Sialic Acid 9-*O*-Acetylesterase Catalyzes the Hydrolyzing Reaction from Alacepril to Deacetylalacepril

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Purpose. In this work, the alacepril thiolesterase, which catalyzes the hydrolyzing reaction of the thiolester linkage in alacepril and the conversion from alacepril to deacetylalacepril, was purified from rat liver cytosol and characterized.

Methods. A purification procedure for the thiolesterase consisted of ammonium sulfate fractionation and chromatographies with phenyl Sepharose CL-4B, Q Sepharose FF, ceramic hydroxylapatite, and phenyl Sepharose HP. The thiolesterase activity was assayed for alacepril as a substrate and the reaction product, deacetylalacepril, was measured using high-performance liquid chromatography.

Results. The purified thiolesterase is heterodimeric with a molecular mass of 29 and 36 kDa subunits as estimated by sodium dodecyl sulfate -polyacrylamide gel electrophoresis. N-terminal amino acid sequence of these subunits reveals that the thiolesterase is identical to sialic acid 9-*O*-acetylesterase. The thiolesterase hydrolyzes not only the thiolester bond in alacepril, spironolactone, and acetyl coenzyme A but also the carboxylester bond in α-naphtyl acetate. The alacepril thiolestrase activity is competitively inhibited by α-naphtyl acetate.

Conclusion. The thiolesterase, i.e., sialic acid 9-*O*-acetylesterase, seems to be involved in the metabolism of certain drugs such as alacepril and spironolactone. However, drugs having ester-type and amide-type linkages, for example dilazep, aniracetam, and benazepril, are not substrates for the thiolestrase.

KEY WORDS: alacepril; thiolesterase; carboxylesterase; hydrolase; sialic acid 9-*O*-acetylesterase.

INTRODUCTION

Alacepril, which is used clinically as an anti-hypertension drug, is a prodrug of captopril, an angiotensin I-converting enzyme (ACE) inhibitor (1). Alacepril is known to be metabolized mainly in the liver and its metabolizing pathway has been reported (2,3). Alacepril first receives the deacetylation of thiolester and is converted to deacetylalacepril. Second, amide linkage in deacetylalacepril is hydrolyzed and phenylalanine is released to produce captopril. Because the sulfhydryl group in captopril chelates zinc that is located in the active center of ACE, a metalloenzyme containing zinc, this group is believed to be essential for the drug effect as an ACE inhibitor (4). Therefore, the conversion from alacepril to deacetylalacepril to hydrolyze the thiolester linkage in alacepril seems to be an important reaction step for alacepril to exhibit the antihypertension effects as an ACE inhibitor.

Esterases are well known to catalyze the hydrolyzing reaction of carboxylester, thiolester, and amide linkages (5). These enzymes are widely distributed in various tissues of animals and hydrolyze a variety of endogenous and exogenous compounds (6–8). Several esterases have been purified from many sources of animals and their properties have been investigated (9–12). In drug metabolism, ester-linkages in many prodrugs are hydrolyzed by esterases, converting prodrugs to active forms (13,14). Other drugs with the linkage are degraded to inactivate forms by the enzymes (15). Many findings on inhibitors (16) and inducers (17,18) for esterases and polymorphism of these enzymes (19,20) have been accumulated and are for analyzing pharmacokinetics of drugs with the ester linkage in human. However, the substrate specificity of each esterase for ester-type drugs are not well known because esterases generally appear to show broad substrate specificity.

Species differences of esterases have not been elucidated sufficiently. However, it is important to speculate the metabolizing pathway of drugs with ester linkage in humans to apply drug metabolism utilizing animal experiments in pre-clinical study of drug development. We previously reported that pranlukast, an antagonist for the leukotriene receptor, was hydrolyzed by rat esterases RL1 and RH1, but not by human esterase HU1 and hCE-2 (21). Indomethacin was hydrolyzed by a pig esterase but not by rat and human esterases (22). Therefore, it is useful to clarify the species differences of esterases for the study of drug metabolism as substrate specificity of esterases for some drugs with the ester-linkage seems to be different between species.

