AD ________________

Award Number: DAMD17-00-1-0518

TITLE: Expression and Purification of a Potential Antidote for Organophosphate Warfare Agents

PRINCIPAL INVESTIGATOR: Kenneth D. Lanclos, Ph.D.

CONTRACTING ORGANIZATION: Augusta Biomedical Research Corporation
Augusta, Georga 30901

REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020913 011

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
July 2002

3. REPORT TYPE AND DATES COVERED
Annual (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Expression and Purification of a Potential Antidote for Organophosphate Warfare Agents

5. FUNDING NUMBERS
DAMD17-00-1-0518

6. AUTHOR(S)
Kenneth D. Lanclos, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Augusta Biomedical Research Corporation
Augusta, Georga 30901

E*Mail: jkl42@comcast.net

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* (abstract should contain no proprietary or confidential information)

Serine-dependent carboxylesterases (E.C.3.1.1.1) are found in a variety of tissues with high activity detected in the human liver. Carboxylesterases (hCaE) hydrolyze aliphatic and aromatic esters, and aromatic amides. Carboxylesterase may play an important role in the detoxification of xenobiotic chemicals that contain organophosphate (OP) compounds. Thus, an injectable form of human hCaE should prove to be a valuable antidote for protecting soldiers from these chemical agents. To this end, clones containing a site-mutated cDNA were prepared and used to stably transform human 293T cells. Transformed 293T cells were grown in a chemostat, and conditions were defined which allow for an optimal time of removal of the culture media for isolation of carboxylesterase. In these studies, enzymatic activity was found to be optimal in cell culture medium at four hours. Enzymatically active carboxylesterase was isolated from the culture media, and the initial steps of enzyme purification were accomplished. Our results indicate that an active recombinant enzyme functions as native enzyme with respect to inhibition by organophosphates and reactivation by oximes.

14. SUBJECT TERMS
carboxylesterase, cDNA cloning, cell culture, protein secretion, enzyme purification, organophosphate detoxification

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover....................1

SF 298.................... 2

Table of Contents.................... 3

Introduction.................... 4

Body.................... 5

Key Research Accomplishments 5-10

Reportable Outcomes....................10

Conclusions....................10-11

References....................11-13

Re: annual progress report: DAMD17-00-1-0518 - Expression and Purification of a Potential Antidote for Organophosphate Warfare Agents

Kenneth D. Lanclos, Ph.D., Principal Investigator

Introduction:

The serine-dependent carboxylesterases (E.C. 3.1.1.1) are found in many different species (1-8), and in a variety of tissues (9-14), with high activities detected in the liver. Generally, carboxylesterases exist as 60 kDa monomers, but a few associate to form homotrimers of approximately 180 kDa (1-4). cDNA clones have been obtained by screening lambda gt11 expression libraries. Their screenings have resulted in the expression of five isoenzymes of rat liver carboxylesterase (5-9) and at least two isoenzymes from the human liver (10-12). The cDNA sequences are, generally, 1.7 to 1.9 kb in size, and they encode mature proteins that range from 507 to 568 amino acids. The different isoenzymes of liver carboxylesterases are all N-linked glycoproteins of the high mannose type (5,6,12,13). Core glycosylation of the carboxylesterases occurs in the endoplasmic reticulum lumen (14,15), and, thus it is necessary to stabilize the active conformation of the protein (6,16). Whereas, most glycosylated proteins are secretory, the carboxylesterases are localized to the luminal side of the endoplasmic reticulum, especially in humans (4-6). Very small amounts of liver carboxylesterase, however, are present in the serum (17). Carboxylesterases can hydrolyze a variety of substrates in the serum, including aliphatic and aromatic esters, and aromatic amides (18). An important function of these enzymes may be the hydrolysis, and subsequent detoxification, of pesticides, insecticides and drugs (18), many of which contain organophosphate compounds that bind covalently to the active site of the enzymes (19,20). In this regard, the carboxylesterases generally function as a high affinity-low capacity detoxification mechanism, in which organophosphates react in an irreversible 1:1 stoichiometry (17). Thus, the detoxifying ability of carboxylesterase is limited by its low concentration in serum where it encounters organophosphate compounds. A treatment for organophosphate toxicity is the administration of oximes, which reactivate inhibited acetylcholinesterase and restore cholinergic neurotransmission (17). Species with high levels of serum carboxylesterase, such as rats and mice, achieve a higher level of oxime-

