



# **Plant Production of Human Cholinesterases for Protection against Nerve Agents**

**Tsafrir Mor**

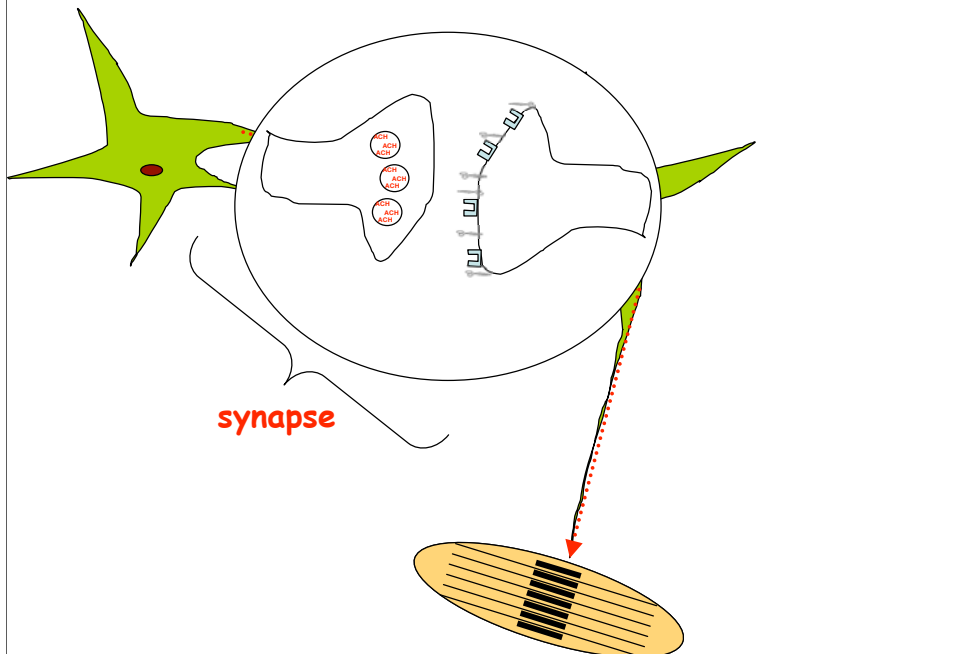
The Biodesign Institute & School of Life Sciences  
Arizona State University

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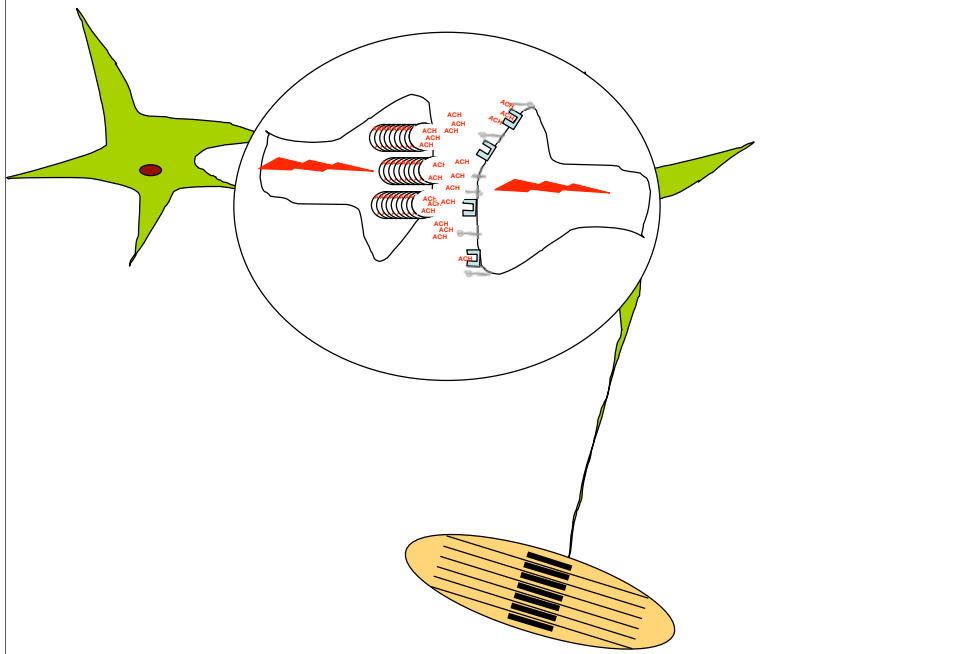
Center for Infectious Diseases and Vaccinology

