Hydrolytic Profile for Ester- or Amide-linkage by Carboxylesterases pI 5.3 and 4.5 from Human Liver

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Received December 2, 1996; accepted April 25, 1997

Carboxylesterases (EC 3.1.1.1) from human liver were purified using Q-Sepharose, Sephadex G-150, isoelectrofocusing and Con A-Sepharose. The calculated molecular mass of the pI 5.3 enzyme was 120 kDa and 61 kDa from the results of Sephadex G-150 gel filtration and SDS-polyacrylamide gel electrophoresis (PAGE), respectively, suggesting that this enzyme is a dimer. On the other hand, carboxylesterase pI 4.5, with a molecular mass of 64 kDa, was a monomer. The activities of both enzymes were inhibited by typical serine enzyme inhibitors. Amino acid sequence analysis of the purified enzymes pI 5.3 and 4.5 showed high homology with rabbit carboxylesterase form 1 and 2, respectively. The results also suggested that carboxylesterase pI 5.3 is identical to the deduced amino acid sequence from cDNA for HU1, and that carboxylesterase pI 4.5 is identical to the deduced amino acid sequence from the cDNA registered as human carboxylesterase (hCE-2) in GenBank. We first purified carboxylesterase pI 4.5 and investigated its hydrolytic activity upon various drugs. The two enzymes differed in substrate specificity. Prodrugs of angiotensin-converting enzyme inhibitors, such as delapril and imidapril, were converted to active metabolites by carboxylesterase pI 5.3, but not by carboxylesterase pI 4.5. The hydrolysis velocity of temocapril by carboxylesterase pI 5.3 was 12-fold faster than by carboxylesterase pI 4.5. In contrast, aspirin, oxybutynin and procaine were hydrolyzed by only carboxylesterase pI 4.5. We also found that an amide-linkage in drugs, except for that in aniracetam, was not a good substrate for the two enzymes. Consequently, carboxylesterases pI 5.3 and 4.5 may be involved in the metabolism of various drugs containing an ester-linkage.

Key words carboxylesterase; amidase; prodrug; biotransformation; drug metabolism

Nonspecific carboxylesterases are widely distributed in animal tissues and appear to be abundant only in liver microsomes. These carboxylesterases contribute to the hydrolysis of various endogenous and xenobiotic compounds and play an important role in drug and lipid metabolism in many mammalian species.[1] Liver microsomal carboxylesterases have been extensively studied and the structures of several isozymes have been elucidated.[2] From the viewpoint of drug metabolism, many prodrugs have been devised to release their active forms upon hydrolysis by carboxylesterases or amidases,[3,4] and some drugs are biotransformed to an inactive form by nonspecific esterases.[5] The specificity of carboxylesterase isozymes is most important for identifying which enzyme hydrolyzes drugs that have ester- and amide-linkages. To reduce the adverse reactions of drugs, information about human drug metabolizing enzymes is necessary, and understanding of the substrate specificity should be put to practical use in clinical trials and the development of new drugs.

In this study, we purified two human liver carboxylesterases and clarified their substrate specificity using therapeutic drugs.

MATERIALS AND METHODS

Materials Q-Sepharose, Sephadex G-150, PBE 94, Con A-Sepharose and Polybuffer 74 were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). α-Naphthyl acetate and *p*-nitrophenyl acetate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bis (*p*-nitrophenyl) phosphate (BNPP), 4-aminoantipyrine and α-methyl-D-mannopyranoside were from Nacalai Tesque (Kyoto, Japan). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Sigma Chemical Co. (St. Louis, U.S.A.). All other reagents were of analytical grade.

Assay of Enzyme Activity Carboxylesterase activity was determined as described using α-naphthyl acetate as a substrate.[6]

Determination of Protein Protein was determined by measuring the absorbance at 280 nm.

Purification of Carboxylesterases pI 5.3 and 4.5 from Human Liver All purification procedures were performed at 4 °C. Liver tissues were obtained during autopsies at the Laboratory of Pathology, Aichi Cancer Center Research Institute. The collected samples were kept frozen at −80 °C. Human liver (64 g) in 260 ml of phosphate buffered saline was homogenized using a Polytron. After ultracentrifugation (105000 × *g*, 1 h), the supernatant was dialyzed against 10 mM phosphate buffer, pH 8.0, containing 0.02% NaN_3, and applied to a column (2.0 × 22.5 cm) of Q-Sepharose equilibrated with the above buffer. The column was washed extensively with the above buffer and the enzyme was eluted with a linear gradient of NaCl (0—0.5 M). The first peak with esterase activity (pI 5.3) was concentrated and applied to a column (3.0 × 71.0 cm) of Sephadex G-150 equilibrated with 25 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.02% NaN_3. The enzyme was further applied to an isoelectrofocusing column (PBE94, 1.0 × 10.6 cm) equilibrated with 25 mM imidazole–HCl, pH 7.4, and eluted