induced reactivation of organophosphate-inhibited acetylcholinesterase than species with lower levels of carboxylesterase; this suggests that oximes also reactivate organophosphate-inhibited carboxylesterase (17). This recycling of organophosphate-inhibited carboxylesterase provides additional protection by making the enzyme available for further binding to organophosphates, and thus, increased detoxification.

Body:

The goals of this project are to over express a functional human liver hCaE from a recombinant cDNA in a human cell line, and isolate and purify the recombinant protein. To accomplish these goals, the cDNA encoding hCaE was altered in order to convert it to a secretory form. Expression of the site-mutated cDNA in cell culture resulted in the secretion of an active hCaE into the growth medium. Thus, the secreted hCaE enzyme will be concentrated and purified using hydrophobic interaction chromatography, Cibacron blue affinity chromatography, antibody-affinity chromatography, and finally HPLC chromatography.

The long-term objective of the project is to isolate quantities sufficient to evaluate its use for protection by enzymatic detoxification of organophosphate nerve agents in an animal model. The short-term goals of this study are to maximize the expression of a functional recombinant secretory form of human liver carboxylesterase using a steady state human cell culture system, and to isolate and purify the recombinant enzyme from the culture media.

Key Research Accomplishments:

Stable clones of 293T cells, stably transfected with the plasmid pRC/mhCaE (***figure 1***), were placed in liquid culture for expression of the secretory form of human liver carboxylesterase.

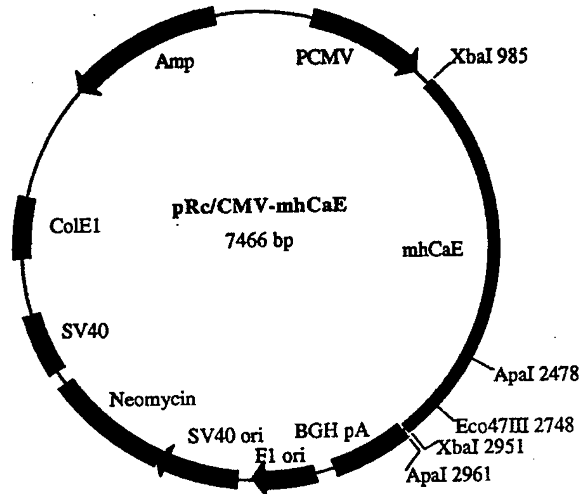

Figure 1: Expression vector containing the cDNA for secretory carboxylesteras

Carboxylesterase activity was observed to increase as early as 24 hours and to reach a maximum at day four (21). Cultured cells were separated from the media by low speed centrifugation and the pooled media from several cultures was used for the isolation of purification enzymatically active enzyme.

Carboxyleasterase was precipitated from the pooled media and precipitated using 70% saturated ammonium sulfate. The precipitate from the ammonium sulfate fraction was dissolved in 20 mM Hepes, pH 7.0 and applied to an octyl Sepharose column (21) for Hydophobic Interaction Chromatography (HIC). Previous observations had shown that HIC chromatography using octyl Sepharose, coupled with octyglucophranoside in the elution buffer resulted in an enhanced partial purification of carboyxlesterase. However, the major contaminant of the preparation, bovine serum abumin, as well as several minor contaminants, were not removed by this procedure. **Figure 2, lane 4**, shows the presence of enzyme along with the bovine serum albumin and other minor contaminants from the HIC column.