### The cholinergic synapse



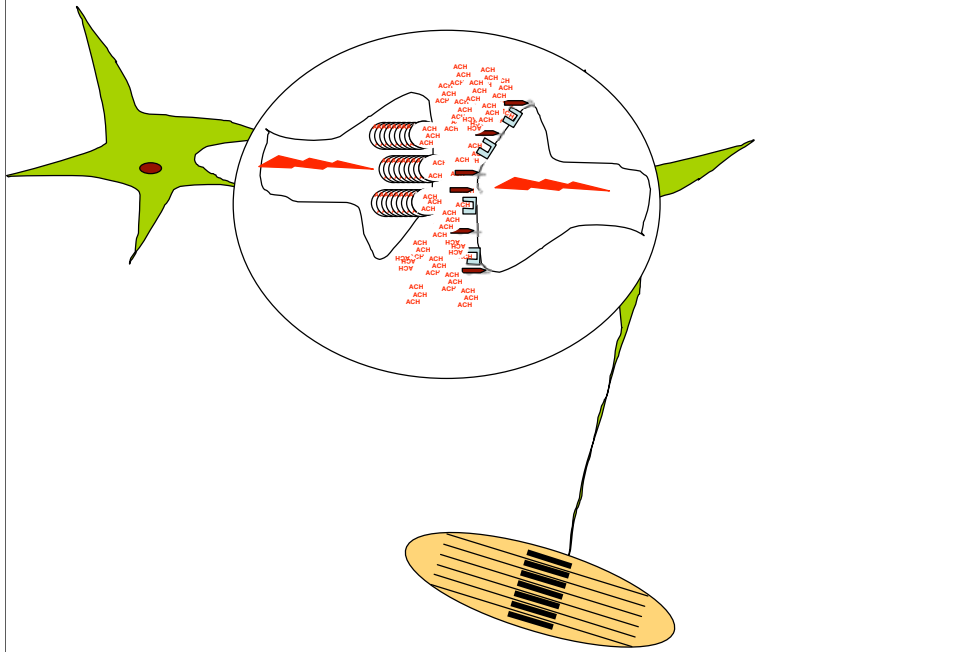
Nerve impulses traveling down the axon are essentially an electric phenomenon, resulting from the transient opening of voltage-gated ion channels. However, across the synaptic cleft, the gap between one nerve cell and another (or between a nerve and a different target cell, such as a muscle cell), the signal is communicated chemically by neurotransmitters, small diffusible molecules like acetylcholine (ACh).

## The cholinergic synapse



In the presynaptic axonal end, the neurotransmitter is packed into synaptic vesicles, which dump their content into the synaptic cleft upon arrival of an impulse. ACh then diffuses to the postsynaptic membrane where it binds to one of several types of ACh receptors and initiate the postsynaptic responses - triggering of another impulse or causing the muscle to contract. Efficient removal of ACh by the hydrolyzing enzyme acetylcholinesterase (AChE) returns the synapse to the ground state in preparation for the next impulse.

## The cholinergic synapse



Inhibiting the activity of AChE by inhibitors would lead to the unregulated accumulation of ACh in the synapse and continuous activation of the postsynaptic cell. In the case of muscles, this leads to a prolonged contraction or tetanus. This so called “cholinergic crisis” is potentially lethal.

