Pergamon

Biochemical Pharmacology, Vol. 48, No. 9, pp. 1747–1755, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00377-7

PURIFICATION AND CHARACTERIZATION OF A HUMAN LIVER COCAINE CARBOXYLESTERASE THAT CATALYZES THE PRODUCTION OF BENZOYLECGONINE AND THE FORMATION OF COCAETHYLENE FROM ALCOHOL AND COCAINE

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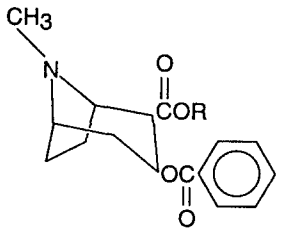
(*Received* 9 *March* 1994; *accepted* 12 *July* 1994)

Abstract—The psychomotor stimulant cocaine is inactivated primarily by hydrolysis to benzoylecgonine, the major urinary metabolite of the drug. A non-specific carboxylesterase was purified from human liver that catalyzes the hydrolysis of the methyl ester group of cocaine to form benzoylecgonine. In the presence of ethanol, the enzyme also catalyzes the transesterification of cocaine producing the pharmacologically active metabolite cocaethylene (benzoylecgonine ethyl ester). The carboxylesterase obeys simple Michaelis–Menten kinetics with K_m values of 116 μM for cocaine and 43 mM for ethanol. The enzymatic activity suggests that it may play an important role in regulating the detoxication of cocaine and in the formation of the active metabolite cocaethylene. Additionally, the enzyme catalyzes the formation of ethyloleate from oleic acid and ethanol. The carboxylesterase was purified from autopsy liver by gel filtration, chromatofocusing, ion-exchange, and hydrophobic interaction chromatography to purity by SDS–PAGE and agarose gel isoelectric focusing. The subunit molecular weight was determined to be 59,000 and the native molecular weight was estimated to be 170,000 from a calibrated gel filtration column, suggesting that the active enzyme is a trimer. The isoelectric point was approximately 5.8. Digestion of carbohydrate residues on the protein with an acetylglucosaminidase plus binding to several lectins indicates that the enzyme is glycosylated. The esterase was cleaved with two proteases, and the amino acid sequences from fourteen peptides were used to search GenBank. Two identical matches were found corresponding to carboxylesterase cDNAs from human liver and lung.

Key words: cocaine; cocaethylene; esterase; ethanol; human; liver

Abuse of the psychomotor stimulant cocaine is a major public health problem in the United States, and increasing numbers of individuals are seeking treatment for cocaine-related problems [1]. Surveys show that about 77% of the time cocaine abusers use a combination of alcohol and cocaine [2]. The reason for this high rate of coabuse is unknown, but the stimulant effects of cocaine may be potentiated by alcohol [3]. Moreover, the toxic and pathologic consequences of cocaine abuse may be exacerbated by concurrent alcohol use [4], which is supported by the increased hepatotoxicity of cocaine with concurrent alcohol use [5, 6].

The short half-life of cocaine in blood is due to its hydrolysis to benzoylecgonine, ecgonine methyl ester, and ecgonine, the major metabolites that eventually appear in urine [7]. Studies examining the effects of ethanol on the distribution of cocaine metabolites in rats indicate that ethanol increases the concentration of cocaine in liver and decreases the concentration of benzoylecgonine in liver and serum [8]. While benzoylecgonine is inactive as a psychomotor stimulant, it may contribute to the vasoconstricting effects of cocaine [9]. In tissues of rats given ethanol and cocaine, two "ethylated" metabolites of cocaine were identified by GC/MS as cocaethylene (benzoylecgonine ethyl ester, Fig. 1) and norcocaethylene (norbenzoylecgonine ethyl ester) [8]. Moreover, cocaethylene was detected in brain, liver, and plasma from individuals who had taken cocaine and alcohol concurrently [10, 11].



Cocaine	R=CH_3
Cocaethylene	R=CH_2CH_3
Benzoylecgonine	R=H

Fig. 1. Structures of cocaine, cocaethylene, and benzoylecgonine.

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Ethanol ingestion is reported to potentiate cocaine-induced myocardial dysfunction [12] and to increase the risk of cocaine-related morbidity and mortality [4]. This may be mediated, in part, by cocaethylene formation since its LD_{50} is less than that of cocaine in mice [13, 14].

Even though cocaine can be hydrolyzed spontaneously to benzoylecgonine at neutral pH, this may not fully account for its rapid elimination *in vivo*. Recent studies in humans and mice suggest that hydrolysis of cocaine to benzoylecgonine is catalyzed by a liver carboxylesterase [15–17]. Serum cholinesterase and hepatic carboxylesterase catalyze the hydrolysis of cocaine to ecgonine methyl ester [15, 18, 19]. In this paper, we report the first characterization of a purified human liver cocaine : benzoylecgonine carboxylesterase that catalyzes the formation of cocaethylene from ethanol and cocaine, as well as the formation of ethyloleate from ethanol and oleic acid. This enzyme may play a key role in regulating tissue levels of cocaine and its metabolites that give rise to the various stimulant, vasoconstricting, and toxic effects of cocaine abuse or the combined use of cocaine and alcohol.