In this study, we identified and characterize an alacepril thiolesterase that catalyzes the hydrolyzing reaction of thiolester linkage in alacepril. The substrate specificity of the thiolesterase for drugs with ester linkage is investigated.

MATERIALS AND METHODS

Materials

Alacepril was a generous gift from Dainippon Pharmaceutical Co. Ltd. (Osaka Japan), and authentic materials of clinically used preparations were kindly supplied by their respective manufactures. Q-Sepharose FF, Phenyl Sepharose CL-4B, and Phenyl Sepharose HP were obtained from AmerSham Pharmacia Biotech (Buckinghamshire, UK). Ceramic Hydroxylapatite was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Cosmosil 5-C_{18} MS, a reversed phase high-performance liquid chromatography (HPLC) column, was from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals and reagents were of analytical grade or liquid chromatographic grade.

Purification of Alacepril Thiolesterase

Livers from Sprague–Dawley male rats were obtained according to a protocol approved by the Review Board in Gifu Pharmaceutical University and stored at –80°C until use.

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Table I. Subcellular Distribution of Alacepril Thiolesterase in Rat Liver

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Cytosol fraction from frozen livers	5,810	21,202	3.649
From fresh livers	5,234	17,849	3.410
Microsomal fraction from frozen livers	13,626	5,062	0.371
From fresh livers	13,455	7,826	0.582

All purification procedures were performed at 0–4°C. Frozen rat livers were homogenized with 6 volumes of 10 mM potassium phosphate, pH 8.0, containing 0.32 M sucrose. The homogenate was centrifuged at 9000 × g for 10 min. and the supernatant was further centrifuged at 105,000 × g for 1 h. The supernatant obtained was designated as the cytosol fraction. The precipitate was suspended with 10 mM potassium phosphate, pH 8.0, containing 0.5% Triton X-100 and then centrifuged at 105,000 × g for 1 h. The supernatant prepared was designated as the solubilized microsomal fraction.

The cytosol fraction was subjected to ammonium sulfate fractionation. The precipitate formed between 45 and 60% ammonium sulfate saturation was collected by centrifugation at 12,000 × g for 20 min and redissolved in 5 mM potassium phosphate, pH 7.0, containing 0.8 M ammonium sulfate and then dialyzed overnight against the same buffer. The dialyzed solution was applied to a Phenyl Sepharose CL-4B column (2.5 × 20 cm) equilibrated with 5 mM potassium phosphate, pH 7.0, containing 0.8 M ammonium sulfate. After washing the column with the same buffer, the alacepril thiolesterase was eluted with 5 mM potassium phosphate, pH 7.0. Fractions with thiolesterase activity were collected and dialyzed overnight against 10 mM Tris-HCl, pH 8.0. The dialysate was loaded to a Q Sepharose FF column (2.5 × 40 cm) equilibrated with 10 mM Tris-HCl, pH 8.0, and the column was washed with 2 volumes of the same buffer. The thiolesterase was eluted with a linear gradient formed from 0 to 0.15 M NaCl in 10 mM Tris-HCl, pH 8.0. The fractions exhibiting the enzyme activity were pooled and dialyzed overnight against 5 mM potassium phosphate, pH 7.0. The dialysate was applied to a Hydroxylapatite column (1.5 × 0.8 cm) equilibrated with 5 mM potassium phosphate, pH 7.0, and the column was washed with 3 volumes of the same buffer. The thiolesterase was eluted with a linear gradient formed from 5 to 100 mM potassium phosphate, pH 7.0, and the fractions with the activity were combined. Ammonium sulfate was added to the fractions to bring the concentration to 1 M and the solution was loaded on a phenyl Sepharose HP column (0.7 × 15 cm) equilibrated with 5 mM potassium phosphate, pH 7.0, containing 1 M ammonium sulfate. After the column was washed with an equal volume of the same buffer, the thiolesterase was eluted with a linear gradient formed from 1 to 0 M ammonium sulfate in 5 mM potassium phosphate, pH 7.0. The fractions containing the enzyme were collected, concentrated by Centricon YM-10 (Amicon-Millipore), and dialyzed overnight against 5 mM potassium phosphate, pH 7.0.