## Organophosphate Toxins

Organophosphate toxicity occurs by inhibition of acetylcholinesterase

Organophosphates have both military and civilian applications:



Sarin, Soman, Tabun, VX



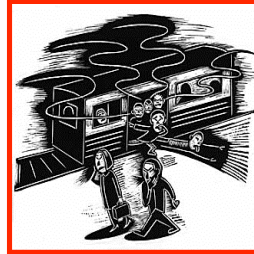
Malathion, Parathion,  
Diazinon, Fenthion,  
Dichlorvos, Chlorpyrifos

Clinical manifestation ranges from common muscarinic symptoms to death resulting from respiratory failure

The catalytic mechanism of AChE and its critical role make the enzyme vulnerable to a variety of inhibitors. While some naturally occurring AChE inhibitors are very potent, human exposure to them is rare. However, manmade anti-AChE compounds, especially organophosphates (OPs), are widely used as pesticides and pose a substantial occupational and environmental risk. Even more ominous is the fear of deliberate use of OPs as chemical warfare agents against individuals or populations by terrorists or by governments which defy international conventions.

Organophosphate nerve-agents are simple to synthesize, store and use.

There's recent history of "successful" use of nerve agents by rogue states and terrorist organizations



Toyko Sarin Attack

Considerable damage is predicted



Halabja, Iraq  
March 17th 1988



Recent use of OP nerve agents as a non-conventional weapon as part of a military campaign occurred in the late 80s during the Iraq-Iran war . At the same time, the Baathist regime of Sadam Hussein used nerve agents in his campaign against civilian Kurds in Northern Iraq. Even more recently, nerve agents were used by the Aum Shinrikyo, a Japanese fundamentalist religious sect and terrorist organization active during the 1990s. Aum Shinrikyo released Sarin in several metro stations and government ministries in Japan.

### Medical Protection

- Antidotes:

- Atropine

- Receptor antagonist

- 2-PAM

- Active-site

- Reactivation

- Prophylactics:

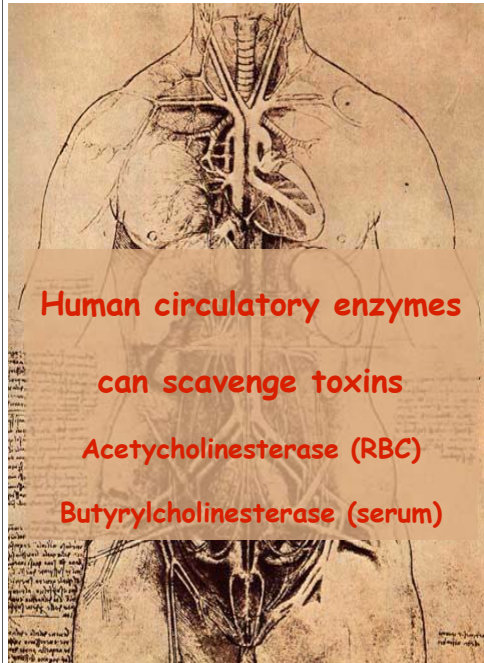
- Pyridostigmine



Atropine self-injection syringe  
from a Chemical and Biological  
Warfare kit

Current medical intervention for organophosphate poisoning consists of a combination of drugs. Pre-treatment with spontaneously reactivating acetylcholinesterase inhibitors, such as the carbamate and pyridostigmine, can protect acetylcholinesterase from irreversible inhibition and induce synthesis of the ACHE gene. Pre-treatments augment the effectiveness in vivo of atropine/oxime therapy but do not alter the acute toxicity of organophosphate poisoning. Moreover pyridostigmine and atropine are poisons, even at allopathic dosages, and it seems pyridostigmine administration is the major contributor to Gulf War Syndrome. Now for the good news: within the last decade, a single protective drug candidate, devoid of pharmacological effects, which would provide protection against the lethality of anti-cholinesterases and prevent post-exposure incapacitation, has been realized.

## Cholinesterases as bioscavengers



Human cholinesterases can be effectively used to prevent or treat nerve-agent poisoning.