MATERIALS AND METHODS

Materials. Reagents were obtained from Sigma (St. Louis, MO), Mallinckrodt (Paris, KY), J. T. Baker Inc. (Phillipsburg, NJ), and other commercial sources. Ultra pure grades of $(NH_4)_2SO_4$ and urea were purchased from Schwarz/Mann Biotech (Cleveland, OH). The enzymes trypsin and *Staphylococcus aureus* V8 protease were obtained from Boerhinger Mannheim (Indianapolis, IN). Ultra pure DTT* was obtained from the United States Biochemical Corp. (Cleveland, OH) and 4-CN was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, NJ). PVDF membrane (Immobilon, 0.45 μm pore size) was from Millipore (Bedford, MA). SDS and the protein standards for SDS–PAGE and IEF were from Bio-Rad (Hercules, CA). Polybuffer 74 and chromatography resins used in the enzyme purification were purchased from Pharmacia (Uppsala, Sweden), except that the DEAE-cellulose was from Whatman Biosystems (Maidstone, U.K.). All solvents were prepared with H_2O treated with a Milli-Q filtration system (Millipore, Bedford, MA). Enzyme pools were concentrated with a Centricon concentrator (YM30 membrane) from Amicon (Beverly, MA).

Carboxylesterase activity. Non-specific acetylesterase activity was determined by incubating 10 μL enzyme (2 μg of the purified carboxylesterase or about 550 μg of the homogenate supernatant) with 20 μL of 4-methylumbelliferyl acetate in dimethyl sulfoxide (5 mM) in 90 mM KH_2PO_4, 40 mM KCl, pH 7.3 (1 mL total volume) at 37°. Formation of the product 4-methylumbelliferone was monitored with a spectrophotometer at 350 nm. Rates of hydrolysis in units of micromoles per minute were calculated by linear regression of absorbance versus time using the extinction coefficient of $12.2\ cm^{-1}\ mM^{-1}$ for 4-methylumbelliferone [15]. Specific activity is expressed as micromoles product formed per minute per milligram protein. Protein concentrations were determined by Bio-Rad protein assay with bovine serum albumin as a standard.

Cocaine : benzoylecgonine esterase and cocaine : cocaethylene ethyl ester transferase activities were determined by incubating 100 μL enzyme (adjusted to approximately 6 U/mL based on the 4-methylumbelliferyl acetate assay) with 3.3 mM cocaine–HCl (dissolved in H_2O) in the presence or absence of 100 mM ethanol at 37°. The assay contained 50 mM NaH_2PO_4, 1 mM benzamidine, 1 mM EDTA, and 1 mM DTT at pH 6.5 in a total volume of 0.5 mL. After 1 hr, the reaction was stopped with 0.5 mL of 5% trichloroacetic acid, and 1 μg of tropacocaine was added as the internal standard. The protein precipitate was centrifuged. Cocaine, benzoylecgonine, and cocaethylene were extracted from the supernatant and analyzed by HPLC as described [15] with the following modifications: the mobile phase contained 18% acetonitrile and 75 mM decane sulfonic acid, the flow rate was 1.2 mL/min, and the reversed-phase column was maintained at 40°.

Fatty acid ethyl ester synthase activity. The enzyme-catalyzed esterification of oleic acid to ethyloleate was determined using 120 μg purified enzyme and previously reported assay conditions [20]. The reaction was stopped by the addition of 5 mL hexane. Following centrifugation at 12,000 *g* for 5 min, the hexane extract was removed, and an additional 5 mL hexane was added to the aqueous layer. The two extracts were pooled and evaporated to dryness under a stream of air. The residue was reconstituted with 100 μL hexane, and 1 μL was injected onto a Hewlett–Packard model 5890 series II gas chromatograph fitted with a Hewlett–Packard special performance cross-linked 5% phenyl methyl silicone capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) and a model 5971 series mass selective detector. The detector was operated in full spectrum mode. The injection port temperature (initially set at 100°) was programmed through the following temperature ramps: ramp 1: rate 15°/min, final temperature of 175° maintained for 2 min; ramp 2: rate 10°/min, final temperature of 190° maintained for 5 min; ramp 3: rate 10°/min, final temperature 270°. Both the interface and detector temperatures were set at 280°.

Human liver cocaine carboxylesterase purification. All buffers were purged with helium and contained 1 mM benzamidine, 1 mM EDTA, and 1 mM DTT. Sixty grams of frozen human liver that was obtained at autopsy was homogenized in 60 mL of 50 mM HEPES at pH 6.8. The homogenate supernatant, prepared by centrifugation at 125,000 *g* for 35 min, was filtered through two layers of Miracloth (Calbiochem Corp., La Jolla, CA). The filtrate was applied to a column of DEAE-cellulose anion exchange resin (150 g) equilibrated with 50 mM HEPES, pH 6.8. Bound protein was eluted with 250 mM KH_2PO_4, pH 6.8, and fractions were assayed for 4-methylumbelliferyl acetate esterase activity. The buffer of the active fractions was exchanged for

* Abbreviations: DTT, dithiothreitol; IEF, isoelectric focusing; PVDF, polyvinylidene difluoride; DAB, diaminobenzidine; and 4-CN, 4-chloro-1-naphthol.