Assay for Enzyme Activities

Forty microliters of the reaction mixture consisted of 50 mM potassium phosphate, pH 7.5, containing 1 mM alacepril, 1.25% DMSO, and the enzyme solution was incubated at 37°C for 60 min. The enzyme reaction was stopped by adding 200 μL of acetonitril. To the reaction mixture, 200 μL of 20 μg/mL *p*-hydroxy benzoic acid *n*-propyl as an internal standard was added and the supernatant was separated by centrifugation. Thirty microliters of the supernatant was subsequently applied to Cosmosil 5-C_{18} MS (4.6 i.d. × 250 mm), a reversed-phase HPLC column, and then alacepril, deacetylalacepril, which is a product of the enzyme reaction, and *p*-hydroxy benzoic acid *n*-propyl were detected by monitoring at a wavelength of 220 nm. The analytical condition of these compounds by HPLC was as follows: the mobile phase consisted of three parts of 10 mM potassium phosphate, pH 2.5, and two parts of acetonitril was used and separation of compounds was performed at 50°C and the flow-rate of 1.5 mL/min. One unit of enzyme activity was defined as the amount to hydrolyze the thiolester bond in alacepril at a rate of 1 nmol per min. The activities of calboxylesterase and acetyl coenzyme A thiolesterase were measured by the methods of Sugiura *et al.* (23) and Prass *et al.* (24), respectively.

Protein Determination

Protein concentration was measured with bovine serum albumin as a standard according to the method of Lowry *et al.* (25).

Protein Blotting and Amino Acid Sequence Analysis

The purified enzyme preparation was resolved by electrophoresis with 12.5% polyacrylamide gel containing 1% so-

Table II. Purification of Alacepril Thiolesterase from Rat Liver

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	5,810	21,202	3.65	100	1.00
Ammonium sulfate fractionation	1,508	9,122	6.05	43.0	1.66
Phenyl Sepharose CL-4B	468	8,174	17.5	38.6	4.79
Q Sepharose FF	25.9	1,348	52.1	6.36	14.3
Hydroxylapatite	1.47	513	349	2.42	95.5
Phenyl Sepharose HP	0.190	132	695	0.62	190

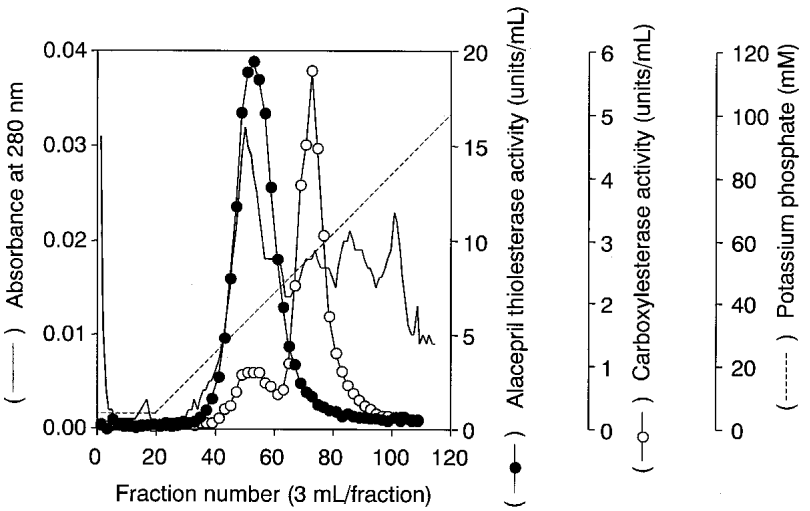

Fig. 1. Hydroxylapatite column chromatography of alacepril thiolesterase from rat liver. The fraction containing thiolesterase, which was obtained from Q Sepharose column chromatography, was applied to a Hydroxylapatite column equilibrated with 5 mM potassium phosphate buffer, pH 7.0. After the column was washed with the buffer, the enzyme was eluted with a linear gradient formed between 5 and 100 mM potassium phosphate. Solid and broken lines indicate the absorbance at 280 nm and the concentration of potassium phosphate, respectively. Open and closed circles indicate the carboxylesterase activity for α-naphtylacetate as a substrate and the alacepril thiolesterase activity, respectively.

dium dodecyl sulfate (SDS) under a reducing condition by 2-mercuptoethanol according to the method described by Laemmli (26). The enzyme in the gel was electroblotted onto a polyvinylidene difluoride membrane according to the method of Towbin *et al.* (27). The protein blotted on the membrane was visualized with coomassie brilliant blue R 250. The protein band was cut out, briefly wash with methanol, and applied to a protein sequencer (model 473 protein sequencer, Applied Biosystems).