Are "disposable molecular sponges": needed in stoichiometric rather than in catalytic quantities

Available sources include:

- outdated human plasma
- cell cultures
- transgenic goats.

These sources are

- Expensive
- supply-limited
- may be contaminated with human pathogens and prions.

The occurrence of cholinesterases in blood, AChE on the surface of erythrocytes, and BChE in the serum, is a long-standing enigma. One of the roles ascribed to cholinesterases in blood is that of circulating scavengers of anticholinesterase inhibitors. Such agents can reach the vital and vulnerable synaptic AChE. The importance of the latter is evident in individuals who are homozygous for the "atypical" mutation that reduces affinity of BChE to many inhibitors and substrates. These individuals exhibit adverse responses to AChE inhibitors.

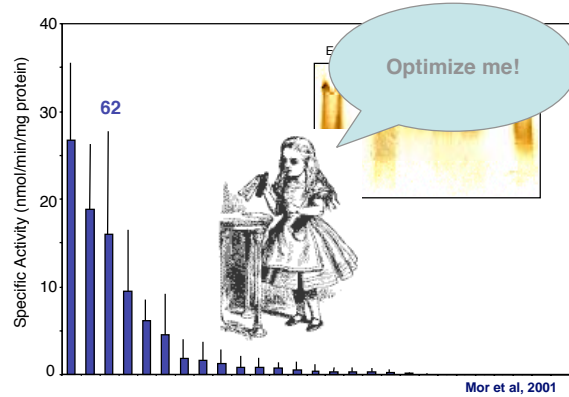
A different approach in treatment and prevention of anti-AChE toxicity seeks to mimic this role of blood cholinesterases in sequestration of anti-AChE agents. Their natural potential to counter-act the toxic effects of anti-cholinergic agents can be boosted by administration of exogenous cholinesterases. The efficacy of this treatment to protect against a challenge of OPs was tested in a variety of animal models such as mice, rats, guinea pigs and primates, and was found to be comparable to or better than the currently used drug regimens in preventing OP-induced mortality without any detrimental side effects. Enzyme therapy has the additional benefit of the relatively long half life time (several days) of the injected enzymes in the

## Why produce cholinesterases in plants?

- **Cholinesterases are effective against OPs BUT you need stoichiometric amounts**
- **Scope:** Unparalleled types of recombinant products that transgenic plants can provide
- **Cost:** Inexpensive to produce and store
- **Scale:** Agriculture *IS* mass production
- **Safety:** No contaminating human pathogens and prions



## Transgenic tomato plants expressing AChE



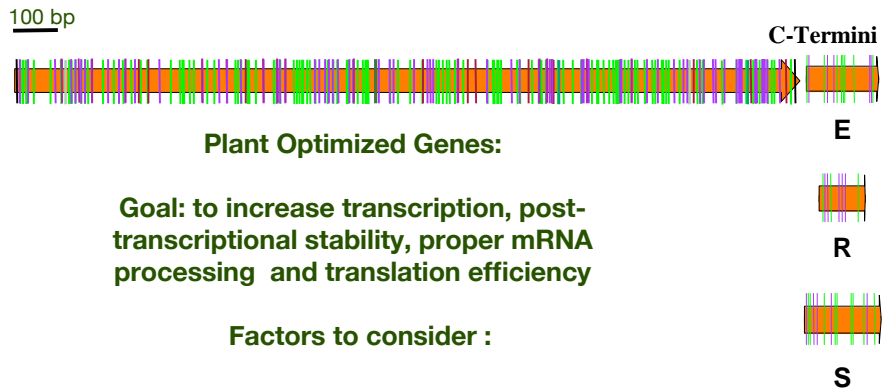
Plants can produce human AChE:



- Active
- Kinetic properties
- Inhibitor binding

We have identified several transgenic tomato lines that expressed relatively high levels of the recombinant human enzyme, which was shown to be kinetically similar to native human AChE. However, expression levels were low and it was clear that the expression has to be optimized.

