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Note

Affinity Purification of Mouse Liver Carboxylesterases with Trifluoromethyl Ketones as Ligands

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INTRODUCTION

Mammalian liver carboxylesterases are one of the microsomal enzymes which have been shown to play an important role in the detoxification of xenobiotics such as pesticides and environmental pollutants.[1] Isoelectric focusing studies have shown the presence of ten or more hepatic isozymes in various mammalian species.[1,2] However, the separation of these isozymes is difficult and only a few have been purified by classical purification techniques such as ion-exchange and gel filtration from rabbit liver[3,4] and several other mammalian species.[5] On the other hand, juvenile hormone esterase (JHE), an esterase in insects which regulates hormones, has been characterized[6] and purified using affinity gels.[7,8] One of trifluoromethyl ketones (TFKs), 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) is a potent inhibitor of JHE, and an OTFP analog 3-(4-mercaptobutylthio)-1,1,1-trifluoro-2-propanone (MBTFP) was utilized as a ligand for affinity purification of JHE. This procedure was also used in partial purification of neuropathy target esterase from chicken brain because this enzyme was inhibited strongly by OTFP.[9] The fact that mouse liver carboxylesterases were also inhibited strongly by OTFP suggested the possibility of using the same procedure in the affinity purification of these enzymes. In this paper, the purification of liver carboxylesterases from solubilized mouse liver microsomes using affinity gels with TFK ligands such as MBTFP and non-sulfide MTFO (8-mercapto-1,1,1-trifluoro-2-octanone, see Fig. 1 for their structures) was investigated. In addition, affinity purification of partially purified commercial porcine liver carboxylesterase was carried out for comparison.

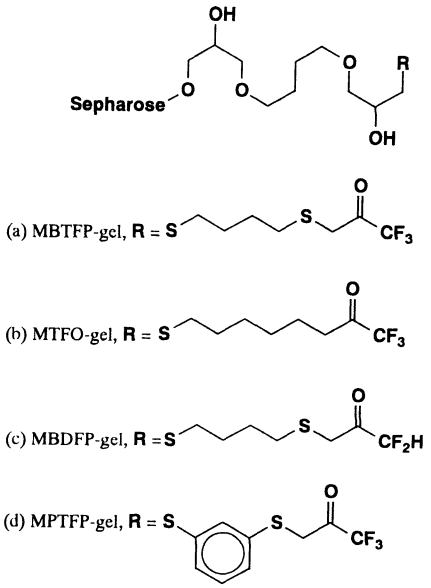

Fig. 1 Structure of ligands of affinity gels for purification of mouse liver carboxylesterases (MLC).

MATERIALS AND METHODS

1. *Enzyme Preparations and Assays*

Unless otherwise stated, 0.1 M Tris-HCl buffer, pH 7.50 containing 0.02% of NaN_3 was used for all experiments. Solubilization of mouse liver microsomal esterases with *n*-octyl β-D-glucopyranoside (OG) was carried out according to a method developed in this laboratory.[10] Briefly, after centrifugation at 100,000×*g* for 1 hr at 4°C the microsomal pellet was resuspended in the buffer containing 1% OG and then gently shaken for 1 hr on a rotating wheel at approx. 50 rpm and again centrifuged at 100,000×*g* for 1 hr. The resulting supernatant fractions were used as a starting enzyme source for affinity purification. Partially purified porcine liver

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carboxylesterase and horse serum butyrylcholinesterase were purchased from Sigma and used in a comparative study with the mouse liver carboxylesterases.

Esterase activity was assayed by monitoring the hydrolysis of methyl *n*-pentylthiopropiothioate (MPTPT) in the presence of the color producing reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent). MPTPT was prepared in this laboratory as a spectrophotometric substrate for esterases, and it was found to be a better substrate for mouse liver carboxylesterase than *p*-nitrophenyl acetate or α-naphthyl acetate in terms of kinetic characteristics.[10] The enzyme reaction was carried out as follows: Enzyme was preincubated in the buffer containing 0.015% DTNB for 2 min at 25°C. MPTPT, in EtOH, was added to give a final concentration of 0.2 mM and the change in absorbance at 405 nm was monitored for 2 min. A series of inhibitors were prepared previously for JHE.[8] For inhibition tests, enzyme was incubated with inhibition for 10 min prior to the addition of substrate. The time-dependent reactivation of inhibited enzymes was investigated as described earlier for JHE.[8] A crude mouse liver carboxylesterase preparation was incubated with inhibitor (40 μM final concentration) for 16 hr at 4°C. The mixture was then dialyzed against buffer at 4°C, and aliquots were removed at appropriate time intervals and assayed for enzymatic activity.

