Stereospecific and Regioselective Hydrolysis of Cannabinoid Esters by ES46.5K, an Esterase from Mouse Hepatic Microsomes, and Its Differences from Carboxylesterases of Rabbit and Porcine Liver

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The properties of ES46.5K, an esterase from mouse hepatic microsomes, were compared with those of carboxylesterases from rabbit and porcine liver. The inhibitory profile with a serine hydrolase inhibitor (bis-*p*-nitrophenylphosphate) and detergents (sodium dodecylsulfate, Emulgen 911) was different between ES46.5K and the carboxylesterases. Bis-*p*-nitrophenylphosphate (0.1 mM) markedly inhibited the catalytic activity of the carboxylesterases but not that of ES46.5K. Emulgen 911 (0.05—0.25%) inhibited the catalytic activity of the carboxylesterases, whereas the detergent conversely stimulated that of ES46.5K by 150%. The two carboxylesterases catalyzed the hydrolysis of acetate esters of tetrahydrocannabinol (THC) analogues with different side chain lengths (C_1—C_5), although ES46.5K showed marginal activity only against the acetate of Δ^8-tetrahydrocannabiorcol, a methyl side chain derivative of Δ^8-THC. ES46.5K hydrolyzed cannabinoid esters stereospecifically and regioselectively. The esterase hydrolyzed 8α-acetoxy-Δ^9-tetrahydrocannabinol (8α-acetoxy-Δ^9-THC, 5.62 nmol/min/mg protein), while the enzyme did not hydrolyze 8β-acetoxy-Δ^9-THC, 7α-acetoxy-, and 7β-acetoxy-Δ^8-THC at all. In contrast, the carboxylesterases from rabbit and porcine liver hydrolyzed 8β-acetoxy-Δ^9-THC efficiently but not 8α-acetoxy-Δ^9-THC. ES46.5K hydrolyzed side chain acetoxy derivatives of Δ^8-THC at the 3′- and 4′-positions, and a methyl ester of 5′-nor-Δ^8-THC-4′-oic acid. The enzyme, however, could not hydrolyze methyl esters of Δ^8- and Δ^9-THC-11-oic acid, while both carboxylesterases hydrolyzed side chain acetoxy derivatives of Δ^8-THC and three methyl esters of THC-oic acids. These differences in stereospecificity and regioselectivity between ES46.5K and carboxylesterases suggest that the configurations of important amino acids for the catalytic activities of these enzymes are different from each other.

Key words ES46.5K; carboxylesterase; regioselective hydrolysis; stereospecific hydrolysis; cannabinoid ester

Carboxylesterases are a family of enzymes that catalyze the hydrolysis of esters and amides.[1—4] Carboxylesterases have an important role in the detoxication of xenobiotics and in metabolic activation of prodrugs in some cases.[5—8] Multiple carboxylesterase isozymes have been purified from various animal species[3,4] including rabbits[9,10] and pigs.[11,12] Although the substrate specificities for many forms of purified carboxylesterases have been well characterized, little is known about their stereospecificity and regioselectivity for the hydrolysis of xenobiotic esters. In relation to the stereospecific hydrolysis of substrates by esterases, enzymatic resolution of enantiomers to obtain a useful chiral starting material has been reported using pig pancreatic lipase.[13,14] Gatley[15] reported that the unusual cocaine enantiomer, (+)-cocaine, was hydrolyzed by butyrylcholine esterase over 2000 times faster than (−)-cocaine. Maksay *et al.*[16] reported that esterases in liver and brain homogenates of mice have opposite stereoselectivity in the hydrolysis of the racemate of oxazepam 3-acetate; the hepatic enzyme preferentially hydrolyzes the (*R*)-enantiomer, whereas the brain enzyme preferentially hydrolyzes the (*S*)-enantiomer. Yang and Lu[17] also reported that esterases in hepatic microsomes and brain homogenate of rats have the same opposite stereoselectivity in catalyzing the hydrolysis of enantiomeric oxazepam 3-acetate. The catalytic triad Ser/Asp/His is suggested to be a part of oxyanion hole as a catalytic site of various esterases and important for the hydrolysis of substrate esters by the enzymes.[18] In addition, structural constraints also affect the interaction of substrates with carboxylesterases.[19]