## Optimization of the human AChE sequence for plant expression

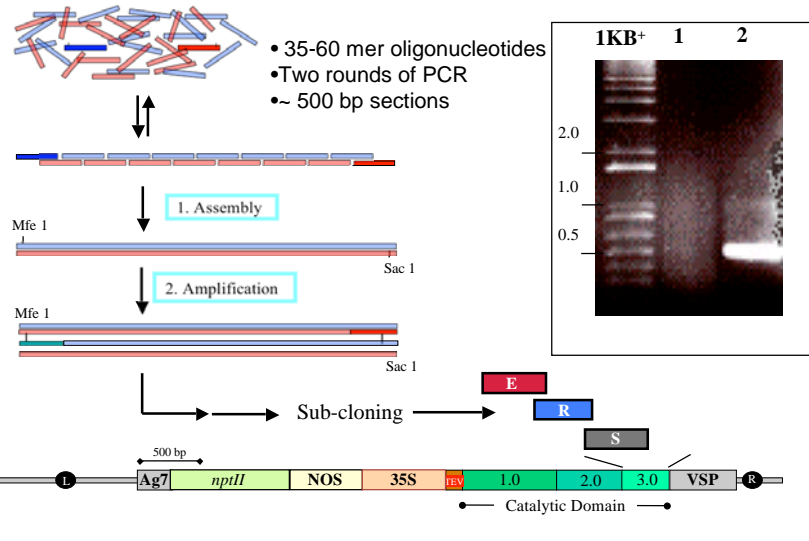


- Potential polyadenylation signals
- Cryptic introns
- Unfavorable codons
- Potential methylation sites

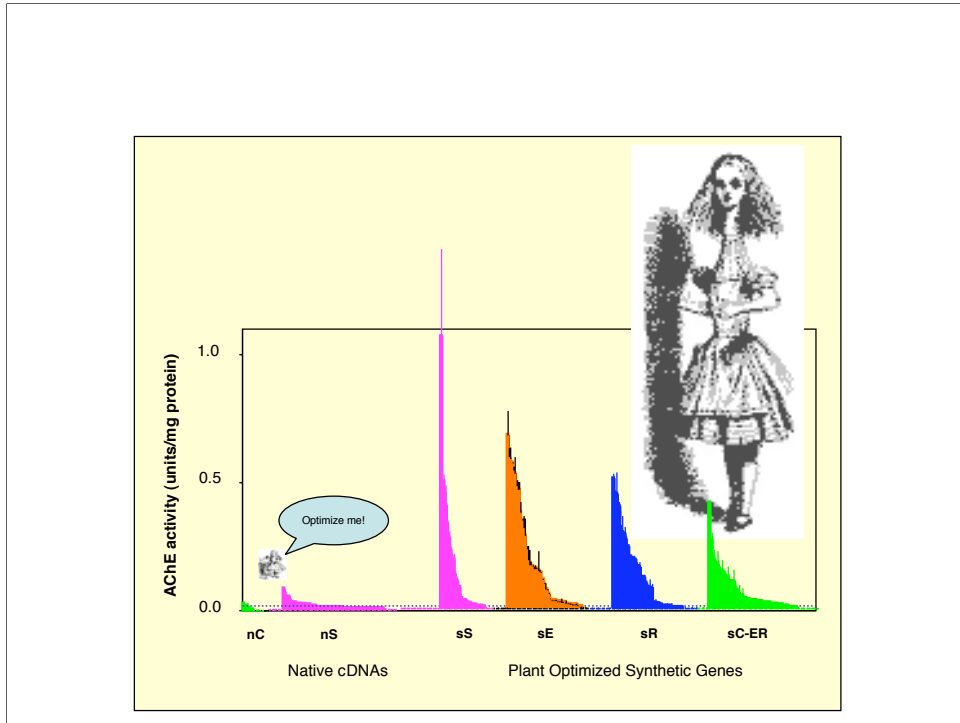
Optimization of the human AChE sequence requires recreating it “from scratch”. Care is taken to conform the codon usage to that of highly expressed plant genes and to eliminate potentially deleterious molecular signals and sequence features.