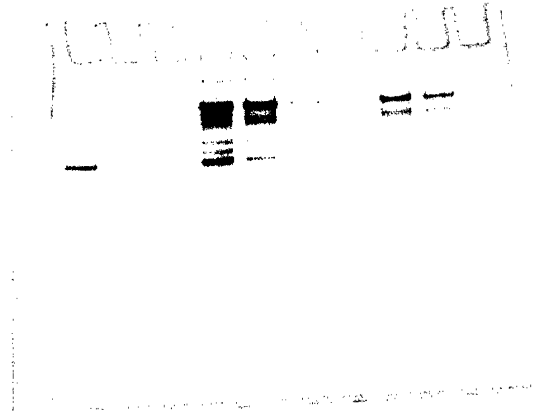

Figure 2. Nondenaturing PAGE. The lanes from left to right are lane 1; BSA standard, lane 2; urease standard, lane 3; blank, lane 4; the ammonium sulfate cut; lane 5-6; the anti-BSA IgG cut; lane 7; blank, lane 8 and 9; before and after a second treatment with anti-BSA IgG. Each lane shows a large molecular weight contaminant at the top of the gel followed by two major bands with significant carboxylesterase activity. The bottom band on the gel is BSA along with two minor bands located just above the BSA band. The gel was stained for enzyme activity using alpha naphthylacetate as substrate coupled to Fast Red TR salt. Following rinsing, the gel was stained for total protein with a 0.1% solution of Coomassie Blue R-250.

Anti-bovine serum albumin IgG was used to remove the bovine BSA during the purification procedure. The results of these experiments are seen in ***figure 2.*** Lane 5 shows partial removal of bovine BSA; lane 6 is a second wash of the preparation to remove any remaining carboxylesterase from the antibody. Lanes 8 and 9 show a second treatment of the enzyme sample with anti bovine serum IgG. These results show that anti-BSA will remove a majority of the contamination BSA; however, there is a considerable loss of enzyme, which probably is a result of the binding to the matrix to which the anti-BSA is attached.

Based on our previous observations (21) that carboxylesterase binds to many matrices used in standard protein purification procedures, we developed a liquid isoelectric focusing preparative system. The strategy was to first remove the bulk of the BSA (pI 4.9) from the carboxylesterase (pI 5.3) by precipitation of the BSA into the anode solution at the bottom of the column ***(figure 3a)***. The peak fractions containing carboxylesterase activity around pI 5.3 were collected and applied to a second column where the anode and cathode solutions were reversed. This resulted in the focusing of the BSA contaminant above the carboxylesterase fraction ***(figure 3b)***. Non-denaturing page gel analysis of these peaks fractions from ***figure 3a and b*** are shown in ***figures 3c and d***, respectively. The zymograms, lanes 1-3, show carboxylesterase activity while the Coomassie blue staining shows the relative amounts of enzyme and contaminating BSA protein. ***Figure 3c*** shows the presence of a small amount of BSA in the first isoelectric focusing fractionation while ***figure 3d***, following the second focusing column, shows almost complete removal of contaminating media proteins from the preparation.