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with a linear gradient of pH (7.4—4.0) by Polybuffer 74–HCl, pH 4.0. Active fractions (pI 5.3) were pooled, concentrated and applied to a Con A-Sepharose column (1.3 × 10.5 cm) equilibrated with 50 mM of a phosphate buffer, pH 7.0, containing 0.2 M NaCl and 0.02% NaN_3. The enzyme was eluted with the above buffer containing 0.2 M α-methyl-D-mannopyranoside. The second peak with esterase activity (pI 4.5) on the Q-Sepharose step was also purified by Con A-Sepharose and Sephadex G-150 column chromatography under the above conditions. After concentration with a membrane filter (YM-10), purified enzymes were stored at 4 °C until use.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) Samples were resolved on 9% polyacrylamide gel according to the method of Laemmli.[7] The protein was stained with Coomassie Brilliant blue R-250. Western blotting proceeded as described by Towbin *et al.*[8] with minor modifications.

Preparation of Antibody A female rabbit was immunized with the purified pI 5.3 enzyme emulsified in Freund's complete adjuvant by injection into each foot pad. The immunization was repeated subcutaneously at two week intervals. Six weeks after the first injection, the rabbit was bled and the antibody was purified by DEAE-cellulose and protein A-Sepharose column chromatographies.

Sequencing of N-terminal Amino Acids Purified carboxylesterases were cleaved by cyanogen bromide. The resulting peptide fragments were separated using reversed-phase C4 HPLC. N-terminal amino acid sequences of the cleaved peptide were analyzed using an automated protein sequencer (model 473A, Applied Biosystems).

Hydrolysis of Drugs by Carboxylesterases The hydrolysis of drugs was determined by HPLC as described.[9] Purified enzyme and drug as a substrate was incubated in 0.1 M Tris–HCl buffer, pH 7.4, at 37 °C, and the enzyme reaction was terminated by adding acetonitrile containing *p*-hydroxybenzoate derivatives as the internal standard. The activity of carboxylesterase for drugs is expressed as μmol/mg protein/min.

RESULTS

Purification and Partial Properties of Carboxylesterases pI 5.3 and 4.5 from Human Liver Carboxylesterases were eluted from the Q-Sepharose column as two peaks. The carboxylesterase activity for α-naphthyl acetate in the first peak, which was eluted at 30 mM NaCl, was about 5-fold higher than that in the second. Both enzymes were further purified and the isoelectric points in the first and second peaks were 5.3 and 4.5, respectively, according to isoelectrofocusing column chromatography. The purification procedures are summarized in Table 1. The purification yields of carboxylesterases pI 5.3 and 4.5 from crude enzyme solutions were 1.2 and 1.0%, respectively.

The purified carboxylesterases migrated as single protein bands by 61 and 64 kDa on SDS-PAGE (Fig. 1). The antibody against carboxylesterase pI 5.3 reacted not only with carboxylesterases pI 5.3 but also with the enzyme pI 4.5. The potential immunoreactivity of this antibody to the carboxylesterase pI 4.5 was weaker than to enzyme pI 5.3.

Table 1. Purification of Human Liver Carboxylesterases

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
a) carboxylesterase pI 5.3				
Crude enzyme	5720.00	16617.0	2.91	100.0
Q-Sepharose	522.20	4768.5	9.13	28.7
Sephadex G-150	26.85	1947.2	72.50	11.7
Isoelectrofocusing	8.89	806.8	90.65	4.9
ConA-Sepharose	2.07	199.4	96.56	1.2
b) carboxylesterase pI 4.5				
Crude enzyme	5720.00	16617.0	2.91	100.0
Q-Sepharose	302.24	893.7	2.96	5.4
ConA-Sepharose	10.35	387.0	37.39	2.3
Sephadex G-150	1.73	175.2	101.21	1.0

