 μmol/min/mg, respectively, when *S*-(4-bromophenyl)-3-thiolactic acid was used as substrate. MLO-I was inhibited by sulphydryl-modifying agents, while MLO-II was not. Both enzymes were strongly inhibited by divalent metal ions. These results indicate that MLO-I and MLO-II are different from L-amino acid oxidase (EC 1.4.3.2), malate oxidase (EC 1.1.3.3), L-α-hydroxy acid oxidase (EC 1.1.3.15) and glycolate oxidase (EC 1.1.3.1). The present enzymes are likely to be involved in the formation of cysteine conjugates from L-3-mercaptolactic acid *S*-conjugates in conjunction with cysteine conjugate aminotransferases.

Conjugation of electrophiles with glutathione and subsequent metabolism that leads to excretion as *N*-acetyl-L-cysteine *S*-conjugates are one of the important pathways for detoxication of xenobiotics. In the last two or three decades, however, several classes of chemicals have been reported to be bioactivated via glutathione conjugation and subsequent metabolism [1–4]. Cysteine conjugates, which are derived from glutathione conjugates after two-step enzymic hydrolysis, are metabolized via two main metabolic pathways initiated with (1) C—S bond cleavage or (2) transamination in addition to N-acetylation. Metabolic activation of some halogenated hydrocarbons via C—S bond cleavage to toxic thiols has recently been established by many investigators [2–4].

Among the enzymes involved in the above three pathways, enzymes responsible for N-acetylation and C—S bond cleavage of cysteine conjugates have extensively been studied [5–8]. In the transamination pathway, 3-mercaptopyruvic acid *S*-conjugates initially formed are reduced to 3-mercaptolactic acid *S*-conjugates or, alternatively, decarboxylated to mercaptoacetic acid *S*-conjugates. These conjugates are reported to be major metabolites of certain xenobiotics in mammals [9–15]. During the course of our study to clarify the enzymes participating in this pathway, we have already purified and characterized the cysteine conjugate aminotransferase [16] and 3-mercaptolactic acid *S*-conjugate reductases [17].

Recently (in a preliminary experiment *in vitro*) we have found that incubation with rat liver homogenate of a 3-mercaptolactic acid *S*-conjugate gave the corresponding cysteine conjugate via enzymic oxidation and transamination. This observation prompted us to examine the responsible oxidase in the rat. In this paper, we describe the purification and characterization of 3-mercaptolactic acid *S*-conjugate oxidases (tentatively designated as MLO†).

MATERIALS AND METHODS

Materials

S-(4-Bromophenyl)-3-thiopyruvic acid was synthesized by the reaction of 4-bromobenzenethiol with 3-fluoropyruvic acid (Aldrich, Milwaukee, MI, U.S.A.) according to the procedure described by Parrod [18]. *S*-(4-Bromophenyl)-3-thio-DL-lactic acid was prepared by the reduction of *S*-(4-bromophenyl)-3-thiopyruvic acid in ethanol with sodium borohydride by the usual method. Other 3-mercaptolactic acid *S*-conjugates, i.e. *S*-phenyl-3-thio-DL-lactic acid, *S*-benzyl-3-thio-DL-lactic acid, *S*-pentyl-3-thio-DL-lactic acid and *S*-cyclohexyl-3-thio-DL-lactic acid, were prepared by the reduction of the corresponding 3-mercaptopyruvic acid *S*-conjugates, which were synthesized by our previous method [18]. L-3-Phenyllactic acid, DL-3-phenyllactic acid, 3-(4-hydroxyphenyl)-DL-lactic acid, DL-2-hydroxycaproic acid, DL-3-hydroxybutyric acid and 4-hydroxybutyric

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† Abbreviations: MLO, 3-mercaptolactic acid *S*-conjugate oxidase; FMN, flavin mononucleotide; DEAE, diethylaminoethyl.

acid were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). L-2-Hydroxyisocaproic acid and DL-2-hydroxyvaleric acid were obtained from Aldrich. DL-2-Hydroxybutyric acid, 2-hydroxyisobutyric acid, L-lactic acid, glycolic acid, L-malic acid, 1-butanol, benzyl alcohol and L-leucine were the products of Wako (Osaka, Japan). DL-Ethyl-2-hydroxybutyrate was purchased from Tokyo Kasei (Japan).

DEAE-cellulose (DE-52) was purchased from Whatman (U.K.). Hydroxyapatite (Biogel-HTP) and Sephadex G-25 and G-200 were obtained from Bio-Rad (Richmond, CA, U.S.A.) and Pharmacia (Uppsala, Sweden), respectively. Standard proteins, i.e. bovine thyroglobulin, bovine γ-globulin, chicken ovalbumin, horse myoglobin and bovine serum albumin were the products of Bio-Rad. Ferritin and catalase were purchased from Pharmacia.

All other reagents used in the present study were obtained commercially and were of analytical reagent grade.

Assay methods

Assay of MLO. For a standard assay of MLO, *S*-(4-bromophenyl)-3-thio-DL-lactic acid was used as substrate and the amount of *S*-(4-bromophenyl)-3-thiopyruvic acid formed was determined by HPLC (conditions described below). The standard assay mixture contained, in a final vol. of 100 μL: *S*-(4-bromophenyl)-3-thio-DL-lactic acid, 50 nmol; potassium phosphate buffer, pH 5.5, 5 μmol; catalase, 500 U; and enzyme solution, 0–50 μL. The mixture was incubated at 37° for 20 min and the reaction was terminated by the addition of 200 μL of 0.625% (w/v) trichloroacetic acid at 0°. After centrifugation at 1500 *g* for 10 min, 20 μL of the supernatant were subjected to HPLC analysis (column: Inertsil ODS 2, 4.6 × 160 mm, GL Science, Japan; eluent: 30% (v/v) acetonitrile in 100 mM aqueous ammonium acetate solution; flow rate: 1.0 mL/min; detection: 254 nm). *S*-(4-Bromophenyl)-3-thio-DL-lactic acid and *S*-(4-bromophenyl)-3-thiopyruvic acid emerged at 3.2 and 4.1 min, respectively. The amount of product was calculated from the peak area. The reaction was linear over the enzyme concentration range of 2–8 U/mL and for up to 40 min with respect to incubation time. One unit of oxidase activity was defined as the quantity of enzyme catalysing the formation of one nmole of product per min under the conditions described above.

Hydrogen peroxide formed in the incubation mixture, from which catalase was omitted, was analysed by the method of Allain *et al.* [19] with Free Cholesterol E-Test (Wako). The amount of blue chromophore formed by the oxidative condensation was determined spectrophotometrically at 600 nm.