We previously purified the esterase ES46.5K from mouse hepatic microsomes and its role in the metabolism of xenobiotic esters has been characterized.[20—22] The enzyme is a 46.5-kDa esterase with considerable homology of the *N*-terminal amino acid sequence to arylacetamide deacetylase purified from human liver.[23] Recently, the enzyme has also been characterized as a microsomal lipase with arylamide deacetylase activity.[24] The mammalian carboxylesterases represent a multigene family, the gene products of which are classified as CES1 to CES4. ES46.5K probably belongs to the CES4 family, as is the case for human acetamide deacetylase.[4]

The present study describes the stereospecific and regioselective hydrolysis of cannabinoid esters as model substrates for ES46.5K and carboxylesterases from rabbit and porcine liver. In addition, differences in the characteristics between ES46.5K and the carboxylesterases were also investigated.

MATERIALS AND METHODS

Chemicals and Enzymes Δ^9-Tetrahydrocannabinol (THC) and Δ^9-tetrahydrocannabivarin (Δ^9-THCV) were purified from cannabis leaves as described.[25] Δ^8-THC,[26] 7α-hydroxy-Δ^8-THC,[23] 7β-hydroxy-Δ^8-THC,[27] 8α-hydroxy-Δ^9-THC,[28] and 8β-hydroxy-Δ^9-THC[29] were prepared by the es-

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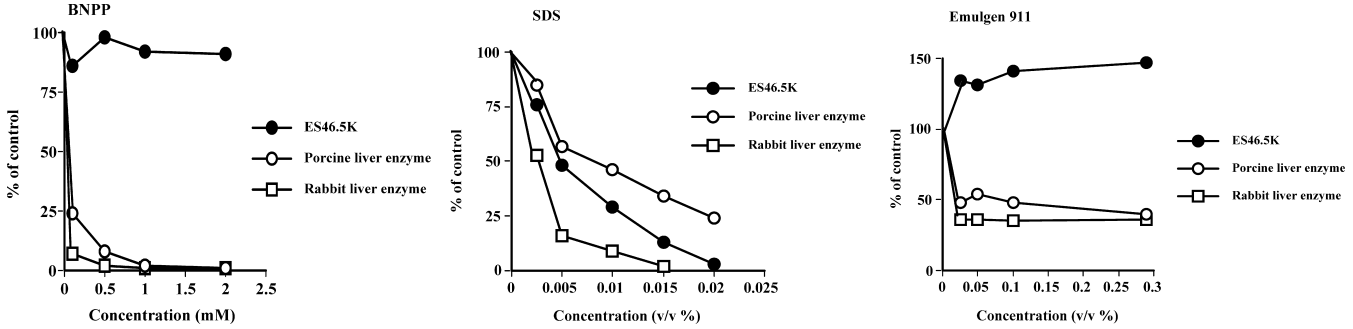


Fig. 1. Effects of Various Compounds on Catalytic Activities of ES46.5K and Two Carboxylesterases for Hydrolysis of *p*-Nitrophenylacetate

The data are means of duplicate incubations.

tablished procedures. Δ^8-Tetrahydrocannabiorcol (Δ^8-THCO) was prepared by condensation of orcinol with *p*-mentha-2,8-dien-1-ol in the presence of *p*-toluenesulfonic acid. The methyl esters of Δ^8-THC-11-oic acid and 5′-nor-Δ^8-THC-4′-oic acid were synthesized using the methods of Mechoulam *et al.*[29] and Ohlsson *et al.*,[30] respectively. 3′-Hydroxy- and 4′-hydroxy-Δ^8-THCs[30] were prepared by the established methods. Δ^9-THC-11-oic acid was supplied by NIDA (U.S.A.). Methyl esters of carboxylic acid derivatives of THCs were prepared by methylation of the carboxylic acids with diazomethane in diethyl ether. Acetoxy derivatives of THCs and their metabolites were prepared by acetylation and/or partial deacetylation of the phenolic hydroxy group of THC metabolites. The purities of the cannabinoids prepared were found to be more than 95% by gas chromatography (GC). Bis-*p*-nitrophenylphosphate (BNPP) was purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Sodium dodecylsulfate (SDS) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Emulgen 911 was supplied by Kao (Tokyo, Japan).