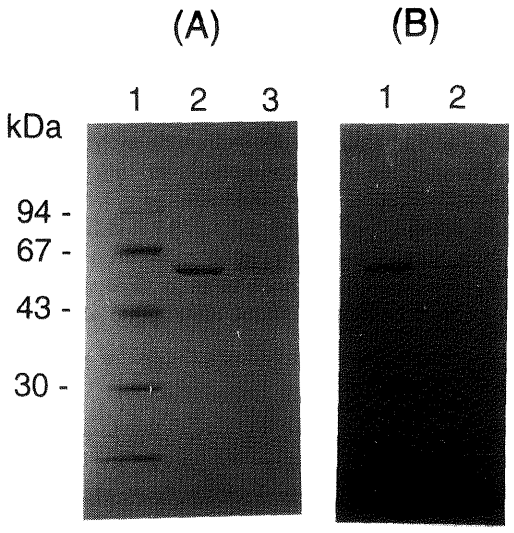

Fig. 1. SDS-PAGE and Western Blots of Purified Carboxylesterases pI 5.3 and 4.5 from Human Liver

(A) Purified carboxylesterase pI 5.3 and 4.5 were resolved by SDS-PAGE (9% polyacrylamide) and proteins were stained with Coomassie Brilliant blue R-250. Lane 1, protein marker; lane 2, purified carboxylesterase pI 5.3; lane 3, purified carboxylesterase pI 4.5. (B) Purified carboxylesterase pI 5.3 and 4.5 were resolved by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane for immunostaining with an antibody against human liver carboxylesterase pI 5.3. Lane 1, purified carboxylesterase pI 5.3; lane 2, purified carboxylesterase pI 4.5.

The specific activity of purified pI 5.3 enzyme for α-naphthyl acetate was almost equal to that of the pI 4.5 enzyme. Both enzymes had an optimum pH of 6—9, were stable at pH 8.0 and 25 °C for 30 min, and were completely inhibited by the serine protease inhibitors, PMSF and BNPP. The pI 5.3 enzyme is a dimer according to its molecular mass of 61 and 120 kDa calculated by SDS-PAGE or Sephadex G-150 gel filtration, respectively, while the pI 4.5 enzyme is a monomer.

Amino Acid Sequencing The results of the amino acid sequencing are shown in Fig. 2. The purified enzymes pI 5.3 and 4.5 have high homology with rabbit carboxylesterase forms 1 and 2, respectively. Carboxylesterases pI 5.3 and 4.5 are identical to the deduced amino acid sequences from cDNAs for HU1[10] and human carboxylesterase (hCE-2).[11]

Substrate Specificity The substrate specificity for purified carboxylesterases pI 5.3 and 4.5 was investigated using commercially available drugs with ester-linkages.