MLO activities in some tissues and in the caecal contents of rats were measured in the presence of 2.5 mM aminooxyacetic acid, which was used as inhibitor of cysteine conjugate aminotransferase and for conversion of the product, 3-mercaptopyruvic acid *S*-conjugate, to the corresponding Schiff's base. Other incubation conditions were the same as for the standard assay. The amount of Schiff's base was subsequently analysed by HPLC [10].

When various α-hydroxy acids other than *S*-(4-bromophenyl)-3-thio-DL-lactic acid were examined as substrates, α-keto acids formed were quantitated spectrophotometrically after derivatization. The incubation conditions were the same as described above except that the incubation mixture was scaled up twice (200 μL) and contained 50 nmol (L-isomer equivalent) of each substrate. The reaction was terminated by the addition of 20 μL of 12.5% trichloroacetic acid at 0°. After centrifugation, 200 μL of the supernatant were mixed with 400 μL of 1.0 M acetate buffer, pH 5.0, and 160 μL of 0.1% (w/v) aqueous solution of 3-methyl-2-benzothiazolinone hydrazone hydrochloride (Wako, Japan). After the mixture was incubated at 50° for 30 min, the azine derivative formed was determined spectrophotometrically at 320 nm [20].

Optical isomers of phenyllactic acid were measured by chiral HPLC (column: TSK-gel enantio L-1, 4.6 × 250 mm, Tosoh, Japan; eluent: 2 mM $CuSO_4$; flow rate: 1.0 mL/min; detection: 259 nm).

Assay of protein. Concentration of protein was determined by the method of Bradford [21] with Protein Assay Kit (Sigma). Bovine serum albumin was used as standard.

Analysis of prosthetic group

For analysis of the prosthetic group, the solution of purified enzyme was heated at 100° for 10 min. After the heat treatment and subsequent centrifugation, the supernatant solution was analysed by HPLC (column: TSK gel ODS-120T, Tosoh, Japan; eluent: 30% (v/v) methanol in 5 mM aqueous ammonium acetate solution, pH 6.5; flow rate: 0.9 mL/min; detection: 260 nm) [22]. Authentic FAD and FMN emerged at 4.2 and 6.3 min, respectively, under the present conditions.

Electrophoresis

The enzymes were analysed for purity by SDS–PAGE on 12.5% polyacrylamide slab gels (Phast Gel Homogeneous 12.5, Pharmacia) by an automated electrophoresis system (Phast System, Pharmacia). Standard proteins used were SDS–PAGE Molecular Weight Standards, Low Range (Bio-Rad). Proteins were stained with Phast Gel blue R (Pharmacia).

Determination of molecular mass by HPLC

The apparent molecular mass was determined by gel permeation chromatography with HPLC equipped with a GPC column (TSK Gel G3000 SWXL, 7.8 × 300 mm, Tosoh, Japan). The molecular mass standards used were: bovine thyroglobulin (670 kDa), ferritin (440 kDa), catalase (232 kDa), bovine γ-globulin (158 kDa), bovine serum albumin (68 kDa), chicken ovalbumin (44 kDa) and horse myoglobin (17 kDa). Proteins were eluted with 50 mM potassium phosphate buffer, pH 7.4, containing 0.2 M NaCl at a flow rate of 0.3 mL/min and were detected at 280 nm.

Purification of MLO from rat liver

All steps of the purification were carried out at 0–4°. Table 1 shows a typical purification of the oxidases.

Step 1: *preparation of rat liver cytosol.* Male

Table 1. Purification of MLOs from rat liver

Step	Protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification (fold)
1. Liver cytosol					
MLO-I + MLO-II	15,100	10,000	100	0.662	1
2. $(NH_4)_2SO_4$					
MLO-I + MLO-II	10,100	8850	88.5	0.876	1.32
3. 1st DEAE-cellulose					
MLO-I	711	5590	55.9	7.86	11.9
MLO-II	587	1040	10.4	1.77	2.67
4. Hydroxyapatite					
MLO-I	30.9	2980	29.8	96.4	146
MLO-II	10.8	900	9.0	83.3	126
5. 2nd DEAE-cellulose					
MLO-I	8.18	1820	18.2	222	335
MLO-II	4.41	441	4.41	100	151
6. Gel filtration					
MLO-I	1.16	377	3.77	325	491
MLO-II	1.45	163	1.63	112	169

One unit of oxidase activity is defined as the quantity of enzyme catalysing the formation of one nmole of the product per minute under the standard assay conditions.

Sprague–Dawley rats (8 weeks old, N = 18) were killed by decapitation and the livers (237 *g*) removed, minced with scissors and homogenized with a glass homogenizer with a teflon pestle in 2 vol. of 10 mM potassium phosphate buffer, pH 7.4, containing 1.15% KCl and 10 μM (4-amidinophenyl)-methanesulphonyl fluoride. The homogenate was centrifuged at 9000 *g* for 20 min. The 9000-*g* supernatant was subsequently centrifuged at 105,000 *g* for 70 min and the cytosol fraction obtained was used for further purification.

Step 2: *ammonium sulphate fractionation.* To 345 mL of the cytosol fraction, 60.7 g of powdered ammonium sulphate were added portion-wise to give 30% saturation. The mixture was stirred for 90 min and centrifuged at 10,000 *g* for 20 min. To the supernatant (365 mL), 59.1 g of powdered ammonium sulphate was added to attain 55% saturation. After 90 min of stirring, active enzyme pellet was obtained by centrifugation at 10,000 *g* for 20 min.

Step 3: *1st DEAE-cellulose.* The active enzyme pellet obtained in Step 2 was dissolved in 145 mL of 10 mM potassium phosphate buffer, pH 7.4 (buffer A). The solution was desalted with a column of Sephadex G-25 equilibrated with buffer A. Eluate protein fractions were applied to a DEAE-cellulose column (2.5 × 40 cm) equilibrated with buffer A. The column was first washed with 720 mL of buffer A and proteins were subsequently eluted with a linear gradient of 0–400 mM KCl in buffer A (1000 mL). Eluate fractions of 11.6 mL were collected. The oxidase activity was eluted as two peaks at KCl concentrations of 100 and 150 mM (Fig. 1). Enzymes eluted in fractions 86–92 and 96–104 were tentatively designated as MLO-I and MLO-II, respectively. Each fraction was separately pooled, concentrated by ultrafiltration with PM-30 Diaflo membrane (Amicon, U.S.A.) and passed through a column of Sephadex G-25 equilibrated with 10 mM potassium phosphate buffer, pH 6.8 (buffer B).