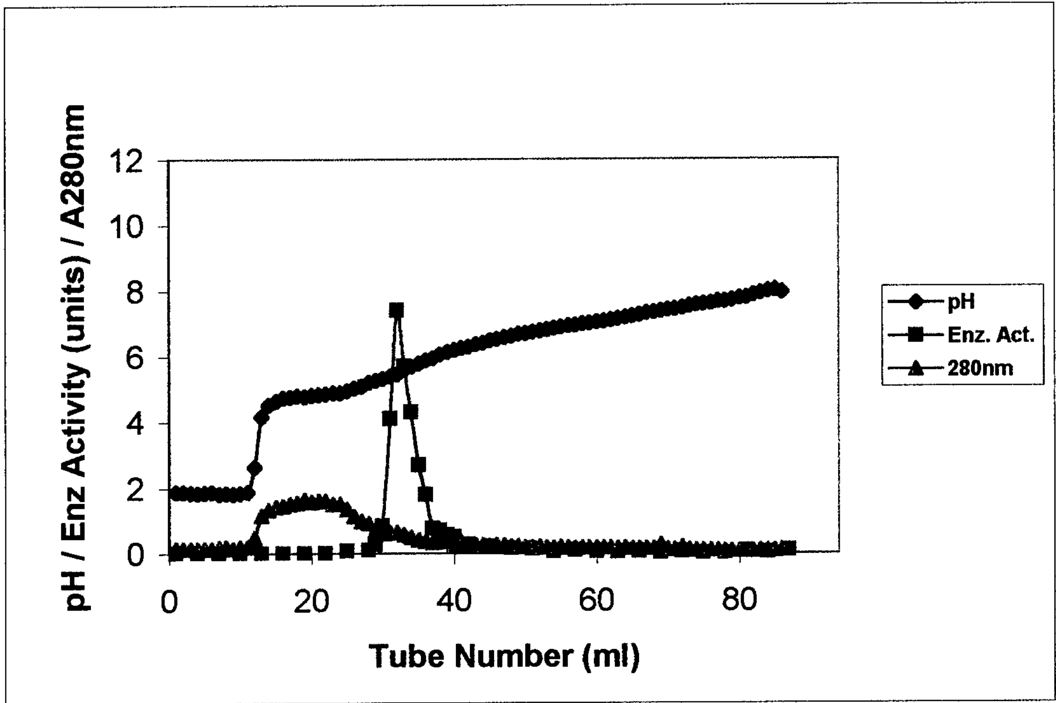

Figure 3a: Preparative Electrofocusing Chromatogram

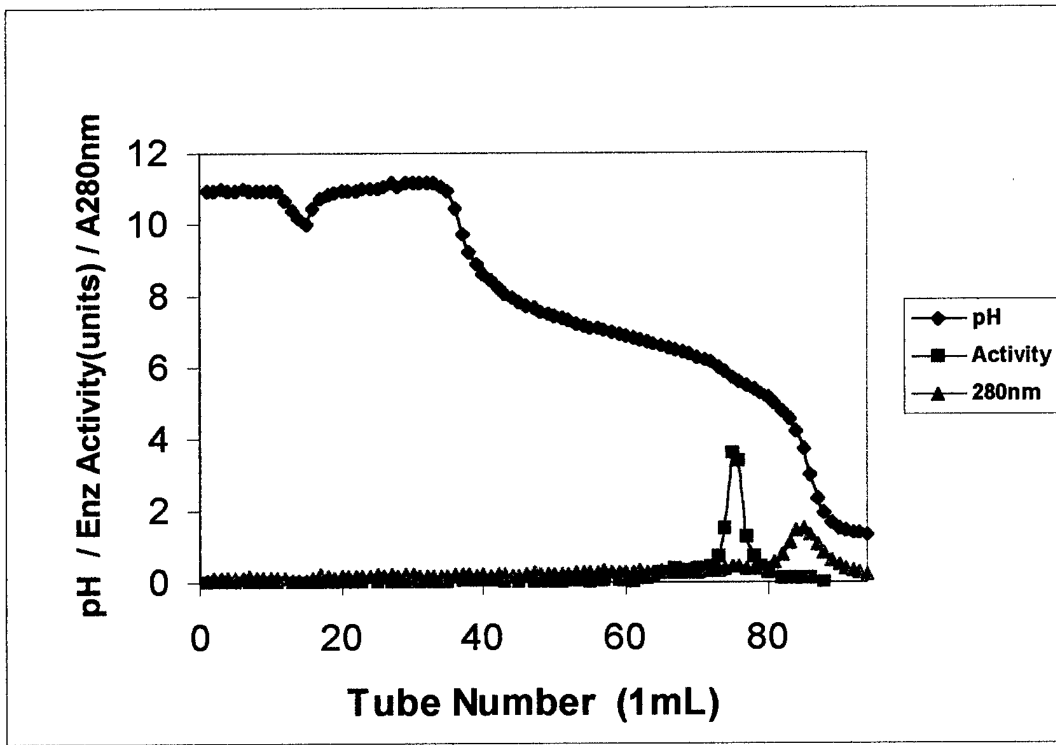

Figure 3b: Preparative Electrofocusing Chromatogram

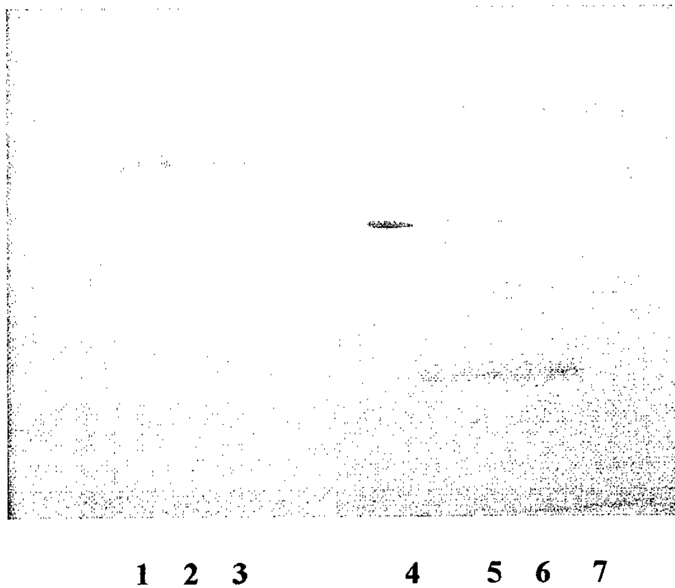

Figure 3c: Nondenaturing Page Gel electrophoresis of fractions from figure 3a. Lanes 1-3; Zymogram showing enzyme activity from peak fractions 31,32,33. Lane 4; albumin standard, Lanes 5-6; Coomassie blue staining of peak fractions 31,32,33.

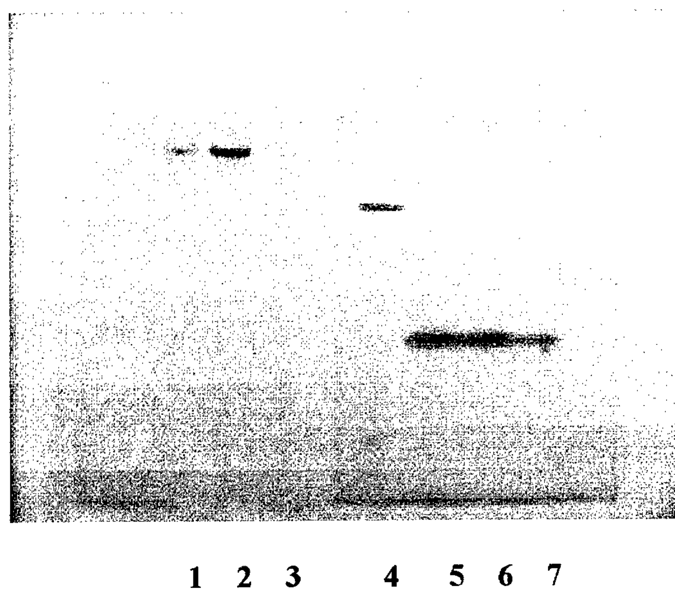

Figure 3d: Nondenaturing Page Gel electrophoresis of fractions from figure 3b. Lanes 1-3; Zymogram showing enzyme activity from peak fractions 74,75,76. Lane 4; albumin standard, Lanes 5-6; Coomassie blue staining of peak fractions 74,75,76.

Table I summarizes the effects of physostigmine and soman on the recombinant human liver carboyxlesterase produced from cDNA in human 293T cells. The data show a lack of inhibition by the general esterase inhibitor, physostigmine. The enzyme was about almost completely inhibited the carboxylesterase inhibitor, soman, and the inhibited recombinant enzyme was reactivated to greater than 90% activity by the oxime, diacetylmonoxime.