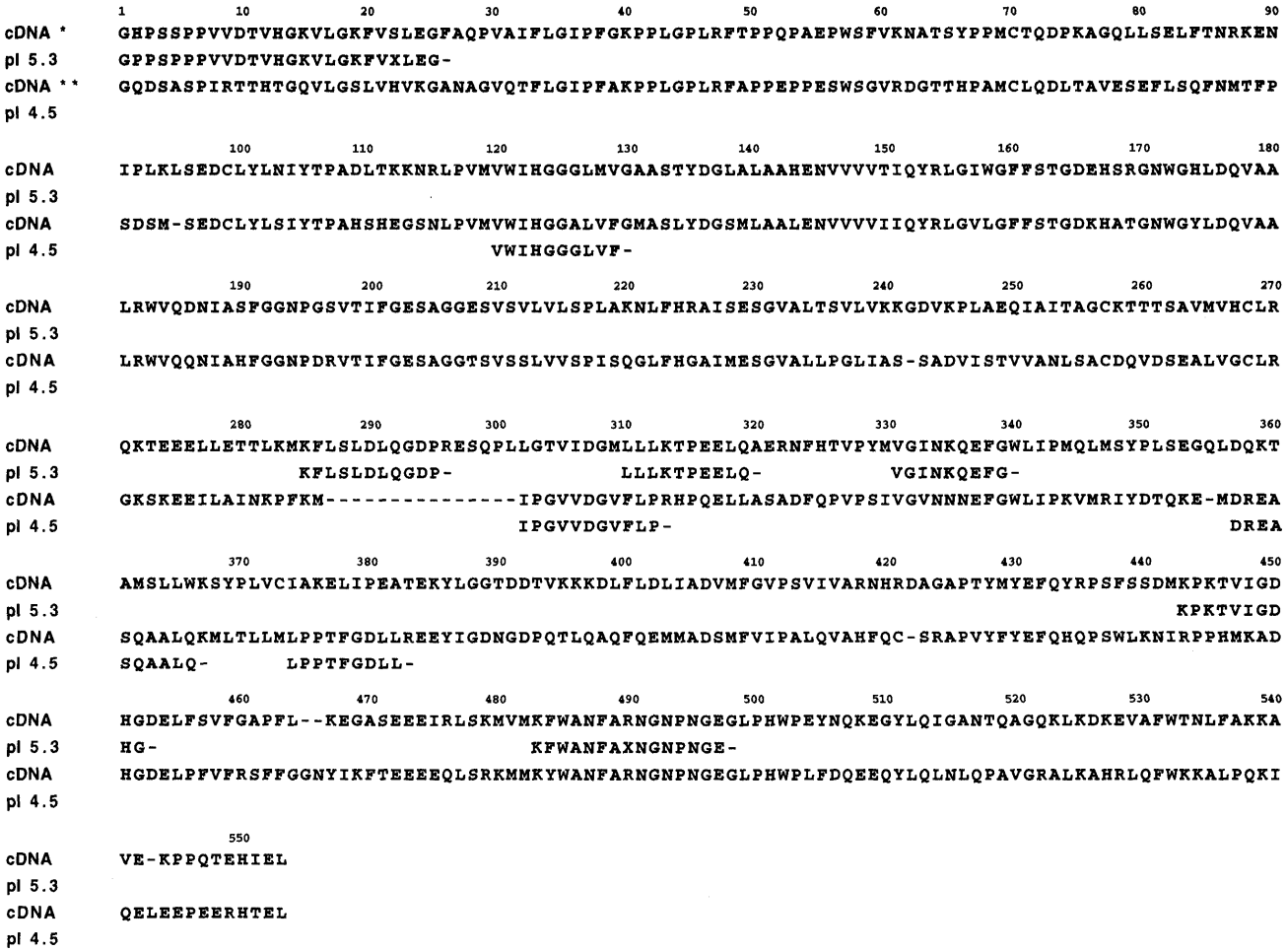

Fig. 2. Comparison of the Amino Acid Sequences of the Peptides Generated by Cyanogen Bromide Cleavage of Carboxylesterases pI 5.3 and 4.5 from Human Liver with the Deduced Amino Acid Sequences from cDNAs for Human Liver Carboxylesterases

a) Data from Kroetz *et al.*[10] *b*) Data from Pindel *et al.*[11]

The results are shown in Table 2. Carboxylesterase pI 5.3 catalyzed the hydrolysis of camostat mesilate and prodrugs for angiotensin converting enzyme (ACE) inhibitors, while carboxylesterase pI 4.5 had weak or no ability to hydrolyze these prodrugs. The ester-linkage of aspirin, procaine and oxybutiyin was hydrolyzed by carboxylesterase pI 4.5 but not by carboxylesterase pI 5.3. Dilazep and irinotecan were also hydrolyzed, mainly by the pI 4.5 enzyme.

Drugs with amide-linkages were also investigated. Only one drug in Table 2, aniracetam, was hydrolyzed by both enzymes. Aniracetam has a unique structure which includes outer and inner amide-linkages of a pyrolidone ring. Aniracetam was hydrolyzed by carboxylesterase pI 5.3 to release anisilic acid and anisamidobutyric acid at a ratio of 3 : 2, respectively, and by pI 4.5 enzyme at a ratio of 1 : 13, respectively.

DISCUSSION

We purified carboxylesterases pI 5.3 and 4.5 from human liver. Microsomal carboxylesterase isozymes in humans and other animals have been extensively studied by Hosokawa *et al.*[12-14] and the properties and structures of three human isozymes (HU1, HU2 and HU3) have been determined.[12]

Their affinity for Con A-Sepharose showed that both enzymes are glycoproteins, like other known human carboxylesterases. Carboxylesterase pI 5.3 is a dimer and the pI 4.5 enzyme is a monomer. Both enzymes were inhibited by PMSF and BNPP but not by EDTA, suggesting that the serine residue in the enzyme is the active center. Carboxylesterase pI 5.3 was classified as form 1 and carboxylesterase pI 4.5 as form 2 because the amino acid sequences of the cleaved peptides were highly homologous to rabbit carboxylesterase forms 1 and 2[16] and to the deduced amino acid sequences from cDNAs for human carboxylesterases.[10,11,17] According to the classification by Shibata *et al.*,[18] carboxylesterase pI 4.5 belongs to group I and carboxylesterase pI 5.3 to group III. These findings thus indicate that carboxylesterase pI 5.3 is different from carboxylesterase pI 4.5 on the basis of substrate specificity and amino acid sequence; however, pI 5.3 has common determinants with pI 4.5 enzyme in terms of the results of the cross-reactivity of the antibody. It is reported that serum carboxylesterase may be partially derived from the liver microsomal enzyme.[19-21] Carboxylesterases pI 5.3 and 4.5 enzymes may be derived from the liver microsome because microsomal carboxylesterases weakly bind with the micosomal membrane. Presently, we cannot explain why the isoelectric points of carboxylesterase pI 5.3 and HU1 are different (pI 5.6).