Step 4: *hydroxyapatite.* The solutions of MLO-I and MLO-II obtained in Step 3 were separately applied to hydroxyapatite columns (1.5 × 44 cm) equilibrated with buffer B. The columns were washed with buffer B (140 mL for MLO-I and 150 mL for MLO-II) and then the enzymes were eluted with a linear gradient of 10–500 mM potassium phosphate buffer, pH 6.8 (500 mL). A fraction of 5.3 and 5.6 mL was collected for MLO-I and MLO-II, respectively. MLO-I and MLO-II gave a single active peak at a buffer concentration of around 250 and 350 mM, respectively (Fig. 2a and b). Fractions 77–84 for MLO-I and 97–106 for MLO-II were pooled, concentrated with PM-30 Diaflo membrane and passed through a column of Sephadex G-25 equilibrated with buffer A.

Step 5: *2nd DEAE-cellulose.* Solutions of MLO-I and MLO-II were separately applied to DEAE-cellulose columns (1.5 × 25 cm) equilibrated with buffer A. Proteins were eluted first with 150 mL of buffer A and subsequently with a linear gradient of 0–400 mM KCl in buffer A (400 mL). A fraction of 5.8 and 5.7 mL was collected for MLO-I and MLO-II, respectively. Active fractions of 61–66 for MLO-I and 50–56 for MLO-II (Fig. 3a and b) were collected separately and concentrated to 5 mL with PM-30 Diaflo membrane.

Step 6: *gel filtration.* Each concentrated solution of MLO-I or MLO-II was applied to Sephadex G-200 column (2.5 × 110 cm) equilibrated with buffer A. The enzymes were eluted with the same buffer and the eluate fractions of 5.2 mL for MLO-I and 5.3 mL for MLO-II were collected. MLO-I and MLO-II were eluted as a single peak (Fig. 4a and b). Active fractions of 47–52 for MLO-I and 42–48 for MLO-II were separately combined, concentrated, subdivided into small portions and stored at −20° until use.

Identification of the products

S-(4-Bromophenyl)-3-thio-DL-lactic acid was incu-

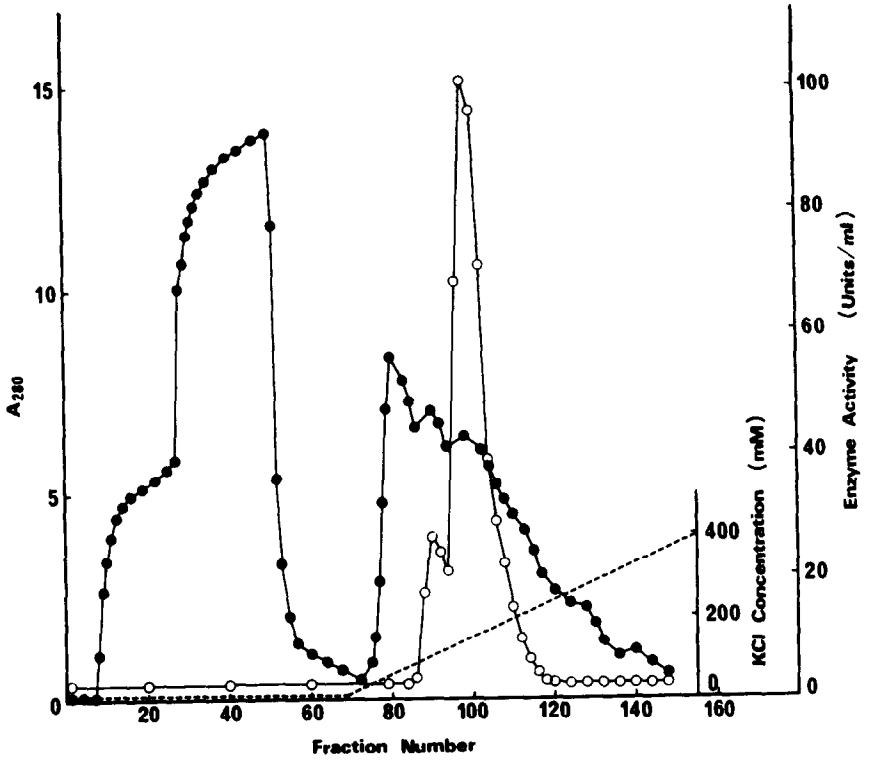

Fig. 1. First DEAE-cellulose column chromatography (step 3). The enzyme solution was applied to a DEAE-cellulose column (2.5 × 40 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.4. The column was first washed with 720 mL of the buffer and proteins were subsequently eluted with 1000 mL of a linear gradient of 0–400 mM KCl in the same buffer (- - - -). Fractions of 11.6 mL were collected. (●) A_{280}, (○) MLO activity.

bated with MLO-I or MLO-II under the conditions described in Assay methods except that the scale was enlarged 20 times and the incubation time was 30 min. After termination of the reaction by adding trichloroacetic acid, the product, *S*-(4-bromophenyl)-3-thiopyruvic acid, was extracted twice with 3 mL of ethylacetate and the organic layer was evaporated to dryness under nitrogen stream. The residue was treated with an excess amount of etherial diazomethane at 0° followed by evaporation to dryness. The product was subjected to GC–MS analysis with a mass spectrometer (Model 5988A, Hewlett Packard, U.S.A.) and a gas chromatograph (Model 5890, Hewlett Packard, U.S.A.) equipped with a DB-17 column (0.25 mm × 10 m, J & W Scientific, U.S.A.). The column temperature was elevated from 50° to 290° at a rate of 30°/min. Helium was used as carrier gas.

RESULTS

Distribution of MLO activity in rats

Liver, kidney, brain, lung, thymus, spleen, testis, small intestinal mucosa, blood and caecal contents of rats were homogenized in 2 vol. of 10 mM potassium phosphate buffer, pH 7.4, containing 1.15% KCl and 10 μM (4-amidinophenyl)-methanesulphonyl fluoride. In the case of the caecal contents, the homogenate was sonicated at 0° for 1 min. Each homogenate was centrifuged at 50 *g* for 7 min and MLO activity in each supernatant was measured in the presence of aminooxyacetic acid. The liver and kidney possessed the highest activity of approximately 40 U/g tissue followed by the testis which showed about 10% of the hepatic activity. It is notable that MLO activity was found in the testis, which was reported to possess high glutathione *S*-transferase activity [23]. This coincidence is indicative of the participation of MLO in the metabolism of certain endogenous sulphur-containing conjugates in this organ. The activity was negligible in other tissues and caecal contents.