Humans and other mammals express several molecular variants of AChE . All share a common core domain and enzymatic properties but possess distinct C-terminal peptides, expression patterns, subcellular localization and protein partner interactions. We optimized the sequence of the three common splice variants of AChE.

## De novo synthesis of plant-expression optimized gene encoding human acetylcholinesterase

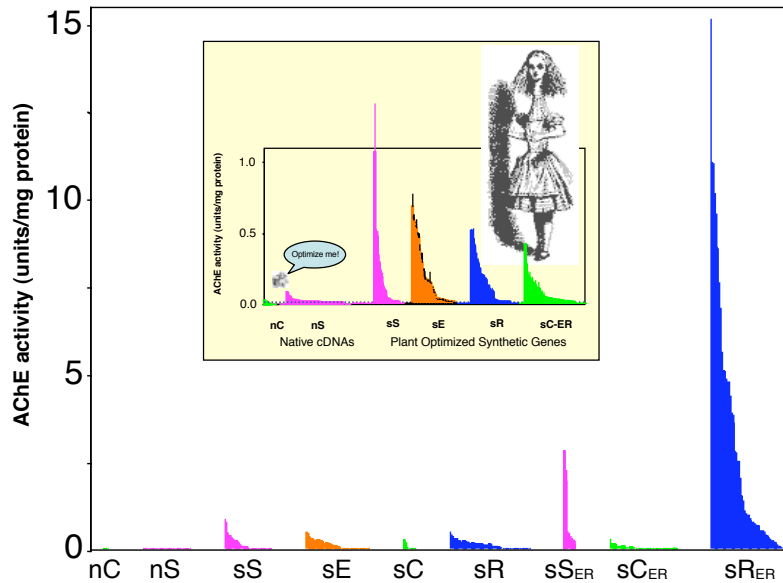


After the sequence was designed for optimal plant expression, we had to build the genes for each isoform using a PCR-based *procedure*. First, oligonucleotides with overlapping complementary sequences are mixed and subjected to a first assembly round of PCR. This produces various sized fragments which can be seen as a smear on an agarose gel – lane 1. Second, the assembly PCR reaction mix is subjected to a second round of PCR using outside primers to amplify the fragment to a workable quantity, as demonstrated in lane 2. It is possible to synthesize sizeable fragments with this technique, however, we found it more advantageous to construct the complete gene sequences in a modular fashion. The synthetic fragments were put together through rounds of sub-cloning and finally, each isoform, including a plant optimized catalytic domain, was put into an expression cassette complete with a selectable marker.



Previous reports from our group focused on plant expression of the common core domain, which accumulated to low levels ( $\sim 0.002\%$  TSP) due to the inefficient translation of the human sequence in plant cells. *De novo* construction of plant-expression optimized genes encoding all three physiological C-terminal variants resulted in  $\sim 50$ -fold higher accumulation levels of the proteins in *N. benthamiana* ( $\sim 0.05\%$  TSP) as compared to the native human sequence (data not shown).