2. Affinity Purification

Affinity gels and inhibitors were prepared as described previously,[8] ligand concentrations on these gels were 27 μmol eq./g of gel. The affinity gel was packed into a column and washed thoroughly with EtOH, 50% EtOH, distilled water, and finally buffer. Enzyme solution (approx. 0.1 mg/ml diluted with buffer) was added and shaken gently for 4 hr. After loading, the gel was washed with buffer, then with the buffer containing 0.1% OG. Enzyme was eluted from the affinity gel by gently shaking with an eluting solution (buffer containing 0.1% OG and 1 mM of 3-pentylthio-1,1,1-trifluoro-2-propanone (PTFP) or 0.5 mM of OTFP) for 8 hr. This was repeated three times with fresh eluting solution, then the eluates were combined, and dialyzed against buffer. For DEAE-ion exchange chromatography, the solution was diluted with 10 mM Tris-HCl, pH 8.5 (DEAE buffer) and gently shaken with DEAE-Sephacryl gel at 4°C. This gel was then packed into a column and proteins were eluted an NaCl gradient (0 to 200 mM). The resulting samples were analyzed by SDS-PAGE (10% gel) stained with Coomassie Brilliant Blue R-250.

RESULTS AND DISCUSSION

1. Inhibition and Reactivation of Mouse Liver Carboxylesterase

Inhibitory potencies (I_{50} values) of fluoromethyl ketones are listed in Table 1. Among the 3-alkylthio-1,1,1-trifluoro-2-propanones, OTFP, which has the longest alkyl chain, showed the

Table 1 Inhibitory activity of fluoromethyl ketones on enzymatic hydrolysis of the spectrophotometric substrate, MPTPT ($CH_3(CH_2)_4S(CH_2)_2C(O)SCH_3$) by solubilized mouse liver microsomes.[a]

Abbreviation	Structure	I_{50}, M
BTFP	$CH_3(CH_2)_3SCH_2C(O)CF_3$	3.35×10^{-7}
PTFP	$CH_3(CH_2)_4SCH_2C(O)CF_3$	4.10×10^{-7}
HxTFP	$CH_3(CH_2)_5SCH_2C(O)CF_3$	9.63×10^{-7}
HpTFP	$CH_3(CH_2)_6SCH_2C(O)CF_3$	1.10×10^{-8}
OTFP	$CH_3(CH_2)_7SCH_2C(O)CF_3$	3.45×10^{-9}
ODFP	$CH_3(CH_2)_7SCH_2C(O)CF_2H$	2.28×10^{-7}
OMFP	$CH_3(CH_2)_7SCH_2C(O)CFH_2$	$>1.0\times10^{-3}$
C_4-bis(TFP)	$CF_3C(O)CH_2S(CH_2)_4SCH_2C(O)CF_3$	2.50×10^{-8}
PhTFP	$PhSCH_2C(O)CF_3$	$>1.0\times10^{-3}$
TFD	$CF_3(CH_2)_9C(O)CF_3$	7.70×10^{-8}

[a] The inhibitor was incubated with the enzyme for 10 min at 25°C prior to the addition of substrate and enzymatic hydrolysis was monitored for 2 min. I_{50} values were determined from regression curves including 5 points (at least 2 points above and below the I_{50} value). All assays were performed at least in triplicate.

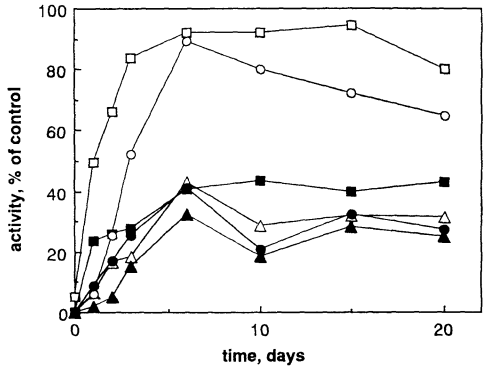

Fig. 2 Time-dependent reactivation of mouse liver carboxylesterase by dialysis following inhibition by 40 μM of fluoromethyl ketones for 16 hr.
□: PTFP, ■: HxTFP, △: HpTFP, ▲: OTFP, ○: ODFP, ●: C_4-bis(TFP).

highest inhibitory activity, and levels of inhibition decreased with a decreases in length of the alkyl chain. The monofluoro analog of OTFP (OMFP) and phenylthio substituent (PhTFP) had no inhibitory activity up to 1×10^{-3} M. The other fluoromethyl ketones, including 3-octylthio-1,1-difluoro-2-propanone (ODFP), 1,4-bis(2-oxo-3, 3, 3-trifluoropropylthio)-butane [C_4-bis-(TFP)], and non-sulfide 1,1,1-trifluoro-2-decanone (TFD), were moderate inhibitors.