Purification of MLO from rat liver

In this study, we chose the liver as enzyme source for purification because as this organ shows the highest total activity it is likely to be the most contributory to the *in vivo* metabolism of 3-mercaptolactic acid *S*-conjugate. More than 90% of the MLO activity in the liver homogenate of rats was recovered in the cytosol fraction. From this fraction, MLO-I and MLO-II were purified 490- and 170-fold, respectively, according to the six-step procedure (Table 1). The purified enzymes showed a single band in SDS–PAGE (Fig. 5).

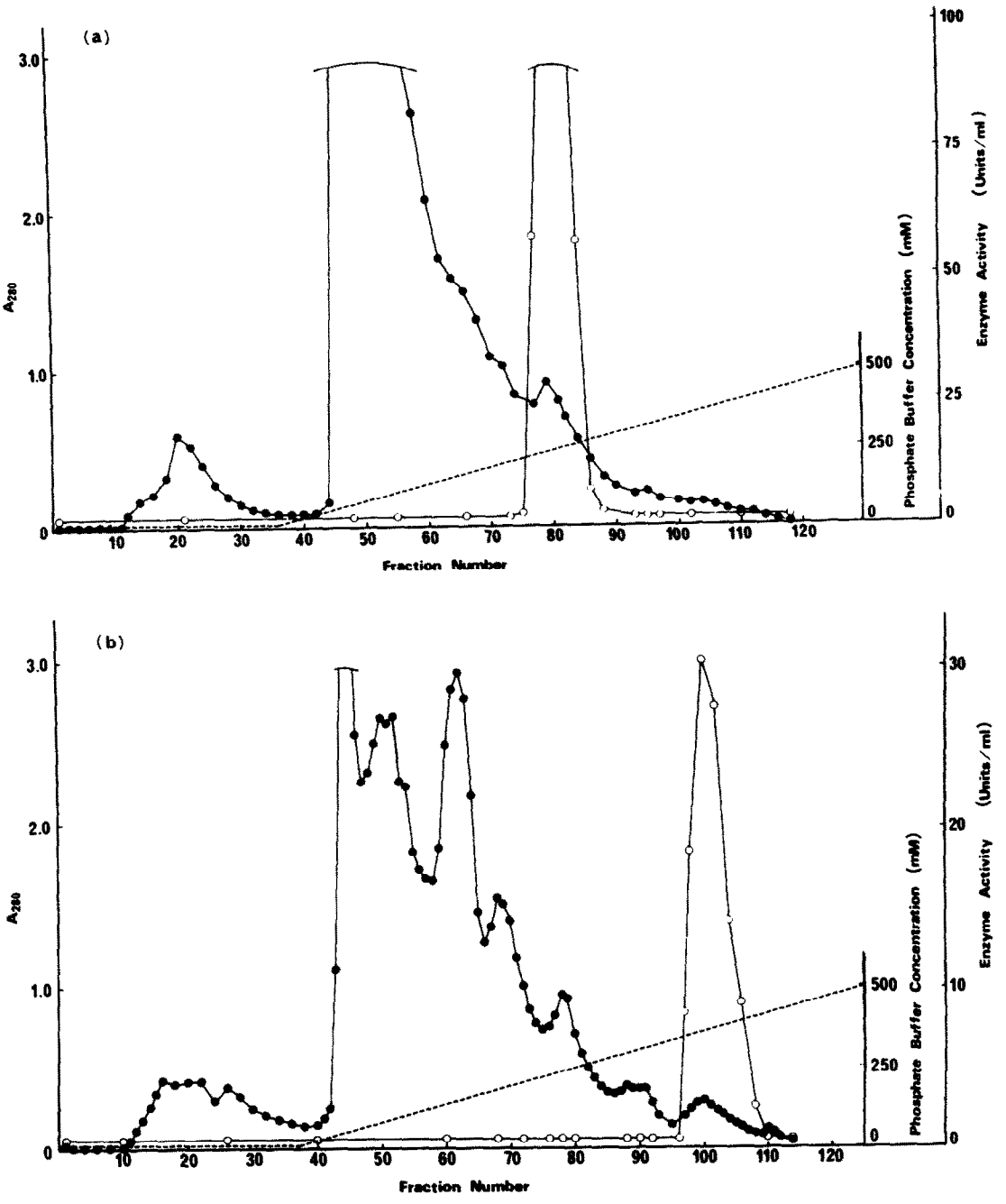

Fig. 2. Hydroxyapatite column chromatography (step 4). The solutions of MLO-I and MLO-II were separately applied to hydroxyapatite columns (1.5 × 44 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.8. The columns were washed with 140 mL (MLO-I) or 150 mL (MLO-II) of the buffer and then the enzymes were eluted with 500 mL of a linear gradient of 10–500 mM potassium phosphate buffer, pH 6.8 (– – –). Fractions of 5.3 (MLO-I) and 5.6 (MLO-II) mL were collected. (●) A_{280}, (○) MLO activity. (a) MLO-I, (b) MLO-II.

Stability

The purified enzyme preparations at step 6 were stable for at least 3 months at −20°. Thermotreatment at 50° for 5 min resulted in 80% (MLO-I) and 40% (MLO-II) loss of the enzyme activities. Treatment at 60° for 5 min abolished the activities completely.

Molecular mass

The apparent molecular mass of MLO-I and MLO-II determined by HPLC was 160 and 250 kDa, respectively. In SDS–PAGE (Fig. 5), MLO-I and MLO-II gave a single band which corresponded to the molecular mass of 41 and 39 kDa, indicating that