ES46.5K was purified from hepatic microsomes of male ddY mice by the method described previously.[20] Carboxylesterases (EC 3.1.1.1) from rabbit liver (E9636, crystalline suspension in ammonium sulfate 3.6 M, Tris 0.01 M, pH 8.5; specific activity, 220 μmol ethyl butyrate hydrolyzed/min/mg protein) and porcine liver (E3019, lyophilized crude powder containing less than 5% buffer salt; specific activity, 110 μmol *p*-nitrophenylbutyrate hydrolyzed/min/mg protein) were purchased from Sigma Chemical.

Enzyme Assay A typical incubation mixture consisted of ES46.5K (0.2—0.4 μg protein), or carboxylesterase from rabbit and porcine liver (2—4 μg protein), and cannabinoid substrates (0.5 μmol) in Tris–HCl 100 mM (pH 8.0) to make a final volume of 0.5 ml. The mixture was incubated at 37 °C for 20 min and then extracted with 4 ml of ethyl acetate after the addition of KH_2PO_4 1 M (0.5 ml) and androstenedione as an internal standard (10 μg). Cannabinoids hydrolyzed were determined by GC after evaporation of the organic solvent.[31] The GC conditions were: apparatus, Shimadzu GC-16A; column, 5% SE-30 on Chromosorb W (60—80 mesh×2 m); column temperature, 230—260 °C; carrier gas N_2, 50 ml/min; and detection mode, flame ionization detector.

Inhibition Studies The enzymatic activity was determined spectrophotometrically as described previously using *p*-nitrophenylacetate as a substrate.[32] A typical incubation mixture consisted of ES46.5K (0.1 μg protein) or carboxylesterases (0.5 μg protein) in Tris–HCl 100 mM (pH 8.0). Reactions were initiated by the addition of *p*-nitrophenylacetate as a substrate (2 mM) at 25 °C, and enzymatic activity was determined based on the increase in absorbance at 400 nm (E_{400}=13 mM^{-1}). Inhibition studies were carried out under similar conditions with various concentrations of BNPP, SDS, and Emulgen 911 as described above.

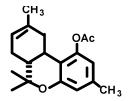
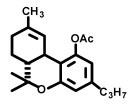
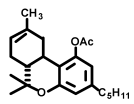
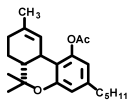
RESULTS

Effects of BNPP, SDS, and Emulgen 911 on Catalytic Activity The effects of BNPP, SDS, and Emulgen 911 on the catalytic acitivities of ES46.5K and carboxylesterases were investigated using *p*-nitrophenylacetate as a substrate. At 0.2 mM, the catalytic activity of carboxylesterases was markedly inhibited by BNPP, a known inhibitor of carboxylesterase. In contrast, the hydrolytic activity of ES46.5K was not inhibited by BNPP at all up to 2 mM of the inhibitor, as shown in Fig. 1. SDS is a detergent known to denature membrane-bound enzyme. As shown in Fig. 1, SDS concentration dependently inhibited the hydrolytic activity of ES46.5K and carboxylesterases. The catalytic activities of ES46.5K and carboxylesterases from rabbit and porcine liver were inhibited by SDS to 13%, 34% and 4%, respectively, as compared with the control activity. Emulgen 911, a nonionic detergent, differently affected the catalytic activity between ES46.5K and carboxylesterases. Emulgen 911 (0.02 v/v%) inhibited the catalytic activity of carboxylesterase to 40% of the control activity, whereas the detergent conversely activated the enzymatic activity of ES46.5K up to 150%, as shown in Fig. 1.

Stereospecific and Regioselective Hydrolysis of Cannabinoid Esters The present study demonstrated that ES46.5K and two carboxylesterases exhibited different catalytic activity for the hydrolysis of acetyl derivatives of THC at the 1-position (Table 1). ES46.5K did not hydrolyze acetyl derivatives of Δ^8-THC, Δ^9-THC, and Δ^9-THCV as reported in the previous report,[16] although the enzyme hydrolyzed an acetyl derivative of Δ^8-THCO (0.23 μmol/min/mg protein), a methyl side chain derivative of Δ^8-THC. However, the catalytic activity of ES46.5K toward the acetate of Δ^8-THCO was about 1/4 and 1/9 of those of carboxylesterases from rabbit and porcine liver, respectively. Carboxylesterases from porcine and rabbit liver hydrolyzed all acetyl derivatives of THC; in particular, the porcine enzyme was more active than the rabbit enzyme for hydrolyzing 1-*O*-acetylcannabinoids.