75 mM Tris–Cl, pH 7.6, using a Minitan ultrafiltration system (Millipore, 30 kDa membrane), and the sample was loaded onto a Q Sepharose Fast Flow column (5.5 × 8 cm). Enzyme was eluted with a 500 mL linear gradient of 75 mM Tris–Cl, pH 7.6, to 250 mM Na_2HPO_4, pH 7.5. The first peak of activity contained the cocaine: benzoylecgonine esterase and the second activity peak contained a cocaine: ecgonine methyl ester hydrolase (see Fig. 2). The protein in the first peak was dialyzed against 50 mM NaH_2PO_4, pH 7.5, concentrated and gel filtered on a Superose 6 column (2.5 × 84 cm). After concentrating the pool of active fractions, the buffer was exchanged for 25 mM histidine, pH 6.2. The sample was applied to a Polybuffer Exchanger chromatofocusing column (1.5 × 23 cm) equilibrated with the pH 6.2 buffer. Enzyme was eluted with Polybuffer 74 diluted 1:10 and adjusted to pH 5.0. The buffer of the activity pool was exchanged for 0.8 M $(NH_4)_2SO_4$, 50 mM NaH_2PO_4 at pH 7.0, and the concentrated enzyme was loaded onto a Phenyl Superose column (HR 5/5, Pharmacia). The bound carboxylesterase was eluted by decreasing the $(NH_4)_2SO_4$ concentration by dilution with a 40 mL linear gradient from 15 to 90% 50 mM NaH_2PO_4, pH 7.0. Active fractions were pooled and concentrated.

SDS–PAGE and IEF. Purity of the esterase was assessed at each step of the purification by SDS–PAGE [21] using the Mighty Small Vertical Slab gel unit (Hoefer Scientific Instruments, San Francisco, CA). Samples containing 1.2 μg or 0.12 μg protein were run on a 10% polyacrylamide gel containing 0.1% SDS. Gels were stained with alkaline silver stain [22].

IEF was performed with Isogel agarose IEF plates, pH 3–10 (FMC BioProducts, Rockland, ME), and an LKB Bromma 2117 multiphor unit (Pharmacia) maintained at 10°. Samples contained 30 μg of protein or 4 mU of acetylesterase activity. The gel was stained for protein with Coomassie Blue according to the instructions provided by FMC Bioproducts. The activity stain contained 0.01% 4-methylumbelliferyl acetate in 0.1 M NaH_2PO_4, pH 7.0, and the fluorescent product 4-methylumbelliferone was visualized by UV light.

Endoglycosidase H treatment and lectin binding. Purified protein (5 μg) was incubated with 2 mU endoglycosidase H for 21 hr at 37° as described [23]. Samples were electrophoresed on a 7.5% polyacrylamide gel with 0.1% SDS, and the gel was stained with alkaline silver stain [22]. To identify carbohydrate groups attached to the esterase, samples of purified protein were run on a 10% polyacrylamide gel with 0.1% SDS. Protein was transferred overnight onto a PVDF membrane using Idea Scientific electrophoretic transfer apparatus (Corvallis, OR) with a 12 V power supply. Transfer buffer contained 25 mM Tris, 190 mM glycine, and 15% methanol. The membrane was cut into strips, each containing 1 lane of purified protein. The membrane strips were treated according to a protocol from Vector Laboratories (Burlingame, CA) using Biotinylated Lectin Kit 1. An avidin-peroxidase conjugate solution (5 μg/mL) was used in a 30-min incubation following the first wash, and the peroxidase color-producing reaction was stopped by rinsing with H_2O. The membrane strips were allowed to air dry.

Kinetics. The K_m values for cocaine and ethanol were obtained from duplicate assays containing about 160 μg enzyme incubated with 5 or 6 different concentrations of substrate for 2 hr at 37° in the assay buffer (total volume = 0.5 mL). When determining the K_m of cocaine, the ethanol concentration was set at 200 mM (about 5 times K_m) and the cocaine concentration ranged from 33 to 400 μM. When evaluating the K_m of ethanol, the cocaine concentration was set at 500 μM (about 5 times K_m) and the ethanol concentration ranged from 10 to 300 mM. The control and standard mixtures contained 0.5 mL of 5% trichloroacetic acid, water, and enzyme in assay buffer. Following incubation, trichloroacetic acid was added to the assays to stop the transesterification reaction, cocaethylene was added to the standards, cocaine was added to the controls, and 1 μg of propylbenzoylecgonine was added to all reaction mixtures as the internal standard (total volume = 1.1 mL). Following thorough mixing, precipitated protein was pelleted by centrifugation. Cocaine and cocaethylene were extracted from the supernatant and analyzed by HPLC [15]. The cocaethylene concentration in sample reaction mixtures was calculated from standard curves where the coefficient of variation for the slope was <2%. The linearity of initial enzyme reaction rates was tested with duplicate sample mixtures, containing 200 mM ethanol and 115 μM cocaine (the approximate K_m of cocaine), that were incubated for 15–120 min.