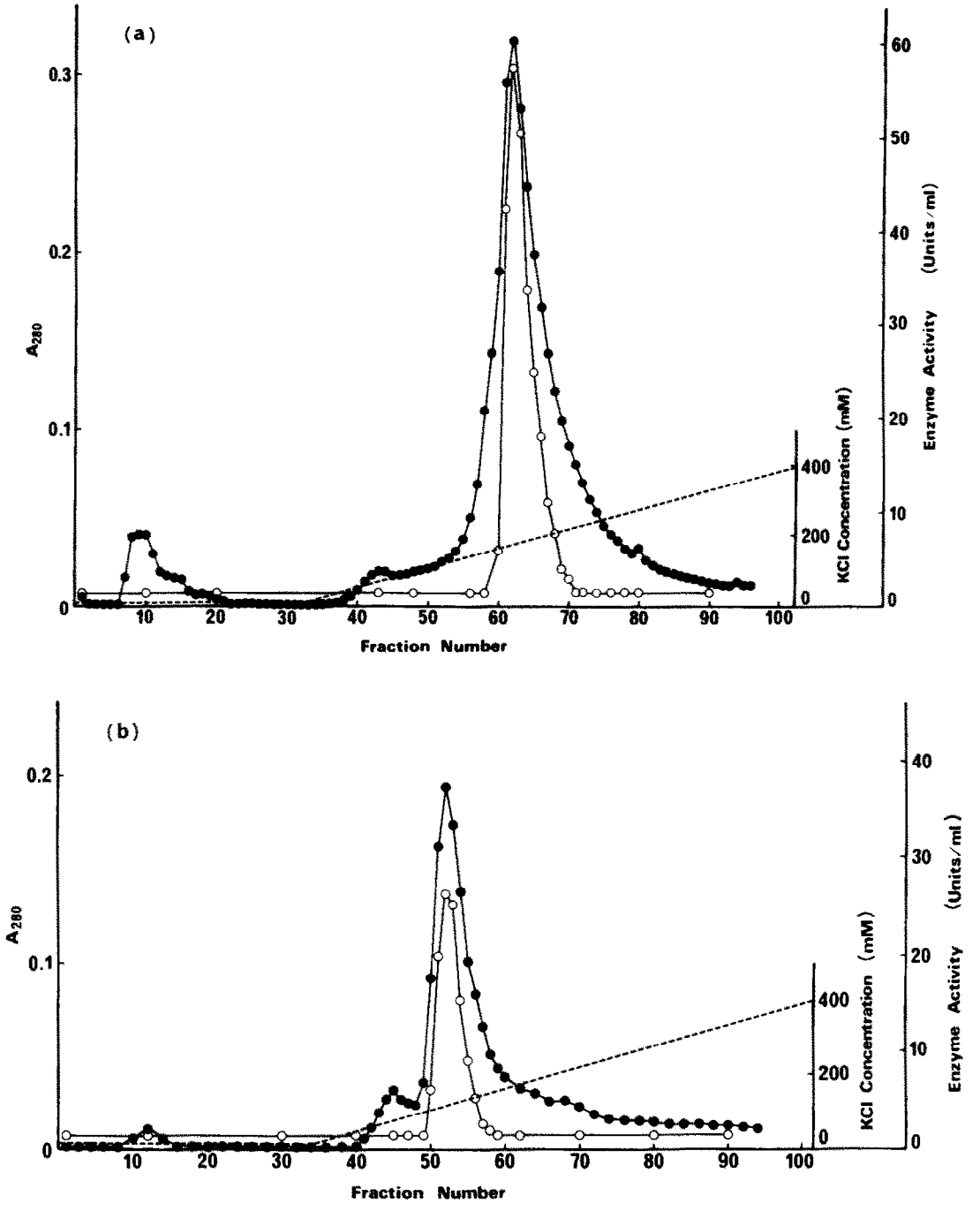

Fig. 3. Second DEAE-cellulose column chromatography (step 5). The solutions of MLO-I and MLO-II were separately applied to DEAE-cellulose columns (1.5 × 25 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.4. Proteins were eluted first with 150 mL of the buffer and then with 400 mL of a linear gradient of 0–400 mM KCl in the same buffer (– – –). Fractions of 5.8 (MLO-I) and 5.7 (MLO-II) mL were collected. (●) A_{280}, (○) MLO activity. (a) MLO-I, (b) MLO-II.

MLO-I and MLO-II were composed of four and six subunits, respectively. N-Terminal amino acid sequence of the two enzymes could not be determined by the usual method [24] probably because of the acylation or alkylation of α-amino moiety.

Spectroscopic properties and prosthetic group

The absorption spectra of the purified MLO-I and MLO-II showed an absorbance peak of around 450 nm characteristic of the flavin chromophore (Fig. 6a and b). In addition, a peak at 350 nm, which was