Molecular Mass Determination

The molecular mass of the native thiolesterase was estimated by gel filtration using a Superdex 200HR column (1 × 25 cm) equilibrated with 10 mM potassium phosphate, pH 7.0, containing 0.15 M NaCl. Ovalbumin (M_r 43,000), bovine serum albumin (68,000), and β-galactosidase (130,000) were used as molecular mass standards. The molecular mass of the denatured thiolesterase was determined by SDS-(12.5%) polyacrylamide gel electrophoresis by the method of Laemmli (26). The sample and standards were treated with 2% SDS and 5% 2-mercuptoethanol at 100°C for 5 min before they were subjected to electrophoresis. The protein standards used were lysozyme (14,000), carbonic anhydrase (31,000), ovalbumin, bovine serum albumin, and phospholylase B (94,000).

Liquid Chromatography-Mass Spectroscopy (LC-MS) Analysis

The product of the thiolesterase reaction using alacepril as a substrate was identified by LC-MS (Hewlett Packard model HP 1100 series LC/MSD). The enzyme reaction was performed in a standard reaction mixture. The separation of the reaction product using HPLC was conducted by the method described above, but the product was eluted form the reversed phase HPLC column by the linear gradient of acetonitril and 1% triethylamine, pH 2.5. The atmospheric pressure chemical ionization method was used as an ionization method of the product in the mass measurement and the

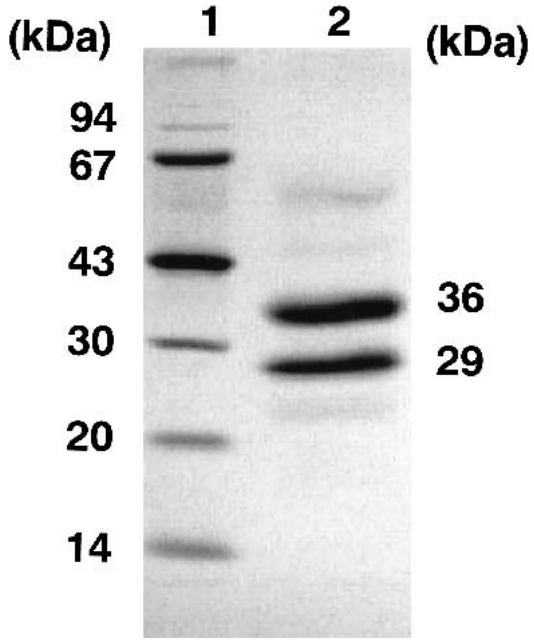

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified alacepril thiolesterase. Lanes 1 and 2 show molecular mass standards and the purified thiolesterase, respectively. About 1 μg of the purified enzyme was resolved with 12.5% sodium dodecyl sulfate polyacrylamide gel under a reducing condition. Proteins on the gel were visualized by staining with coomassie brilliant blue R-250.

analysis condition was optimized by the flow injection analysis function equipped in the LC-MS system.

RESULTS

Subcellular Distribution of Thiolesterase in Rat Liver

The cytosol and solubilized microsomal fractions were prepared from fresh and frozen rat livers and the alacepril thiolesterase activity in these fractions was assayed for alacepril as a substrate (Table I). The specific activity of the thiolesterase in the cytosol fraction was found to increase by freezing rat livers, whereas it decreased in the microsomal fraction. This implies that a part of the thiolesterase is released from the microsomal fraction to the cytosol fraction by freezing and thawing. Because the total and specific activities of the thiolesterase in the cytosol fraction from frozen livers were observed to be about four and ten times higher, respectively, than those in the solubilized microsomal fraction, the thiolesterase was purified using the cytosol fraction from frozen livers.