Peptide mapping and amino acid sequencing. For tryptic peptide mapping, purified protein (25 μg) was treated for 4 hr with 1 M urea in 200 μL of 0.1 M Tris–Cl, pH 8.5, at −20°. After thawing the reaction mixture, 1.25 μg of trypsin was added and the digestion proceeded overnight at 37° [24]. The reaction was stopped with 20 μL of 10% trifluoroacetic acid. For Glu-specific cleavage, purified protein (25 μg) was treated overnight with 0.17 M urea in 200 μL of 25 mM $(NH_4)_2CO_3$, pH 7.8, at −20°. After thawing the reaction mixture, 1.25 μg of *S. aureus* V8 protease was added and the digestion proceeded for 5 hr at 25° [24]. The reaction was stopped with trifluoroacetic acid. The peptides were separated by reversed-phase chromatography as described [25], and fourteen of the peptides were sequenced by Edman degradation on a Porton automated amino acid sequencer from Beckman Instruments (Palo Alto, CA).

RESULTS

A carboxylesterase that hydrolyzes cocaine to form benzoylecgonine and methanol, and catalyzes the ethyl transesterification of cocaine with ethanol to form cocaethylene (Fig. 1) and methanol was isolated from human liver by a five-step purification procedure. Anion exchange chromatography, gel filtration, chromatofocusing, and hydrophobic interaction chromatography were used. Non-specific acetylesterase activity (4-methylumbelliferone forming), cocaine esterase activity (benzoylecgonine

Table 1. Purification of cocaine esterase: measurement of products formed in three different assays

Steps	4-Methylumbelliferone		Benzoylecgonine		Cocaethylene	
	Total units	Specific activity (U/mg)	Total units (10^{-2} U)	Specific activity (10^{-4} U/mg)	Total units (10^{-2} U)	Specific activity (10^{-4} U/mg)
Liver extract	590	0.17	32	0.90	19.6	0.56
DEAE-cellulose	420	0.29	27	1.9	13.4	0.95
Q Sepharose	110	0.36	12.7	4.2	11.0	3.6
Superose 6	81	0.45	13.5	7.5	11.6	6.5
Polybuffer exchanger	31	1.22	7.3	28	10.5	40
Phenyl Superose	19	6.8	3.8	126	2.7	90

Assays for the production of 4-methylumbelliferone, benzoylecgonine, and cocaethylene were performed as described in Materials and Methods.

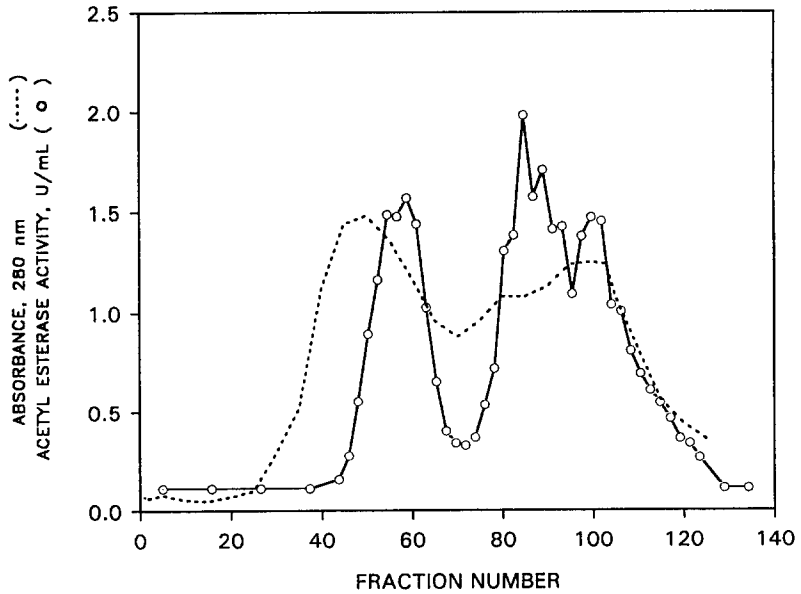

Fig. 2. Ion exchange chromatography of carboxylesterase. The pool of esterase activity from the DEAE-cellulose step was applied to a Q Sepharose Fast Flow column as described in Materials and Methods. Absorbance of fractions at 280 nm and levels of 4-methylumbelliferyl acetate activity are indicated by the dashed line and solid line with open symbols, respectively. Cocaine: benzoylecgonine hydrolase eluted in the first peak of activity (fractions 45–65) and cocaine: ecgonine methyl ester hydrolase eluted in the second activity peak (fractions 75–115).

forming), and ethyl ester transferase activity (cocaethylene forming) were measured at each step of the isolation procedure (Table 1). Two separate acetylesterase activities were separated by anion exchange chromatography on Q Sepharose (Fig. 2), but only the enzyme in the first peak (fractions 45–65) catalyzed the formation of benzoylecgonine [15]. The enzyme in the second peak (fractions 75–115) catalyzed the formation of ecgonine methyl ester* [15]. Increases in specific activity were observed using either cocaine or 4-methylumbelliferyl acetate as the substrate at each step following separation of esterase activities on Q Sepharose (Table 1). The yield of cocaine esterase activity (benzoylecgonine forming) was 12%, and a 140-fold purification was achieved.