Time-dependent reactivation of mouse liver carboxylesterase by dialysis, following inhibition by the fluoromethyl ketones, resembled the pattern seen with JHE.[8] The mouse liver carboxylesterase inhibited by PTFP was reactivated readily (>80% within 3 days), and ease of enzyme reactivation correlated with the shortness of the alkyl chain (Fig. 2). Surprisingly, the mouse liver carboxylesterase inhibited by ODFP showed rapid reactivation (>80% within 6 days). OTFP and PTFP were used as eluting agents in the affinity purification study.

2. *Affinity Chromatography*

Horse serum butyrylcholinesterase failed to bind to the MBTFP- and MTFO-gels using the ligand concentrations and conditions described above (data not shown). Partially purified porcine liver carboxylesterase bound to the MBTFP-gel, whereas the affinity for MTFO-gel was not strong enough and most of the activity was eluted by washing. Although enzyme was tightly bound to MBTFP-gel, it was possible to elute it with OTFP in the presence of 0.1% OG. After this purification, impurities in the partially purified commercial sample were eliminated (Fig. 3(a)). However, following elution with OTFP it was not possible to recover esterase activity using the dialysis conditions described above.

The results for the affinity binding of mouse liver carboxylesterase were different from those of porcine liver carboxylesterase, because esterase activity from crude mouse liver microsomes

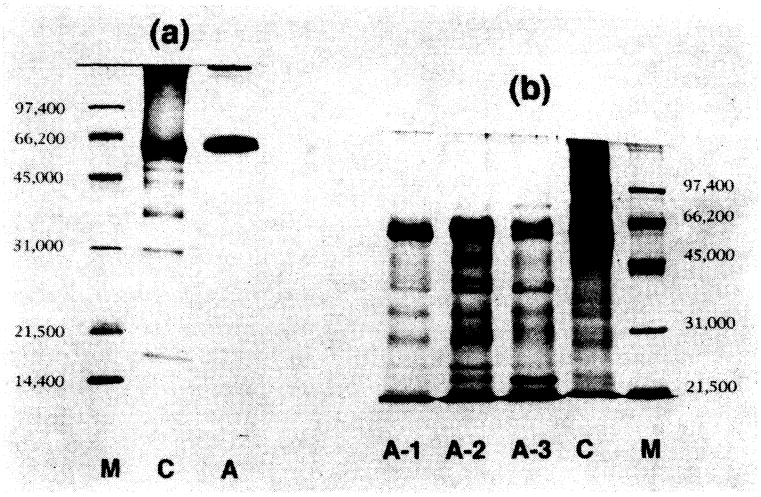

Fig. 3 SDS-PAGE of affinity purified microsomal esterases, from (a) partially purified porcine liver esterase, and (b) solubilized mouse liver microsomes.
M: marker, C: crude preparation, A: protein-OTFP complex eluted from the affinity gel, A-1, A-2, A-3: protein-PTFP complex eluted from the affinity gel.

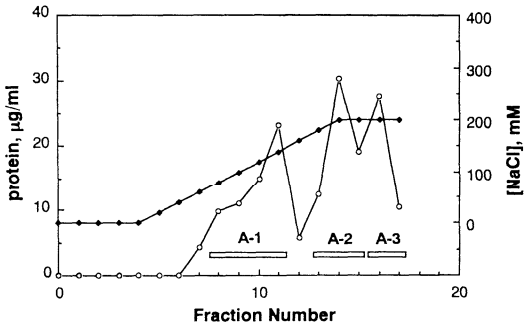

Fig. 4 DEAE-ion exchange chromatography.