Purification of Thiolesterase from Rat Liver

The alacepril thiolesterase was purified by sequential application of ammonium sulfate precipitation and four serial chromatographies. About 190 μg of the aracepril thiolesterase was obtained from 67 g of frozen rat livers. The overall yield was approximately 0.62%. Using aracepril as a substrate, a 190-fold purification was obtained (Table II). The yield of the enzyme activity was shown to be relatively low in the step of Q-Sepharose column chromatography. This was caused by the enzyme eluating broadly from the column and the part of the fractions exhibiting the activity were collected by raising the purification efficiency. Although the alacepril thiolesterase activity coeluted with the carboxylesterase activity for α-naphtyl acetate as a substrate in the hydroxylapatite column chromatography, the thiolesterase was separated from the carboxylesterase, shown as a major fraction of the activity for α-naphtyl acetate (Fig. 1). The chromatographies with PBA-30, concanavaline A (Con A) Sepharose, and wheat germ lectin (WGA) agarose were performed to determine whether or not the thiolesterase contained sugar chains. The solution of the purified thiolesterase was dialyzed against 25 mM HEPES-NaOH, pH 7.5 containing 1 mM $MgCl_2$ and then applied to a PBA-30 column equilibrated with the same buffer. The thiolesterase was retained on this column and eluted with 25 mM HEPES-NaOH, pH 7.5, containing 100 mM sorbitol. However, the thiolesterase was not absorbed by Con A Sepharose or WGA agarose, although the enzyme solution was dialyzed against 10 mM potassium phosphate, pH 7.4, containing 0.15 M NaCl and then applied to these columns.

The native thiolesterase eluted as a single peak by gel filtration with Superdex 200HR and the molecular mass was estimated as about 60 kDa. Two stained protein bands corresponding to the molecular mass of 29 and 36 kDa were observed by SDS-polyacrylamide gel electrophoresis under a reducing condition (Fig. 2). When the thiolesterase was incubated at room temperature for 8 h with 10 mM 2-mercaptothanol or dithiothreitol, the enzyme activity completely disappeared.

Identification of Enzymatic Reaction Product

The enzymatic reaction product with alacepril as a substrate for thiolesterase was identified by LC-MS analysis. Alacepril, deacetylalacepril (product), and internal standard were completely separated by HPLC with a reversed-phase column. Alacepril and deacetylalacepril were confirmed by the

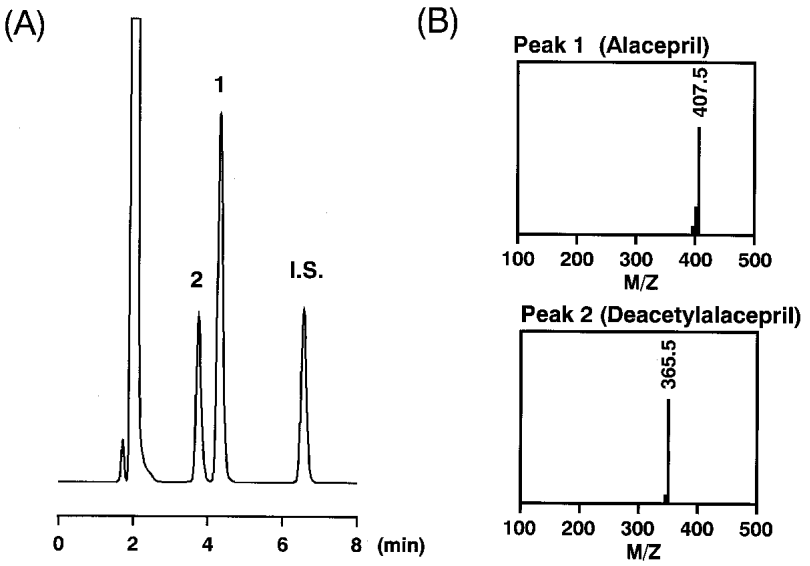

Fig. 3. (A) High-performance liquid chromatography profile and (B) mass spectra of alacepril and deacetylalacepril. The standard reaction mixture containing the purified alacepril thiolesterase was incubated at 37°C for 60 min. Acetonitril and n-propyl p-hydroxybenzoate as an internal standard were added to the mixture. After centrifugation, 30 μL of the supernatant was analyzed by high-performance liquid chromatography and liquid chromatography-mass spectroscopy under the conditions described in Materials and Methods section.

mass of the molecular ion peak, which were estimated as 407.5 and 365.5 (m/z), respectively (Fig. 3).