Purity of the enzyme was examined by SDS–PAGE and IEF. A single protein band was observed on SDS–PAGE (Fig. 3a). The subunit molecular weight of 59 kDa for the cocaine: benzoylecgonine esterase was estimated from the migration distance using a standard curve generated with high range SDS–PAGE markers. The following equation was used for the calculation:

$$\text{Log mol. wt} = (-1.52 \times 10^{-1} \pm 1.3 \times 10^{-2})(\text{cm of migration}) + (5.4 \pm 4 \times 10^{-2}).$$

* Abraham T, unpublished results.

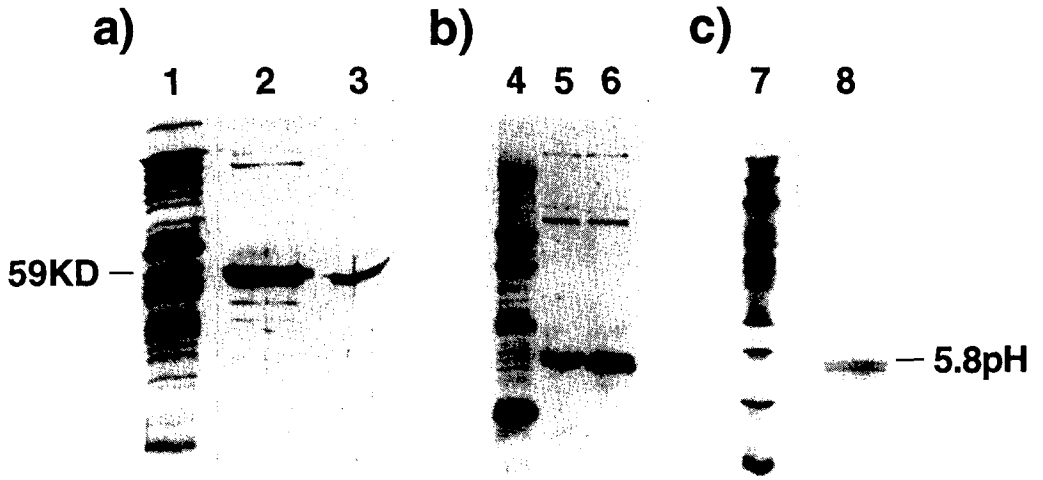

Fig. 3. SDS–PAGE and IEF analysis of carboxylesterase. (a) Samples of the esterase pool were separated by SDS–PAGE (10% polyacrylamide gel) and detected by silver stain as described in Materials and Methods. Aliquots are shown from the initial DEAE-cellulose step (lane 1, 1.2 μg) and the final Phenyl Superose step (lanes 2, 1.2 μg and 3, 0.12 μg). The migration of the predominant band in lane 2 corresponded to a molecular weight of approximately 59,000 based on a standard curve generated by high range SDS–PAGE markers. (b) A sample of purified esterase was digested with endoglycosidase H and 1 μg was analyzed by SDS–PAGE (7.5% polyacrylamide gel) as described in Materials and Methods. Lane 4 contains high range mol. wt standards consisting of myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). Lane 5 contains an untreated esterase sample; a predominant protein band at 59 kDa is apparent. Traces (<5%) of high molecular weight contaminants that were not removed during the purification are also visible. The decrease in apparent subunit molecular weight to about 57 kDa for enzyme sample treated with endoglycosidase H is shown in lane 6. (c) A 30-μg sample of purified esterase and prestained IEF standards were focused until the standards appeared as discrete bands in the gel. The agarose gel was stained for protein with Coomassie Blue as described in Materials and Methods. Lane 7 contains IEF standards and the first 4 from the bottom of the gel are: phycocyanin (pI 4.65), β-lactoglobulin B (pI 5.10), bovine carbonic anhydrase (pI 6.00), and human carbonic anhydrase (pI 6.50). Lane 8 shows the multiple bands produced by the enzyme sample.

A Superose 6 gel filtration column was calibrated with four protein markers to determine the native polymeric molecular weight of the esterase. The following equation was generated from the standard curve:

$$\text{Log mol. wt} = (-1.49 \times 10^{-2} \pm 3.8 \times 10^{-3})(\text{elution volume}) + (9.6 \pm 1.2).$$

A molecular weight of 170,000 was calculated from the esterase elution volume, suggesting that the active enzyme is a trimer. The subunit size and polymeric structure are similar to those reported for non-specific carboxylesterases from rat [23, 26] and pig [27]. An isoelectric point of approximately 5.8 that was determined for the cocaine : benzoylecgonine esterase by agarose gel IEF agrees with previously reported pI values for liver carboxylesterases [28]. The protein stain on the IEF gel from the pure esterase sample showed a series of closely spaced bands (Fig. 3c) whose positioning corresponded to that of a broad esterase activity band (data not shown).