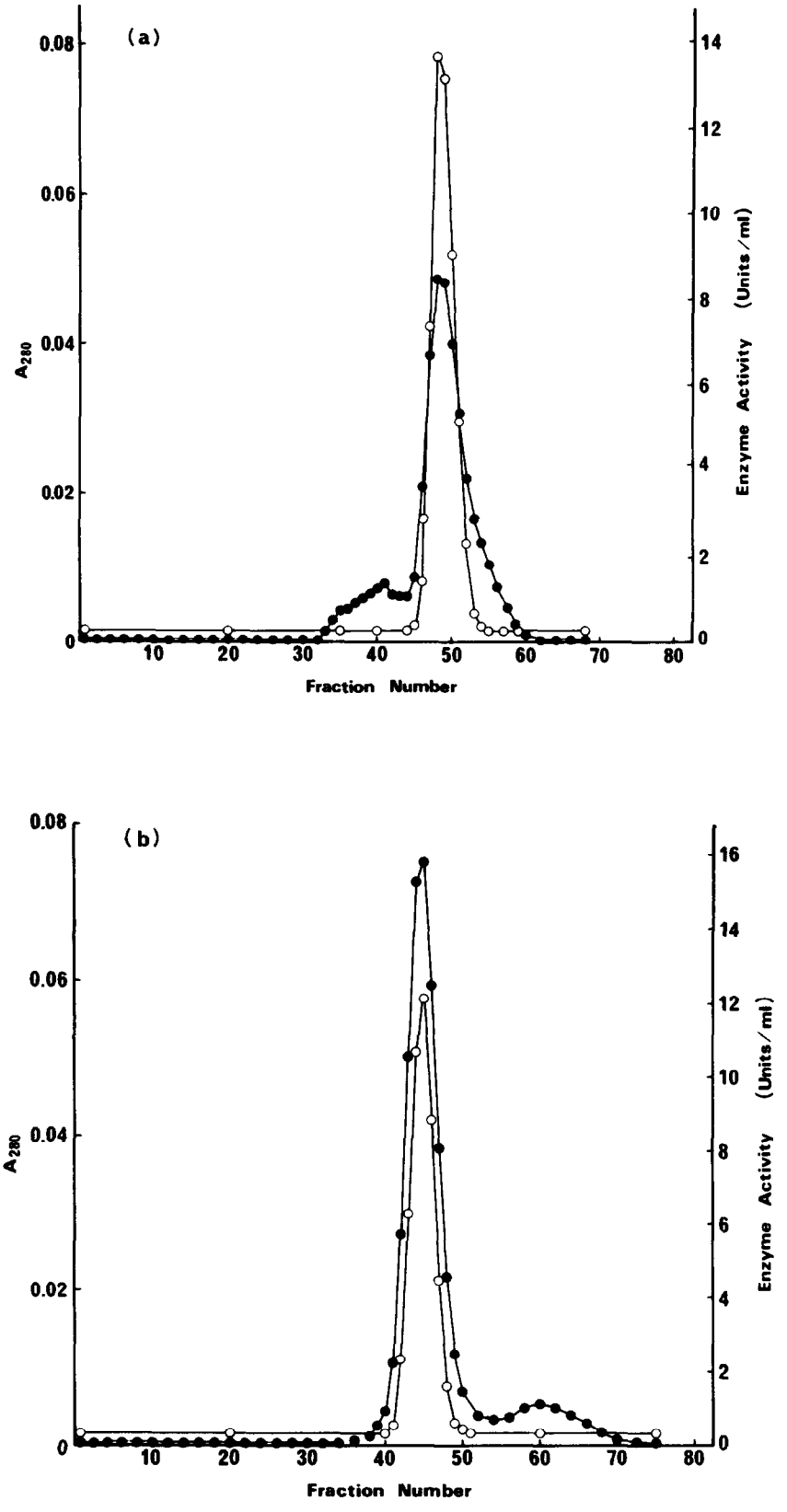

Fig. 4. Gel filtration (step 6). Concentrated solutions of MLO-I and MLO-II were separately applied to Sephadex G-200 column (2.5 × 110 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.4. Enzymes were eluted with the same buffer and eluate fractions of 5.2 (MLO-I) and 5.3 (MLO-II) mL were collected. (●) A_{280}, (○) MLO activity. (a) MLO-I, (b) MLO-II.

Table 2. Effect of various reagents on the enzyme activity

Reagent	Concentration (mM)	Residual activity (%) MLO-I	MLO-II
Control		100	100
N-Ethylmaleimide	0.1	93	100
	1.0	30	108
Iodoacetic acid	0.1	99	102
	1.0	84	97
FMN	0.1	98	88
	1.0	78	70
FAD	0.1	96	92
	1.0	99	91
Hg^{2+}	0.1	8	12
	1.0	0.6	0.4
Cu^{2+}	0.1	9	63
	1.0	3	34
Fe^{2+}	0.1	36	80
	1.0	7	38
Zn^{2+}	0.1	83	96
	1.0	26	77
Co^{2+}	0.1	85	94
	1.0	82	92
Mg^{2+}	0.1	109	102
	1.0	100	108

Dithiothreitol, EDTA, NAD and NADP had no effect on the activity of MLO.

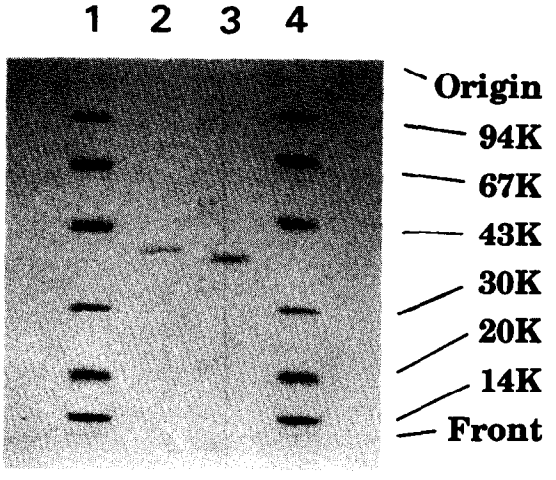

Fig. 5. SDS–PAGE of MLOs. Lane 2, MLO-I; Lane 3, MLO-II; Lanes 1 and 4, standard proteins.

likely attributable to the bound flavin [25] (cf. 375 nm for free flavin), was observed in MLO-II. The two peaks at 350 and 450 nm were reduced by the addition of sodium dithionite as reductant. The presence of FMN in these two enzymes was confirmed by HPLC analysis; supernatant solution of heat-denatured proteins gave a single peak at 6.3 min, which co-migrated with authentic FMN.

GC–MS analysis of the product and stoichiometry

GC–MS spectra (Fig. 7) of the methylated product gave a molecular ion at m/z 302 (isotope ion at m/z 304), consistent with the dimethylated product of *S*-(4-bromophenyl)-3-thiopyruvic acid. The ion at m/z 188 (base peak, isotope ion at m/z 190) was derived by the cleavage of the S—C bond. The origin of the ion at m/z 201 (isotope ion at m/z 203) was assumed to be the cleavage of the C=C bond. The spectrum is identical with that of the methylated authentic compound.

Amounts of *S*-(4-bromophenyl)-3-thiolactic acid converted and *S*-(4-bromophenyl)-3-thiopyruvic acid produced during the enzymic oxidation were 310 and 309 nmol/min/mg, respectively, for MLO-I, and 110 and 108 nmol/min/mg, respectively, for MLO-II. The amount of hydrogen peroxide generated in the reaction mixture was 300 and 112 nmol/min/mg for MLO-I and MLO-II, respectively. Formation of the two products stoichiometrically accounted for the decrease in substrate for MLO-I and MLO-II.

Kinetic properties

When *S*-(4-bromophenyl)-3-thio-DL-lactic acid was used as substrate, K_m values were 0.5 and 0.25 mM (L-isomer equivalent) for MLO-I and MLO-II, respectively. The respective V_{max} values for MLO-I and MLO-II with this substrate were 0.9 and 0.2 μmol/min/mg.

pH optimum

The pH dependence of MLO-I and MLO-II for *S*-(4-bromophenyl)-3-thiolactic acid was examined in citrate-phosphate buffer (pH 4.0–7.6), potassium phosphate buffer (pH 5.7–7.8) and Tris–HCl buffer (pH 7.8–9.5) (Fig. 8a and b). MLO-I exerted its maximum activity at pH 5.2–5.5 and MLO-II at pH 5.5. About 40% of the maximum activity of MLO-