Table 1: Inhibition and reactivation of recombinant Carboxylesterase

	Carboxylesterase Activity (% of Control)[a]
Treatment	**293T cells**
Physostigmine[b]	96+/-2
Soman[c]	8+/-4
Oxime[d]	91+/-4

[a] Activities (means +/- std. err.) are expressed relative to untreated extracts.
[b] Incubation with 10μM physostigmine for 30 minutes at 25°C (pH 7.4).
[c] Incubation with 1μM soman for 30 minutes at 25°C (pH 7.4).
[d] Incubation of soman-inhibited carboxylesterase with 1 mM diacetylmonoxime for two hours at 25°C (pH 7.4).

Reportable Outcomes:

Manuscript, abstract, and presentation

Conclusion:

Clones containing the secretory form of human liver carboxylesterase were used to establish culture conditions where the enzyme is produced maximally in the 293T human cell line. This objective was accomplished by growing cells in a chemostat where the cells are maintained in a constant exponential phase of growth. In addition, conditions were defined which allow for an optimal time of removal of the culture media for isolation of carboxylesterase. Enzyme was isolated from the culture media, and the initial steps of enzyme purification using ammonium sulfate precipitation, HIC chromatography, anti-bovine BSA IgG chromatography, and preparative liquid isoelectric focusing were accomplished. It was observed that anti-bovine BSA IgG

chromatography removed greater than 90 % of the contaminating protein, but resulted in a loss of enzyme and was not effective in removing minor contaminants from the cell culture media. The use of preparative liquid electrofocusing columns, however, was found to be efficient and resulted in a preparation that was free of bovine serum albumin and other minor contaminants as measure by polyacrylamide gel electrophoresis.

A major goal of this study was to produce a recombinant carboxylesterase that has the potential for use as an antidote against the action of soman and other toxic organophosphorus agents. For this purpose it was important that the recombinant enzyme, unlike the cholinesterases, possess a relatively specific esterase activity and that the recombinant enzyme be regenerable by oxime agents. In this regard, carboxylesterase can be differentiated from cholinesterase [20] by the inability of carbamates to inhibit carboxylesterase at concentrations that readily inhibit cholinesterase [23]. Additionally, carboxylesterase can be differentiated by its ability to be reactivated by oximes after inhibition by organophosphorus compounds; cholinesterase cannot be reactivated after inhibition by organophosphorus compounds. Physostigmine is the carbamate most commonly used to differentiate carboxylesterase from cholinesterase [22] and soman is the organophosphorus compound that has the greatest difference in oxime reactivation to differentiate inhibition of carboxylesterase and cholinesterase [23]. Thus, our findings show that the recombinant cDNA of human liver carboxylesterase codes for an enzyme that is processed in the Golgi to produce an active form of the enzyme in human cells. Most importantly, the recombinant carboxylesterase is not inhibited by physostigmine and it is almost completely regenerable by an oxime.

References:

1. Junge, W., Heymann, E., Krisch, K. and Hollandt, H. (1974) Human liver carboxylesterase. *Arch. Biochem. Biophys.* **165**, 749-763.

2. Miller, S. K., Main, A. R. and Rush, R. S. (1980) Purification and physical properties of oligomeric and monomeric carboxylesterases from rabbit liver. *J. Biol. Chem.* **255**, 7161-7167.

3. Mentlein, R., Ronai, A., Robbi, M., Heymann, E., and Deimling, O. (1987) Genetic identification of rat liver carboxylesterases located in different laboratories. *Biochim. Biophys. Acta* **913**, 27-38.

4. Ozols, J. (1989) Isolation, properties, and the complete amino acid sequence of a second form of a 60-kDa glycoprotein esterase. *J. Biol. Chem.* **264**, 12533-12545.

5. Long, R. M., Satoh, H., Martin, B., Kimura, S., Gonzales, F. J. and Pohl, L. R. (1988) Rat liver carboxylesterase: cDNA cloning, sequencing, and evidence for a multigene family. *Biochem. Biophys. Res. Commun.* **156**, 866-873.

6. Robbi, M., Beaufay, H. and Octave, J. N. (1990) Nucleotide sequence of cDNA

coding for rat liver pI-6.1 esterase (ES-10), a carboxylesterase located in the lumen of the endoplasmic reticulum. *Biochem. J.* **269**, 451-458.