Catalytic Properties of Thiolesterase

Substrate specificity of the alacepril thiolesterase are shown in Table III. The thiolesterase catalyzed the hydrolyzing reaction of not only the thiolester bond in alacepril, spironolactone, and acetyl coenzyme A but also the carboxylester bond in α-naphthyl acetate. α-Naphthyl acetate was found to be a competitive inhibitor for this enzyme when the activity was assayed for alacepril as a substrate. The inhibition constant of α-naphthyl acetate was calculated to be about 10 mM. The thiolester bond in palmitoyl coenzyme A was not hydrolyzed by this enzyme. Whether the thiolesterase catalyzes the hydrolysis of thiolester-, carboxylester-, and amide-type bonds in various drugs clinically used was investigated, as alacepril is a drug administered frequently in Japan. As shown in Table IV, this enzyme was not observed to hydrolyze the bonds in all the drugs examined.

Optimal pH of the enzymatic reaction was determined to be between 7.5 and 8.0 in potassium phosphate and Tris-HCl buffers when alacepril was used as a substrate, and similar optimal pH was observed with α-naphtyl acetate as a substrate.

Effects of Metal Ions and Inhibitors

Table V lists metal ions and compounds which affected the thiolesterase activity. The enzyme activity was inhibited by diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride, which are well known as serine residue modifiers, and the enzyme was also inactivated strongly by Hg^{2+} and Cu^{2+}.

N-Terminal Amino Acid Sequence of Thiolesterase

After two subunits, corresponding to the molecular mass of 29 and 36 kDa, of the thiolesterase were electroblotted on a polyvinylidene difluoride membrane, the N-terminal amino acid sequences of these subunits were analyzed separately. As shown in Fig. 4, sequences of 29 and 36 kDa subunits partly agreed with those of the small and large subunits of sialic acid 9-*O*-acetylesterase from rat, although several unidentified amino acids were observed in these sequences.

DISCUSSION

Alacepril thiolesterase, which catalyzes the hydrolyzing reaction of the thiolester bond in aracepril and the conversion to deacetylalacepril, was purified to apparent homogeneity from the cytosol fraction of frozen rat livers. Molecular masses of the native and the denatured thiolesterase were calculated as 60 and, 29 and 36 kDa, respectively, indicating that this enzyme is heterodimeric. Both subunits of the thiolesterase appear to be necessary for the enzyme activity because the thiolesterase activity disappeared by the reduction of the enzyme with the reducing reagents for the disulfide bond, 2-mercaptoethanol and dithiothreitol. Thiolesterase seems to be different from calboxylesterases RL1, RH1, and RL2 (28–31) from the result of the hydroxylapatite column chromatography; the thiolesterase activity for alacepril and the major calboxylesterase activity for α-naphtyl acetate eluted separately from this column. N-terminal amino acid sequences of 29- and 36-kDa subunits of the purified thiolesterase were found to be identical to those of the sialic acid 9-*O*-acetylesterase reported as a membrane associated esterase, LSE (32,33).

Sialic acid 9-*O*-acetylesterase was reported to be distributed widely to variety of rat tissues including the liver, kidney, heart, lung, spleen, intestine, testis, and brain (34). Acetylesterase activity was observed in influenza C virus and mammals such as human and mouse (35). Two acetylesterases were reported to show a similar substrate specificity but different subcellular distribution (34). One was a membrane associated intralumenal sialic acid 9-*O*-acetylesterase localized mainly in lysosome (LSE) and the other was a cytosolic sialic acid 9-*O*-acetylesterase (CSE). The alacepril thiolesterase was purified from the cytosol fraction prepared from frozen

Table III. Substrate Specificity of Alacepril Thiolesterase

Substrate	K_m (mM)	V_{max} (μmol/min/mg)
Alacepril	22.0	28.6
Spironolactone	0.652	0.0156
α-Naphtyl acetate	1.71	143
Acetyl coenzyme A	1.28	0.998
Palmitoyl coenzyme A	ND[a]	ND[a]

[a] ND, not detected.