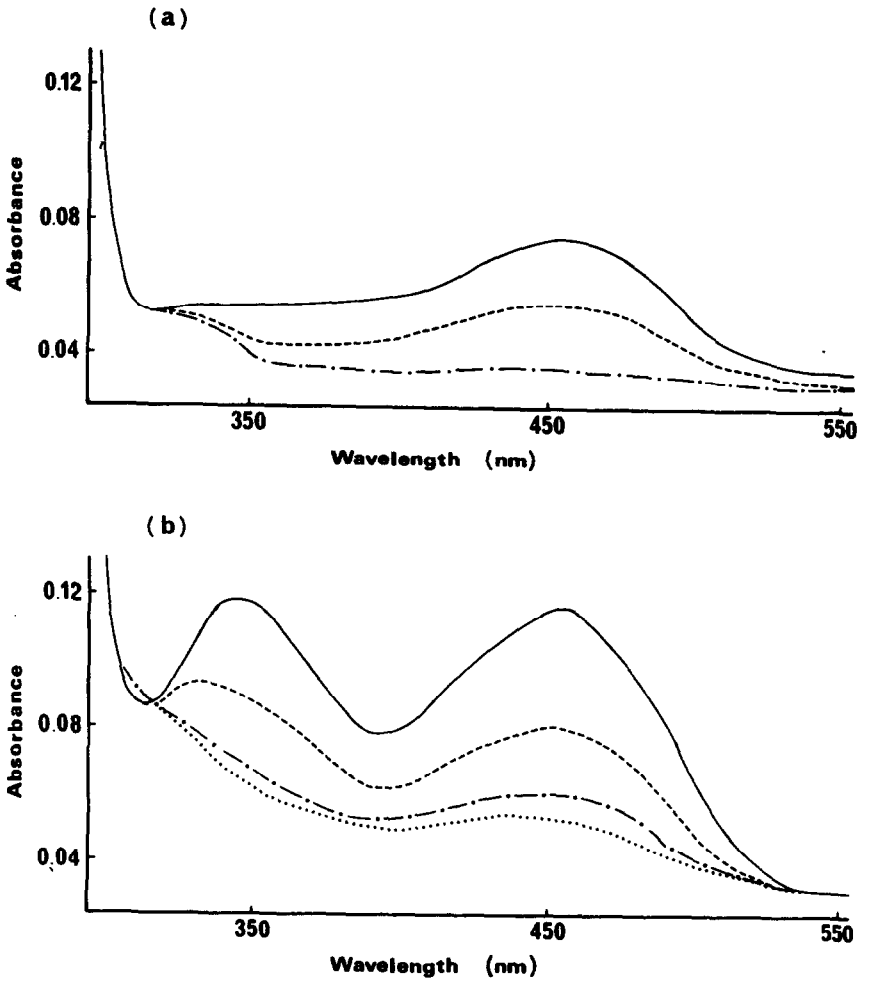

Fig. 6. Absorption spectrum of the purified MLOs. Step 6 preparations of the enzymes (0.3 mg/mL for MLO-I and 0.5 mg/mL for MLO-II) were examined in the presence of 0 (——), 10 (- - - -), 100 (— · — · —) and 1000 μM (·····) sodium dithionite. (a) MLO-I, (b) MLO-II.

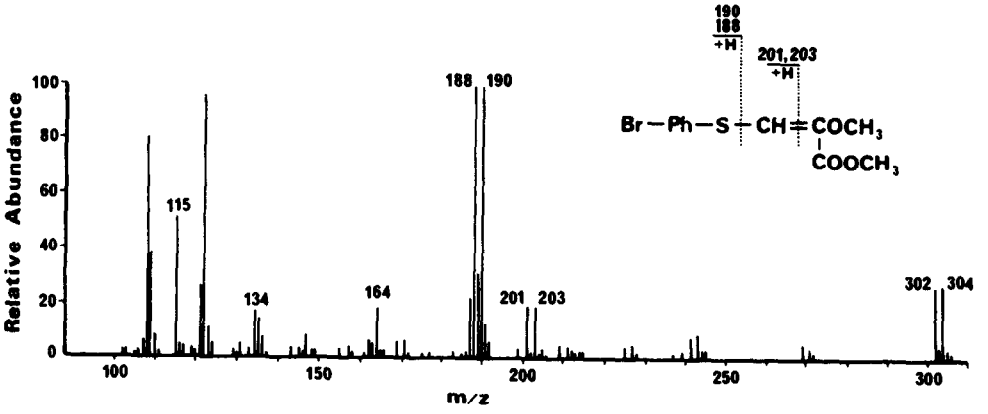

Fig. 7. GC–MS spectrum of dimethylated *S*-(4-bromophenyl)-3-thiopyruvic acid. *S*-(4-Bromophenyl)-3-thiolactic acid was incubated with MLO under the standard conditions and the product was collected by HPLC according to the procedure described in Materials and Methods. The product was methylated with etherial diazomethane and subjected to GC–MS analysis.

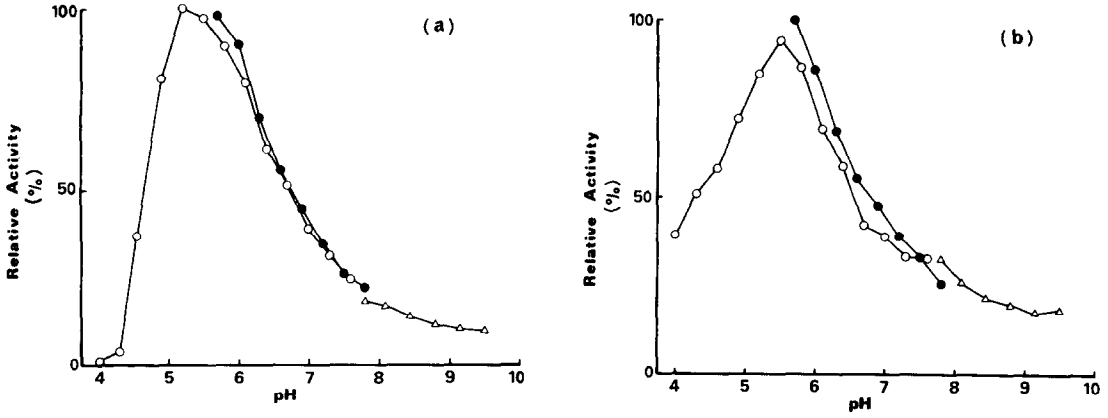

Fig. 8. pH dependence of MLOs. (○) Citrate-phosphate, (●) potassium phosphate, (△) Tris–HCl. (a) MLO-I, (b) MLO-II.