The specificity of the two human carboxylesterases is

Table 2. Kinetic Parameters of the Hydrolysis of Various Drugs and Endogenous Substances by Purified Carboxylesterases pI 5.3 and 4.5 from Human Liver

Substrates	Carboxylesterase pI 5.3			Carboxylesterase pI 4.5		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Ester-type drugs						
Camostat mesilate	0.707	0.958	1.356	2.700	0.090	0.015
Dilazep	0.154	0.112	0.727	0.089	0.119	1.326
Irinotecan	1.453	0.082	0.056	0.241	0.074	0.307
ONO-5046	1.060	0.132	0.125	2.080	0.476	0.229
Benazepril	0.734	0.886	1.207	0.785	0.330	0.420
Cilazapril	1.295	2.168	1.674	1.349	0.667	0.494
Quinapril	0.134	0.184	1.373	0.122	0.034	0.279
Temocapril	0.786	4.762	6.059	0.325	0.402	1.237
Delapril	1.502	1.569	1.045	—		
Imidapril	0.287	0.195	1.679	—		
Alacepril	—			—		
Aspirin	—			2.270	0.244	0.107
Procaine	—			3.330	0.029	0.009
Oxybutynin	—			1.124	0.358	0.319
Diltiazem	—			—		
Flavoxate	—			—		
Propiverine	—			—		
Amide-type drugs						
Aniracetam						
to anisic acid	0.085	0.009	0.106	0.301	0.020	0.066
to anisamidobutyric acid	0.095	0.007	0.074	0.412	0.349	0.847
Capsaicin	—			—		
Captopril	—			—		
Flutamide	—			—		
Fominoben	—			—		
Indomethacin	—			—		
Lisinopril	—			—		
Nefiracetam	—			—		
Phenacetin	—			—		
Phenobarbital	—			—		
Pranlukast	—			—		
Prazosin	—			—		
Procainamide	—			—		
Sultopride	—			—		
Tiaramide	—			—		
Endogenous substance						
Acetyl coenzyme A	—			—		

Specific activity of carboxylesterases pI 5.3 and 4.5 for α-naphthyl acetate was 96.6 and 101.2 units/mg protein, respectively. K_m: mM, V_{max}: μmol/mg protein/min, —: below 1.0 nmol/mg protein/min.

very different for substrates with an ester-linkage. Prodrugs for ACE inhibitors were hydrolyzed to release the active metabolites by carboxylesterases pI 5.3 and/or pI 4.5, except for alacepril. The hydrolysis of thioesters may be catalyzed by another enzyme(s), since the thioester-linkage of alacepril was not hydrolyzed by either carboxylesterase. On the other hand, the ester-linkages in aspirin, procaine and oxybutynin were hydrolyzed only by carboxylesterase pI 4.5. Camostat mesilate contains two ester-linkages and the ester-linkage between two benzene rings was hydrolyzed only by carboxylesterase pI 4.5, while the other linkage was hydrolyzed by both enzymes. Except for aniracetam, amide-linkages in the therapeutic drugs listed in Table 2 were not hydrolyzed by the two human carboxylesterases. Aniracetam was hydrolyzed to produce anisilic acid and anisamidobutyric acid at the ratio of 3 : 2 and 1 : 13 by carboxylesterases pI 5.3 and 4.5, respectively. The amide-linkage in the pyrolidone ring is preferably hydrolyzed by carboxylesterase pI 4.5. However, the amide-linkage in many drugs may cause them to be generally poor substrates for human liver carboxylesterases. We reported that the rat hydrolases A and B, which are form 1 enzymes, hydrolyze the amide-linkage in capsaicin and pranlukast, respectively.[9] The amide-linkage in indomethacin is also hydrolyzed by human liver homogenate.[22] An enzyme similar to the indomethacin hydrolyzing enzyme, which we found in the pig liver microsomal fraction,[22] might catalyze the reaction because both carboxylesterases described here did not hydrolyze indomethacin. Substrate specificity is important for understanding the metabolism of drugs or xenobiotics.[1,5] However, it cannot be explained by the genetic classification of enzymes.[18,23]

Consequently, ester-type drugs are biotransformed to deacyl derivatives by carboxylesterases, and understanding the substrate specificity of these enzymes provides useful information about the metabolism of drugs and xenobiotics.

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