7. Robbi, M. and Beaufay, H. (1994) Cloning and sequencing of rat liver carboxylesterase ES-3 (Egasyn). *Biochem. Biophys. Res. Commun.* **203**, 1404-1411.

8. Yan, B., Yang, D., and Parkinson, A. (1995) Cloning and expression of hydrolase C, a member of the rat carboxylesterase family. *Arch. Biochem. Biophys.* **317**, 222-234

9. Robbi, M., Van Schaftingen, E. and Beaufay, H. (1996) Cloning and Sequencing of rat liver carboxylesterase ES-4 (microsomal palmitoyl-CoA hydrolase). *Biochem. J.* 313, 821-826.

10. Long, R. M., Calabrese, M. R., Martin, B. M. and Pohl, L. R. (1991) Cloning and sequencing of a human liver carboxylesterase isoenzyme. *Life Sci.* **48**, PL-43-PL-49.

11. Shibata, F., Takagi, Y., Kitajima, M., Kuroda T. and Omura, T. (1993) Molecular cloning and characterization of a human carboxylesterase gene. *Genomics* **17**, 76-82.

12. Kroetz, D. L., McBride, O. W., and Gonzales, F. J. (1993) Glycosylation-dependent activity of baculovirus-expressed human liver carboxylesterases: cDNA cloning and characterization of two highly similar enzyme forms. *Biochemistry* **32**, 11606-11617.

13. Robbi, M. and Beaufay, H. (1988) Immunochemical characterization and biosynthesis of pI-6.4 esterase, a carboxylesterase of rat liver microsomal extracts. *Biochem. J.* **254**, 51-57.

14. Robbi, M. and Beaufay, H. (1986) Biosynthesis of rat liver pI-5.0 esterases in cell-free systems and in cultured hepatocytes. *Eur. J. Biochem.* **158**, 187-194.

15. Robbi, M. and Beaufay, H. (1987) Biosynthesis of rat liver pI-6.1 esterase, a carboxylesterase of the cisternal space of the endoplasmic reticulum. *Biochem. J.* **248**, 545-550.

16. Robbi, M. and Beaufay, H.. (1992) Topogenesis of carboxylesterases: a rat liver isoenzyme ending in -HTEHT-COOH is a secreted protein. Biochem. Biophys. Res. Commun. **183**, 836-841.

17. Maxwell, D. M. (1992) Detoxication of organophosphorus compounds by carboxylesterase *in "Organophosphates: Chemistry, fate and effects"* (Chambers, J. E. and Levi, P. E., Eds.), pp.183-199, Academic Press , San Diego.

18. Leinweber, F. J. (1987) Possible physiological roles of carboxylic ester hydrolases. *Drug Metab. Rev.* **18**, 379-439.

19. Sterri, S. H., and Fonnum, F. (1987) Carboxylesterases in guinea pig plasma and liver. *Biochem. Pharmacol.* **36**, 3937-3942.

20. Sterri, S. H. (1989) The importance of carboxylesterase detoxification of nerve agents. *Proc. 3rd Int. Symp. Protection Against Chemical Warfare Agents, Sweden*, pp.235-240.

21. Miller, A..D., Scott, D.F.,Chacko, T.L., Maxwell, D.M., Schlager, J.J.,and Lanclos, K.D.(1999), Expression and Purification of a Recombinant Secretory Form of Human Liver Carboxylesterase. *Protein Expression and Purification* **17**, 16-25

22. Sterri S.H., Johnsen B.A. and Fonnum F., (1985) A radiochemical assay method for carboxylesterase, and comparison of enzyme activity toward the substrates methyl [1-^{14}C] butyrate and 4-nitrophenyl butyrate. *Biochem Pharmacol* **34:** 2779-2785, 1985.

23. Maxwell D.M., Lieske C.N. and Brecht K.M.(1994) Oxime-induced reactivation of carboxylesterase inhibited by organophosphorus compounds. *Chem Res Toxicol* **7:** 428-433, 1994