Table IV. List of Drugs Not Hydrolyzed by Aracepril Thiolesterase

Linkage	Drugs
Ester type	Dilazep, Flavoxate, Propiverine, Oxybutynin
Amide type	Aniracetam, Flutamide, Indomethacin, Posatireline, Pranlukast, Prazosin, Tiaramide
Mixed type	Camostat mesilate, Benazepril, Ditiazem, Irinotecan, Quinapril, Temocapril

Table V. Effects of Metal Ions and Compounds on Alacepril Thiolesterase Activity

Metal ion and compound	Concentration (mM)	Remaining activity (%)
Hg^{2+}	1	0
	0.1	3.9
	0.01	85.9
Cu^{2+}	1	0.6
	0.1	34.9
Ni^{2+}	1	73.3
Co^{2+}	1	81.3
Fe^{2+}	1	89.8
Mg^{2+}	1	92.7
Zn^{2+}	1	97.0
Mn^{2+}	1	101.4
Cd^{2+}	1	115.3
Diisopropyl fluorophosphate	1	0
	0.1	5.4
	0.01	28.8
	0.001	69.1
Phenylmethylsulfonyl fluoride	10	46.4
	1	77.9
Ethylendiaminetetraacetic acid	1	93.4

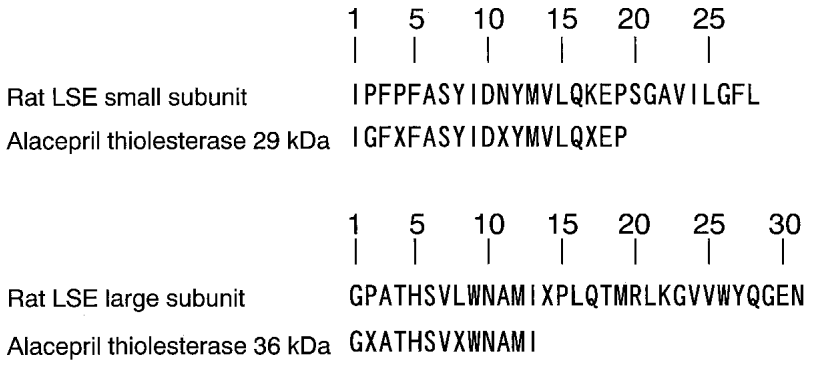

Fig. 4. N-terminal amino acid sequences of two subunits of the purified alacepril thiolesterase from rat liver. Two subunits of the purified thiolesterase was electroblotted on a polyvinylidene difluoride membrane after electrophoresis with sodium dodecyl sulfate polyacrylamide gel. The amino acid sequence of the blotted protein was analyzed by automated Edman degradation using a gas-phase protein sequencer. LSE, lysosomal sialic acid 9-*O*-acetylesterase (33); X, unidentified amino acid

rat livers despite the consistency of N-terminal amino acid sequence between this enzyme and the membranous LSE. This contradiction may be solved by the following explanations. The molecular mass of CSE was reported as about 82 kDa with a single polypeptide. The N-terminal amino acid sequence of CSE reported, MAQQTLGTMNFTLRV-, was apparently different from that of LSE, but LSE and CSE are suggested to be encoded by one gene via differential usage of a single peptide-encoding exon at the N terminus (36). The sialic acid 9-*O*-acetylesterase from mammalian sources appeared to be a soluble form even though two enzymes of LSE and CSE, which were localized in different subcelluar fractions, were found (35). Because frozen rat livers were used as the starting material for purifying the thiolesterase, this enzyme could efficiently traverse the damaged membrane from lysosome to cytosol. Therefore, the alacepril thiolesterase is believed to be identical to the sialic acid 9-*O*-acetylesterase of LSE, from the result of the N-terminal amino acid sequencing of the thiolesterase.