The cocaine : benzoylecgonine esterase was treated with an acetylglucosaminidase called endoglycosidase H to test for the presence of carbohydrate groups. Endoglycosidase H hydrolyzes Asn-linked high mannose oligosaccharides of various sizes. From SDS–PAGE (Fig. 3b), a lower molecular weight band of 57 kDa appeared, suggesting that the enzyme is a glycoprotein. There was no apparent shift in the isoelectric point following the endoglycosidase H treatment nor decrease in multiplicity of protein bands. To evaluate the type of carbohydrate bound to the esterase, seven lectins that show specificities toward different carbohydrate groups were screened for binding the esterase. After blotting the SDS–PAGE gel to a PVDF membrane and incubating the membrane with different lectins, the lectin–enzyme complex was visualized with an avidin-peroxidase conjugate and the substrates DAB and 4-CN. Concanavalin A displayed the most intense band, while *Dolichos biflorus* agglutinin, peanut agglutinin, and *Ulex europaeus* agglutinin I gave weaker bands (data not shown).

For steady-state kinetic analysis, the rate of cocaethylene formation was measured at different substrate concentrations and data were fit to the Michaelis–Menten equation. The apparent K_m for cocaine was 116 ± 17 μM, and the apparent K_m for ethanol was 43 ± 2 mM. Initial reaction rates for cocaethylene formation remained linear for 2 hr of incubation (data not shown).

The purified cocaine : benzoylecgonine esterase also exhibited fatty acid ethyl ester synthase activity

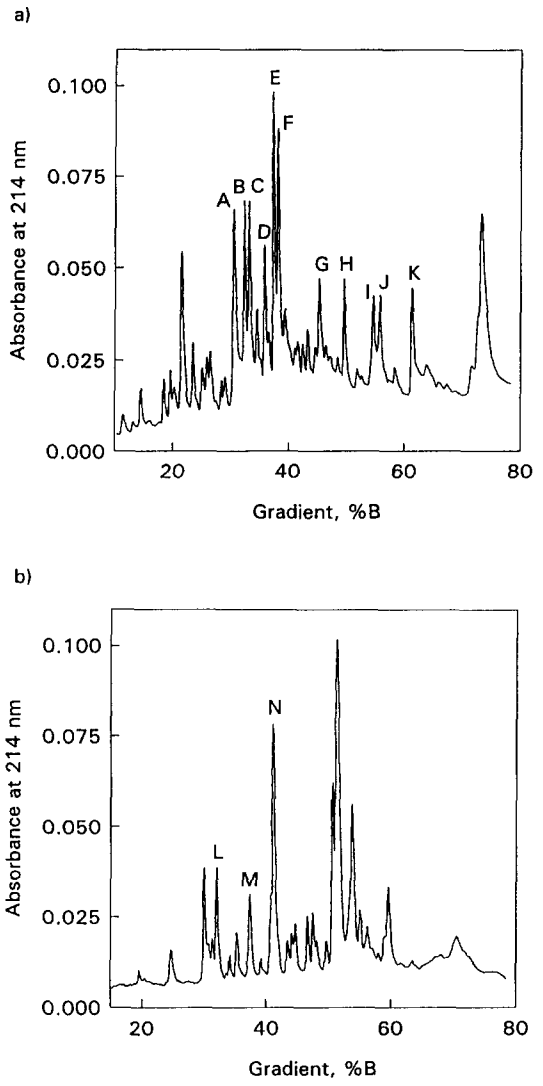

Fig. 4. HPLC elution profiles of peptides from purified carboxylesterase. Samples of purified enzyme were cleaved with (a) trypsin or (b) Glu-specific V8 protease, and peptides were separated by reversed-phase HPLC using a gradient of increasing acetonitrile in 0.1% trifluoroacetic acid, as described in Materials and Methods. Fractions indicated by the alphabetical labels were collected manually and stored at −20° until sequencing. The corresponding amino acid sequences are presented in Table 2.

Table 2. Peptide sequences of the carboxylesterase

Peptides	Amino acid sequence	Esterase sequence number*
Tryptic		
A	NGNPNGEXL	489–497
B	QKTEEELLETT	271–281
	FXANFA	482–487
C	GNWGHLDQVAAL	170–181
	QKTEEEL	271–277
D	TAMSLL	360–365
E	DAGAPTYMYXFQYRPSFS	422–439
	KGDVKPLAEQIAITAG	241–256
F	FTPPQPAEPWS	48–58
	DAGAPTYMYXFQY	422–434
G	LKDKXXAF	522–529
H	TVIGDXGDELF	446–456
I	ESQPLL	297–302
J	NFXTVPYMVGINK	323–335
K	FVSLEGFAQPVAIFLG	20–35
V8 protease		
L	FQYRPSFSSDMKP	432–444
M	GHPXSPPXVD	1–10
N	VAFWTNLFAKKAVE	527–540

The major peptide sequence is listed first and the minor sequence, if present, is listed second. X indicates cycles in which an amino acid could not be positively identified. Sequences where less than 5 cycles could be assigned are not reported.