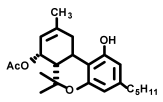
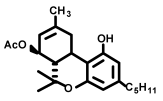
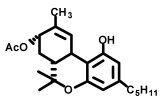
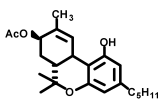
ES46.5K stereospecifically hydrolyzed acetyl derivatives

Table 1. Hydrolysis of 1-Acetylcannabinoids by ES46.5K and Carboxylesterases

Structures of cannabinoid esters	Catalytic activity (μmol/min/mg protein)		
	ES46.5K	Carboxylesterases	
		Rabbit liver	Porcine liver
 1-Acetyl-Δ^8-THCO	0.23	0.96	1.98
 1-Acetyl-Δ^9-THCV	ND	0.68	1.02
 1-Acetyl-Δ^8-THC	ND	0.46	0.58
 1-Acetyl-Δ^9-THC	ND	0.37	0.48

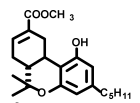
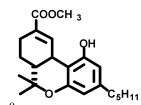
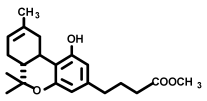
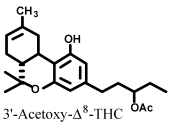
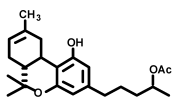
ND: Not detectable (<0.01). The data are means of duplicate determinations.

Table 2. Hydrolysis of Acetyl Derivatives of 7-Hydroxy-Δ^8-THC and 8-Hydroxy-Δ^9-THC by ES46.5K and Carboxylesterases

Structures of cannabinoid esters	Catalytic activity (μmol/min/mg protein)		
	ES46.5K	Carboxylesterases	
		Rabbit liver	Porcine liver
 7α-Acetoxy-Δ^8-THC	ND	ND	ND
 7β-Acetoxy-Δ^8-THC	ND	ND	ND
 8α-Acetoxy-Δ^9-THC	5.62	ND	ND
 8β-Acetoxy-Δ^9-THC	ND	0.20	0.87

ND: Not detectable (<0.01). The data are means of duplicate determinations.

Table 3. Hydrolysis of Methyl Esters of THC-oic Acids and Side Chain Acetoxy Derivatives of Δ^8-THC

Structures of cannabinoid esters	Catalytic activity (μmol/min/mg protein)		
	ES46.5K	Carboxylesterases	
		Rabbit liver	Porcine liver
 Δ^8-THC-11-oic acid methyl ester	ND	0.05	0.10
 Δ^9-THC-11-oic acid methyl ester	ND	0.10	0.12
 5'-Nor-Δ^8-THC-4'-oic acid methyl ester	2.28	0.16	0.31
 3'-Acetoxy-Δ^8-THC	2.92	0.10	0.17
 4'-Acetoxy-Δ^8-THC	11.7	0.28	0.68

ND: Not detectable (<0.01). The data are means of duplicate determinations.

of 7-hydroxy-Δ^8-THC and 8-hydroxy-Δ^9-THC (Table 2). ES46.5K efficiently hydrolyzed 8α-acetoxy-Δ^9-THC (5.62 μmol/min/mg protein) but not 8β-acetoxy-Δ^9-THC, whereas carboxylesterases from rabbit and porcine liver hydrolyzed 8β-acetoxy-Δ^9-THC but not 8α-acetoxy-Δ^9-THC. On the other hand, none of the three enzymes used in the present study hydrolyzed 7α-acetoxy-Δ^8-THC and 7β-acetoxy-Δ^8-THC under the present conditions.

Differences in the catalytic activity of ES46.5K and the carboxylesterases were also demonstrated in the hydrolysis of methyl esters of THC-oic acids (Table 3). ES46.5K hydrolyzed the methyl ester of 5′-nor-Δ^8-THC-4′-oic acid but not methyl esters of Δ^8- and Δ^9-THC-11-oic acids, although both carboxylesterases hydrolyzed all of the methyl esters of THC-oic acids examined in the present study. ES46.5K also catalyzed the hydrolysis of the acetates of side chain hydroxylated derivatives of Δ^8-THC more efficiently as compared with the carboxylesterases (Table 3).