LSE was observed to bind to Con A Sepahrose because of the N-linked sugar chains in the enzyme, but CSE did not, as it lacks the sugar chains (34). Therefore, LSE seemed to be a glycoprotein with high mannose-type and complex-type sugar chains. It was suggested that these esterases were involved in regulation of membrane sialic acid *O*-acetylation and acted as a scavenger of recycled free *O*-acetylsialic acid (37). The alacepril thiolesterase was not retained on Con A Sepharose and WGA agarose in our conditions, although the thiolesterase was identified to LSE. Nonetheless, the thiolesterase bound to PBA-30 which couples agarose with *m*-aminophenyl boronic acid. Because a boronate group in PBA-30 was reported to form a complex with hydroxyl groups in a sugar, this suggests the possibility that the thiolesterase contains sugar chains. It is unfortunately unclear whether N-linked sugar chains proposed in LSE are involved in the thiolesterase.

The alacepril thiolesterase activity was shown to be sensitive for serine modifiers, diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride, and heavy metal ions, Hg^{2+} and Cu^{2+}, suggesting that this enzyme is one of the serine hydrolases. The optimal pH of the thiolesterase activity was observed to be around pH 7.8. Although these properties seem to resemble partly those of LSE reported, the effects of the fluoride and the metal ions on the thiolesterase were different from those on LSE (34). The reason why this difference occurs is not clear at present. However, the result that the thiolesterase was inhibited at a concentration range from 1 to 10 μM diisopropyl fluorophosphate is consistent with the report about the sialic acid 9-*O*-acetylesterase (38). The sialic acid 9-*O*-acetylesterase including LSE was also reported to not contain the active site sequence Gly-X-Ser-X-Gly commonly found in serine hydrolases and its putative sequence Gly-Asp-Ser-Arg-Thr in the acetylesterase was proposed.

It seems certain that thiolesterase hydrolyzes both thiolester and carboxylester bonds since alacepril, coenzyme A, and α-naphtyl acetate were apparent substrates for the thiolesterase and the thiolesterase activity with alacepril as a substrate was inhibited competitively by α-naphtyl acetate. However, the thiolesterase did not catalyze the hydrolyzing reaction of the thiolester bond in palmitoyl CoA and the carboxylester bonds in dilazep, flavoxate, proplverine, and oxybutynin. These results suggest that the ester bonds consisted of an acetyl group, a small molecule, which are only hydrolyzed by this enzyme. Schauer *et al.* (35,39) described that the substrate specificity of sialic acid 9-*O*-acetylesterase seemed to be relatively specific for acetic acid esters, such as *N*-acetyl-9-*O*-acetylneuraminic acid and 4-methylumbelliferyl acetate. It was demonstrated especially that this acetylesterase from rat liver had strict substrate specificity for *O*-acetylsialic acids and broad specificity for small synthetic esters, such as *p*-nitrophenyl acetate, α-naphthyl acetate, and 4-methylumbelliferyl acetate (34). The alacepril thiolesterase seems to show similar substrate specificity for synthetic acetylesters to LSE, although 9-*O*-acetyl-*N*-acetyl-neuraminic acid was not examined at present because of its commercial unavailability.

The alacepril thiolesterase was demonstrated to hydrolyze alacepril and spironolactone, but carboxylester-type and amide-type linkage in the drugs tested were not substrates for this enzyme in this report. The apparent K_m value for the endogenous substrate, 9-*O*-acetyl-*N*-acetyl-neuraminic acid, in LSE was estimated as 8.8 mM with a V_{max} of 48 nmol/min/mg (34) and the value for alacepril in the thiolesterase was 22 mM with 28.6 μmol/min/mg. It is likely for this enzyme to

metabolize alacepril at a similar rate to the endogenous substrate in liver, although it may not be able to compare these values directly because of the different assay system. Thus, the alacepril thiolesterase, i.e., LSE, is suggested to take part in the metabolism of certain drugs with acetylester and acetylthiolester bonds. It is very beneficial to clarify what enzymes are involved in metabolism of drugs and prodrugs with thiolester, carboxylester, and amide linkages for studying the pharmacokinetics of these drugs. LSE was distributed in a variety of rat organs and its specific activity was observed to be high in the brain, testis, kidney, and colon mucosa (34). Therefore, thiolesterase is expected to be applied to the development of acetylester- and acetylthiolester-type prodrugs targeting organs in which the enzyme is contained specifically, if the organ distribution of this enzyme in humans is examined fully.

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