* Sequence numbers are based on the deduced sequence of a human lung carboxylesterase cDNA [31].

catalyzing the ethyl esterification of oleic acid to ethyloleate. The formation of ethyloleate was confirmed by GC/MS. The total ion chromatogram obtained from the sample containing esterase, oleic acid, and ethanol revealed a prominent well-resolved peak with a retention time and mass spectrum virtually identical to those obtained for a pure standard of ethyloleate (data not shown). The ethyl ester peak was not detected in control samples lacking either enzyme or ethanol.

To determine the amino acid sequence of the esterase, the purified protein was digested with trypsin that cleaves at Lys and Arg residues and *S. aureus* V8 protease that cleaves at Glu residues at pH 7.8. Peptides were separated by reversed-phase HPLC, and fourteen peptides that eluted in well-resolved peaks (Fig. 4) were sequenced by automated Edman degradation (Table 2). A search was performed in GenBank, and the fourteen peptide sequences obtained for the enzyme perfectly matched the deduced sequence for carboxylesterase cDNAs from human liver [29, 30] and lung [31]. Fifty-eight residues that were positively identified from homogeneous peptides were consistent with the published carboxylesterase sequence. In some HPLC peaks, major and minor peptide sequences were detected due to similar retention times of the peptides. In these peaks, an additional 82 amino acids were found to be consistent with the published carboxylesterase cDNA sequence. Overall, the sequences determined for the enzyme represent 27% of the total residues deduced from the human lung carboxylesterase cDNA [31].

DISCUSSION

Carboxylesterases catalyze the hydrolysis of ester, thioester, and amide bonds in the catabolism of a variety of endogenous and exogenous substrates. Broad substrate specificity carboxylesterases appear to exist primarily to detoxify xenobiotics, although

specific physiological roles also have been proposed [32]. It was suggested that these enzymes serve to prepare lipophilic compounds for elimination as more hydrophilic carboxylic acids or alcohols [32]. For example, B-type esterases prefer more lipophilic esters as compared with charged or polar substrates [33]. The carboxylesterases are widely distributed in animal species, and the relatively high content in different tissues may compensate for their relatively low catalytic efficiency for substrates [32]. Multiple isozymes of non-specific carboxylesterases have been isolated from the livers of rat, pig, and rabbit and differentiated according to isoelectric point, substrate specificity, and inhibitor specificity. Most of these enzymes are microsomal glycoproteins [28, 34–36]. It was proposed that amino acid substitutions in esterase substrate binding sites enable the family of isoenzymes to catalyze the wide range of hydrolytic reactions necessary for survival of the organism [33].

While many human non-specific carboxylesterases have been described, this is the first report of the purification and structural characterization of a human liver carboxylesterase that catalyzes the hydrolysis of cocaine to benzoylecgonine and the ethanol-dependent transesterification of cocaine to cocaethylene. The enzyme is one of two distinct cocaine carboxylesterases identified in human liver [15]. The second carboxylesterase catalyzes the hydrolysis of cocaine to ecgonine methyl ester which, like benzoylecgonine, is devoid of cocaine-like psychomotor activity [37, 38]. Both enzymes catalyze the hydrolysis of 4-methylumbelliferyl acetate, a reaction used to follow esterase activity during the purification with a spectrophotometric assay (Table 1). Following separation of the two cocaine carboxylesterases by ion-exchange chromatography (Q Sepharose, Fig. 2), the cocaine : benzoylecgonine esterase was purified to homogeneity by column chromatography. The protein exhibited greater than 95% homogeneity judging by SDS–PAGE analysis (Fig. 3a). The specific activities calculated at various stages of enzyme purity for the cocaine : benzoylecgonine, cocaine : cocaethylene, and 4-methylumbelliferyl acetate assays increased with enzyme purification (Table 1). Based on the results of the cocaethylene assay, the protein was purified from the liver homogenate-supernatant about 160-fold with a 14% recovery. Several factors influence the enzymatic assays and the calculation of yield, specific activities, and fold-purification reported in Table 1. The activity in the first steps of purification may be underestimated, since the cocaine concentration in the assay decreases during initial rate measurements due to the presence of competing hydrolases that produce ecgonine methyl ester from cocaine. In the methylumbelliferyl acetate assay, substrate may be hydrolyzed by non-specific carboxylesterases other than the hydrolase that produces benzoylecgonine [39]. With respect to formation of cocaethylene, both the hydrolytic and transesterification reactions occur simultaneously. In the presence of a high concentration of ethanol (100 mM), transesterification occurs at a faster rate than hydrolysis, since approximately 3.5 times more cocaethylene than benzoylecgonine product is formed from this reaction during the 1-hr incubation period.