DISCUSSION

Differences in the enzymatic properties of ES46.5K and two carboxylesterases from rabbit and porcine liver have been demonstrated. The enzymatic activities of the carboxylesterases from rabbit and porcine liver were almost completely inhibited by BNPP up to 0.5 mM, which is a typical inhibitor of carboxylesterase,[33] although that of ES46.5K was not inhibited even up to 2 mM. Probst *et al.*[23] reported that the enzymatic activity of arylacetamide deacetylase purified from human liver, a 45-kDa esterase with considerable homology of the *N*-terminal amino acid sequence with ES46.5K, was inhibited noncompetitively by BNPP. Thus the catalytic site of ES46.5K may have a different structure from that of arylacetamide deacetylase and not react efficiently with the organophosphate. None of acetylcholine esterase, trypsin, and nonspecific serum choline esterase, which are

serine hydrolases, have been reported to be not inhibited by BNPP.[33] The results of the present study suggest that ES46.5K may have a catalytic structure similar to that of those hydrolases.

Most membrane proteins are denatured by a high concentration of detergents. The anionic detergent SDS is known to induce alterations in the conformation of proteins and to denature membrane-bound enzymes.[34] Microsomal carboxylesterases are membrane-bound enzymes and thus their enzymatic activities may be modulated by the detergent. SDS inhibited the activities of all three enzymes in the same manner. The present results indicated that ES46.5K and carboxylesterases are modulated differently by the detergent Emulgen 911. Emulgen 911 is a nonionic detergent and has been used to solubilize a number of membrane-bound enzymes such as cytochrome P450 without loss of their biological activities.[35] Emulgen 911 (0.02—0.3 v/v%) inhibited the activity of carboxylestreases. In contrast, the activity of ES46.5K was increased up to 150% of control activity with the addition of the detergent. This result suggests that the two carboxylesterases have their active conformations modulated by Emulgen 911 and lose their catalytic activities, while the substrate accessibility of ES46.5K may be stimulated by the detergent and its enzymatic activity is increased.

Carboxylesterases hydrolyze a variety of esters, and therefore their substrate specificities are not clearly distinguished. The present results indicated that ES46.5K hydrolyzed only an acetate of the methyl side chain analogue of Δ^8-THC, Δ^8-THCO. Both carboxylesterases hydrolyzed all (methyl, propyl, and pentyl) the THC derivatives examined in the present study. These results suggest that the active-site interaction of ES46.5K with the acetate of the THC analogues was different from those of the carboxylesterases. The longer side chains (C_3 and C_5) at the 3-position of cannabinoids may interfere with the interaction with the active site of ES46.5K but not those of the carboxylesterases.

The stereospecific hydrolysis of cannabinoid esters by the esterases was observed in 8-acetoxy-Δ^9-THC. ES46.5K hydrolyzed 8α-acetoxy-Δ^9-THC but not 8β-acetoxy-Δ^9-THC, while two carboxylesterases conversely hydrolyzed 8β-acetoxy-Δ^9-THC but not 8α-acetoxy-Δ^9-THC. In addition, ES46.5K and the carboxylesterases could not hydrolyze 7α- and 7β-acetoxy-Δ^8-THC. These results indicate that the position of acetate esters is critical for substrates of these esterases. A few reports described the stereospecific hydrolysis of ester substrates by microsomal esterases.[11,13]

Some differences in the regioselective hydrolysis of cannabinoid esters between ES46.5K and carboxylesterases were also demonstrated in methyl esters of carboxylic acid derivatives of THCs as substrates. Two carboxylesterases hydrolyzed both carboxylic acid esters at the 11-position and the side chain of THCs. In contrast, ES46.5K hydrolyzed only the side chain carboxylic acid ester of THC. Thus methyl esters of Δ^8-THC-11-oic acid and Δ^9-THC-11-oic acid are not good substrates for ES46.5K. In addition, the acetates of side chain hydroxylated metabolites of Δ^8-THC were favorably hydrolyzed by ES46.5K.

The present study demonstrated that ES46.5K, an esterase from mouse hepatic microsomes, hydrolyzes cannabinoid esters stereospecifically and regioselectively, and that some properties of the esterase are different from those of carboxylesterases from rabbit and porcine liver. Carboxylesterases are known to be serine hydrolases that have the common active site sequence motif of –Gly–X–Ser–X–Gly–.[36] The catalytic triad Ser/Asp/His is also known as important amino acids for the catalytic activity of the enzymes. The differences in stereospecificity and regioselectivity between ES46.5K and the carboxylesterases therefore suggest that the configurations of important amino acids for the catalytic sites of ES46.5K and the carboxylesterases are different.

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