Proteins eluted from the MTFO-gel were dialyzed and incubated with DEAE-Sephacryl. Bound proteins eluted (○) with a gradient of NaCl (●, 0 to 200 mM). Fractions from each protein peak were combined as A-1, A-2, and A-3. However, no enzyme activity was recovered.

bound to both the MTFO- and MBTFP-gels. Little or no activity was eluted during washing with buffer in the absence or presence of 0.1% OG. The amount of protein eluted from the MTFO-gel was larger than from the MBTFP-gel. PTFP was selected for further experiments because the PTFP-enzyme complex was reactivated more easily than the complex with OTFP, as mentioned above. The PTFP eluate was then chromatographed on DEAE-Sephacryl gel with a concentration gradient of NaCl to remove excess PTFP and OG as well as to fractionate proteins.

Three major peaks of protein were found with ion-exchange chromatography (Fig. 4), and the fractions from each peak were combined, concentrated, and dialyzed against buffer. Each peak still contained several proteins after the ion-exchange chromatography (Fig. 3(b)). It was hoped that esterase activity could be regenerated after dialysis to remove PTFP. However, active enzyme could not be recovered, possibly due to low stability of the enzyme after purification. Addition of stabilizing reagents such as sugars may allow the recovery of active enzyme.

This report describes initial experiments in the affinity purification of mammalian carboxylesterases. Since the amounts of starting material used in this study were small, there was insufficient purified protein for further analysis by isoelectrofocusing or HPLC. However, this study shows that the affinity chromatography system can be used to purify mammalian carboxylesterases. Based on these results, we are attempting to optimize conditions for enzyme stability and investigating the use of other affinity ligands for the selective purification of carboxylesterase isozymes.

REFERENCES

1) T. Satoh: *Rev. Biochem. Toxicol.* **8**, 155 (1987)
2) C. W. Young & E. S. Bittar: *Cancer Res.* **34**, 2675 (1974)
3) J. Ozoles: *J. Biol. Chem.* **262**, 15316 (1987)
4) H. A. van Lith, M. Haller, B. F. M. van Zutphen & A. C. Beynen: *Eur. J. Biochem.* **206**, 545 (1992)
5) M. Hosokawa, T. Maki & T. Satoh: *Arch. Biochem. Biophys.* **277**, 219 (1990)
6) B. D. Hammock: "Comprehensive Insect Physiology, Biochemistry, and Pharmacology," ed. by G. A. Kerkut & L. I. Gilbert, Pergamon Press, New York, pp. 431–472, 1985
7) Y. A. I. Abdel-Aal & B. D. Hammock: *Science* **233**, 1073 (1986)
8) T. Shiotsuki, T. L. Huang, T. Uematsu, B. C. Bonning, V. K. Ward & B. D. Hammock: *Protein Express. Purif.* **5**, 296 (1994)
9) T. C. Thomas, A. Szekacs, S. Rojas, B. D. Hammock, B. W. Wilson & M. G. McNamee: *Biochem. Pharmacol.* **40**, 2587 (1990)
10) T. L. Huang, T. Uematsu, T. Shiotsuki, B. Borhan & B. D. Hammock: Abstr.; 207th Am. Chem. Soc. Natl. Meet., San Diego, MEDI-269, 1994

要　約

トリフルオロメチルケトンをリガンドにもつアフィニティークロマトグラフィーによるマウス肝カルボキシルエステラーゼの精製

塩月孝博，Tien L. HUANG, Bruce D. HAMMOCK

3-Octylthio-1,1,1-trifluoro-2-propanone (OTFP) に代表されるトリフルオロメチルケトンはその遷移状態アナログとしてエステラーゼによる加水分解反応を阻害する．この部分構造 3-(4-mercaptobutylthio)-1, 1, 1-trifluoro-2-propanone (MBTFP) と 8-mercapto-1,1,1-octanone (MTFO) をリガンドにもつアフィニティーゲルによるマウス肝カルボキシルエステラーゼの精製を試みた．両アフィニティーゲルは，いずれも可溶化マウス肝ミクロゾームのエステラーゼ活性を吸着した．溶出に用いるフリーリガンド検索のため，阻害後の酵素活性回復を調べたところ，OTFP で阻害された酵素の活性は回復しなかったが，短い炭素鎖をもつ 3-pentylthio-1,1,1-trifluoro-2-propanone (PTFP) で阻害された場合ではほぼ完全に回復した．そこで活性の吸着したゲルから，界面活性剤存在下 PTFP で溶出を行なうと MTFO-ゲルからのみ分子量の異なる数種のタンパクが得られた．続く透析による酵素活性回復は認められなかったが，条件の検討により十数種存在するカルボキシルエステラーゼのアイソザイムの分離・精製の可能性が示唆された．