The subunit molecular weight of human liver carboxylesterase was estimated by SDS–PAGE analysis to be 59 kDa (Fig. 3a). The native molecular weight was approximately 170,000 from a calibrated gel filtration column, consistent with a value obtained by PAGE that was reported previously [28]. The combined results suggest that the active enzyme is a trimer. From agarose gel IEF, a pI value of 5.8 was determined. The protein stain on the IEF gel showed several closely spaced bands (Fig. 3c), which may be due to isozymic forms of the carboxylesterase or some type of non-specific interaction with ampholytes in the IEF gel as reported previously [28].

To compare the structure of this carboxylesterase to other non-specific carboxylesterases that have been sequenced or cloned, samples of purified enzyme were digested with trypsin and a Glu-specific endoproteinase from *S. aureus* and peptide fragments were separated (Fig. 4) and sequenced (Table 2). Two identical matches to the sequences were found in GenBank, which corresponded to the deduced amino acid sequence of a human lung carboxylesterase cDNA [31] and the deduced sequence of a human liver carboxylesterase cDNA fragment [29]. The peptide sequences also matched the partially deduced sequence of a human liver carboxylesterase [30]. Overall, the amino acid sequences reported in Table 2 represent approximately 27% of the total residues deduced from the lung and liver enzyme cDNAs. The human liver cocaine carboxylesterase [29] displays the following sequence identities: 69% to rat liver carboxylesterase called E1 [40], 76% to rat liver carboxylesterase called ES-10 [41], 77% to rabbit liver carboxylesterase called form 1 [42], and 48% to rabbit liver carboxylesterase called form 2 [36]. It was reported that isoenzymes of pig liver esterase [27] and the two forms of rat liver carboxylesterase [23, 26] are trimeric in the native state and the subunit molecular weight for each enzyme ranges from 58,000 to 61,500, like the human liver enzyme reported here.

The major rat liver esterases are glycoproteins [34], and both forms of rabbit liver esterase are glycosylated [35, 36]. Within the amino acid sequence of the human lung carboxylesterase [31], one potential Asn-Ala-Thr glycosylation site exists. Experiments showing a decrease in subunit molecular weight of the enzyme after endoglycosidase H digestion and binding of lectins to purified human liver esterase indicate that it is a glycoprotein. Concanavalin A exhibited the strongest interaction of the seven lectins screened, suggesting that the esterase contains an α-linked mannose residue.

Non-specific carboxylesterases are abundant in mammalian liver microsomes. The human liver esterase purified in this study is undoubtedly a microsomal enzyme since the cDNA reported by Munger *et al.* [31], which encodes a protein identical to the sequence reported here, has an amino terminal leader sequence. Additionally, the four carboxy-terminal residues have the sequence His-Ile-Glu-Leu, often associated with proteins retrieved from secretory traffic that remain in the microsomal

fraction [43]. It was reported recently that the enzymatic transesterification of cocaine to cocaethylene occurs exclusively in the microsomal fraction of mouse liver [16].

Carboxylesterases have been suggested to play important roles in the metabolism of many compounds, including steroid [44] and lipid esters [45]. It was demonstrated that a carboxylesterase contributes to the non-oxidative metabolism of alcohol in various organs by catalyzing the esterification of oleic acid and ethanol to form ethyloleate [20]. This esterase was purified from rat adipose tissue by monitoring both carboxylesterase activity and fatty acid ethyl ester synthase activity. The amino acid sequence of the first 27 N-terminal residues is identical to that of rat liver carboxylesterase form ES-10 [41], which shares 76% homology with the purified human liver esterase reported here. Our human hepatic enzyme exhibits fatty acid ethyl ester synthase activity producing ethyloleate from oleic acid and ethanol. However, we could not detect the direct esterification of benzoylecgonine with ethanol to form cocaethylene. Thus, it appears that cocaethylene is only formed by transesterification.

The human liver carboxylesterase characterized in this paper may play an important role in detoxication and metabolism of cocaine. The K_m value of cocaine (116 μM) is much higher than concentrations of cocaine, which are reported to be about 3 μM in blood immediately after a 100-mg intravenous dose [46]. However, the carboxylesterase K_m is similar in magnitude to the value of 50 μM observed for the hydrolysis of cocaine to ecgonine methyl ester by serum cholinesterase [18]. Clearly, both enzymes would exhibit first-order kinetics with respect to cocaine at physiological concentrations. The product of the reaction, benzoylecgonine, is devoid of psychomotor stimulant activity [38] making the purified enzyme a potential reagent to increase cocaine hydrolysis and shorten its duration of action in the body, as suggested in a study of a cocaine catalytic antibody [47].

The formation of cocaethylene by the carboxylesterase in the presence of alcohol and cocaine may contribute to increased toxicity of cocaine when taken with alcohol [10]. The K_m for ethanol of 43 mM is equivalent to a blood level of about 180 mg/100 mL. Blood alcohol levels were reported to range from 30 to 460 mg/100 mL in postmortem blood from individuals whose deaths were cocaine related [10]. Hence, the carboxylesterase catalyzed transesterification of cocaine with ethanol to form cocaethylene could occur at ethanol concentrations observed in individuals abusing both alcohol and cocaine.

Acknowledgements—This work was supported by RO1 DA06836 and a predoctoral fellowship to M.R.B. from T32 AA07462.

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