Table 3. Substrate specificity of MLO in rat liver

Substrate	Relative activity	
	MLO-I	MLO-II
S-(4-Bromophenyl)-3-thio-DL-lactic acid	100	100
S-Phenyl-3-thio-DL-lactic acid	80	130
S-Benzyl-3-thio-DL-lactic acid	128	165
S-Pentyl-3-thio-DL-lactic acid	98	107
S-Cyclohexyl-3-thio-DL-lactic acid	43	121
L-3-Phenyllactic acid	29	116
3-(4-Hydroxyphenyl)-DL-lactic acid	ND	122
DL-2-Hydroxycaproic acid	34	203
L-2-Hydroxyisocaproic acid	159	189
DL-2-Hydroxyvaleric acid	55	251
DL-2-Hydroxybutyric acid	13	274
DL-Ethyl-2-hydroxybutyrate*	ND	ND
DL-3-Hydroxybutyric acid*	ND	ND
4-Hydroxybutyric acid*	ND	ND
2-Hydroxyisobutyric acid	ND	ND
L-Malic acid	ND	ND
L-Lactic acid	ND	81
Glycolic acid	311	ND
1-Butanol*	ND	ND
Benzyl alcohol*	ND	ND
L-Leucine	ND	ND

Each substrate was incubated with MLO under the conditions described in the text. α-Keto acid formed was determined spectrophotometrically after derivatization with 3-methyl-2-benzothiazolinone hydrazone hydrochloride.
ND, lower than the detection limit (5%).
* Formation of hydrogen peroxide was measured.

II still remained at pH 4.0, while MLO-I did not catalyse the reaction at all at this pH.

Effect of various reagents on enzyme activity

MLO-I and MLO-II were assayed in the presence of sulphydryl-modifying agents, a sulphydryl-protecting agent, redox coenzymes, divalent metal ions and a metal-chelating agent (Table 2). Sulphydryl-modifying agents showed different effects on MLO-I and MLO-II; *N*-ethylmaleimide (1.0 mM) and iodoacetic acid (1.0 mM) inhibited MLO-I by 70% and 16%, respectively, although dithiothreitol showed no effect. Interestingly, MLO-II was not affected by these reagents. In the redox coenzymes tested, only FMN (1.0 mM) showed inhibitory effects on MLO-I and MLO-II by 20–30%. MLO-I was

strongly inhibited by Hg^{2+}, Cu^{2+} and Fe^{2+}, and MLO-II by Hg^{2+}. EDTA, NAD and NADP did not show any significant effect on these enzymes.

Substrate specificity

S-substituted 3-mercaptolactic acids, other hydroxy acids, alcohols and L-leucine were examined as substrates for MLO-I and MLO-II (Table 3). Both MLO-I and MLO-II oxidized *S*-aryl, *S*-aralkyl and *S*-alkyl-3-thiolactic acids, the result indicating the low specificity of these enzymes for *S*-substituents of 3-mercaptolactic acid *S*-conjugates. Regarding other α-hydroxy acids, 2-hydroxyisocaproic acid worked as substrate comparably for the two enzymes, whereas MLO-II showed a higher activity than MLO-I for 3-phenyllactic acid, 2-hydroxycaproic acid, 2-hydroxyvaleric acid and 2-hydroxybutyric acid. 3-(4-Hydroxyphenyl)-lactic acid and glycolic acid were the specific substrates for MLO-II and MLO-I, respectively. Neither of the enzymes oxidized ethyl-2-hydroxybutyrate, 2-hydroxyisobutyric acid, malic acid, 1-butanol, benzyl alcohol or L-leucine. Both β- and γ-hydroxy acids such as 3- and 4-hydroxybutyric acid were inert as substrates.

After enzymic oxidation of DL-3-phenyllactic acid by the present oxidases, the incubation medium was analysed for the remaining substrate by chiral HPLC. The L-isomer substrate was specifically consumed during incubation with either MLO-I or MLO-II. When DL- and L-3-phenyllactic acid were separately incubated with the present enzymes, the rate of formation of 3-phenylpyruvic acid was virtually the same. Therefore, D-isomer was unlikely to compete with L-isomer. These results indicate that α-hydroxy acid moiety with L-configuration is essential for the substrate of MLO.

DISCUSSION

When *S*-(4-bromophenyl)-3-thiolactic acid, one of the urinary metabolites of *S*-(4-bromophenyl)-L-cysteine, was incubated with homogenate of rat liver or kidney, a considerable amount of the substrate was converted to the cysteine conjugate (data not shown). The result indicates that MLO(s) as well as the transaminases [9] are present in these tissues and that 3-mercaptolactic acid *S*-conjugates can be converted to reactive thiols via 3-mercaptopyruvic acid *S*-conjugates and cysteine conjugates. This consideration prompted us to examine the enzyme involved in the oxidation of 3-mercaptolactic acid *S*-conjugates.

The enzymes purified in this study oxidized α-hydroxy acids including *S*-substituted 3-mercaptolactic acids, specifically (Table 3). To our knowledge, at least four enzymes that oxidize certain kinds of α-hydroxy acid have been reported: (a) L-amino acid oxidase (EC 1.4.3.2), (b) glycolate oxidase (EC 1.1.3.1), (c) malate oxidase (EC 1.1.3.3) and (d) L-α-hydroxy acid oxidase (EC 1.1.3.15). Among these oxidases, L-amino acid oxidase [26] and malate oxidase [27] are distinct from MLO, because MLO did not oxidize L-leucine and L-malic acid, respective substrates for the two former enzymes. Glycolate oxidase, which was reported to be identical to L-α-hydroxy acid oxidase in rat liver [28], is located in rat liver peroxisomes and has a similar specificity for α-hydroxy acid substrates as compared with cytosolic MLO [29]. However, MLO-II was reported to be distinct from glycolate oxidase because glycolic acid was not oxidized by MLO-II. We considered that MLO-I was also different from glycolate oxidase/α-hydroxy acid oxidase: MLO-I oxidized 3-phenyllactic acid, which was an inert substrate for L-α-hydroxy acid oxidase [30]. Moreover, MLO-I and L-α-hydroxy acid oxidase have different molecular masses of 160 and 300 kDa, respectively [30].

In the present study, we demonstrated by using phenyllactic acid as substrate that substrate oxidized by MLO was restricted to the L-isomers of α-hydroxy acids. Coupled with our unpublished results that 3-mercaptopyruvic acid *S*-conjugate reductases produced only L-isomers, this result indicates the fully reversible reaction between 3-mercaptopyruvic acid *S*-conjugates and the L-isomers of 3-mercaptolactic acid *S*-conjugates *in vivo*.

C—S bond cleavage of cysteine conjugates to form thiols has been considered to be the key step in the toxication of halogenated hydrocarbons [2–4]. On the other hand, the transamination pathway has been assumed to be a mere detoxication pathway. As described above, however, 3-mercaptolactic acid *S*-conjugate was easily converted back to cysteine conjugate *in vitro* via enzymic oxidation and transamination. The present study provides a new insight into the formation of cysteine conjugates: the conjugates can be generated via oxidation of 3-mercaptolactic acid *S*-conjugates and subsequent transamination in addition to hydrolysis of glutathione or *N*-acetylcysteine *S*-conjugates.

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