### Optimization of AChE coding region enhances expression in plants



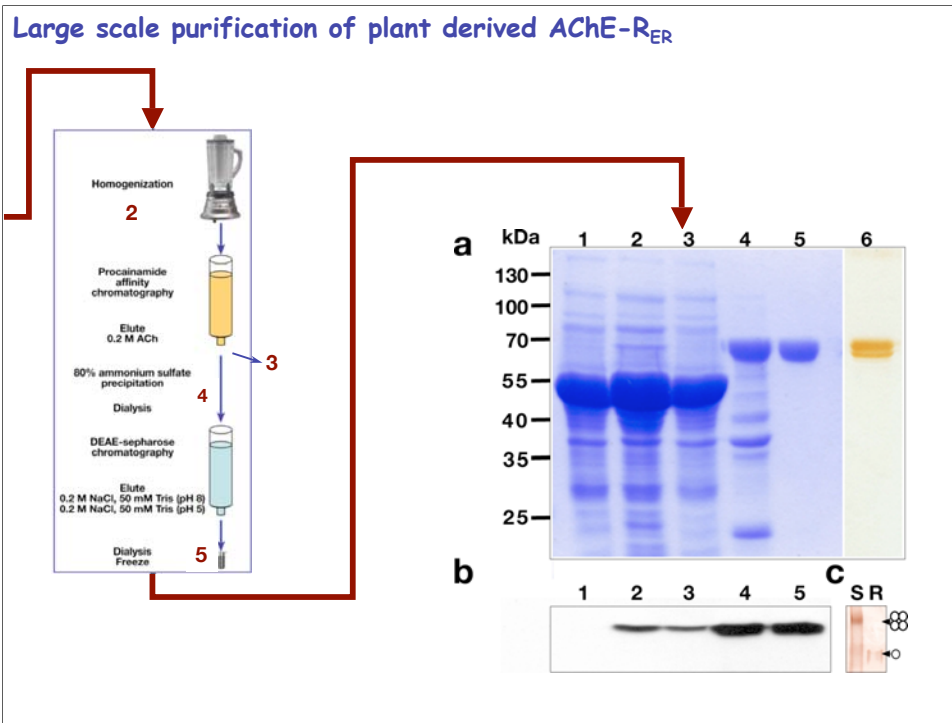
Plants, like other heterologous expression systems, may produce recombinant human proteins that are decorated with non-human complex glycans, predicting their fast circulatory clearance. In addition, plant glycans may contain unique glyco-epitopes that may be allergenic in some cases. We therefore engineered AChE-R to contain the C-terminal peptide SEKDEL (AChE-R-ER), allowing its retrograde transport from the *cis*-Golgi back to the ER. These modifications enabled us to select a plant line, harboring at least four copies of the transgene that accumulated AChE-R<sub>ER</sub> to 0.5%-1.0% TSP (~19 U/mg protein, or ~30 mg/kg fresh weight).

Large scale purification of plant derived AChE-R<sub>ER</sub>



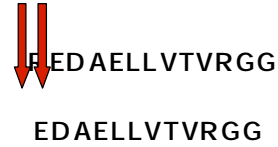
Transgenic *N. benthamiana* plants  
expressing AChE-R<sub>ER</sub> at the ASU  
Pharming Greenhouse.

The selected line was propagated for seed and biomass production in a USDA-approved containment greenhouse. The plants appeared phenotypically indistinguishable from WT plants under normal growth conditions.

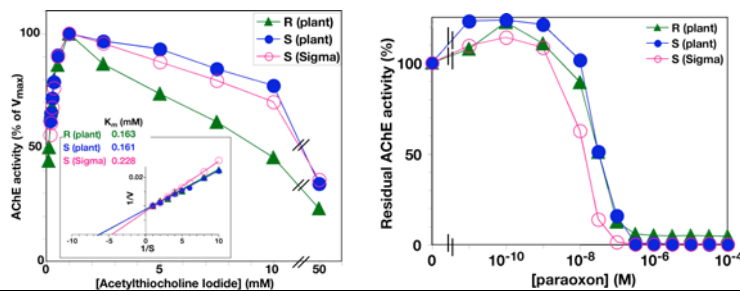


The AChE-R<sub>ER</sub> protein can be detected in crude plant extracts by SDS-PAGE, revealing a 67 kDa band absent from the WT's extract. AChE-R<sub>ER</sub> was purified to homogeneity from plant extracts using procainamide-affinity chromatography followed by anion-exchange chromatography with final specific activity of the purified protein > 3000 U/mg. When resolved by SDS-PAGE and visualized either by Coomassie or silver staining, the purified protein appeared as a doublet that strongly reacts with AChE-specific Abs (Fig. 2A,B). As predicted by its retention in the ER, the protein contains high-mannose glycans (data not shown) and the two bands apparent in the gel possibly represent two differently glycosylated

## N-terminal sequencing



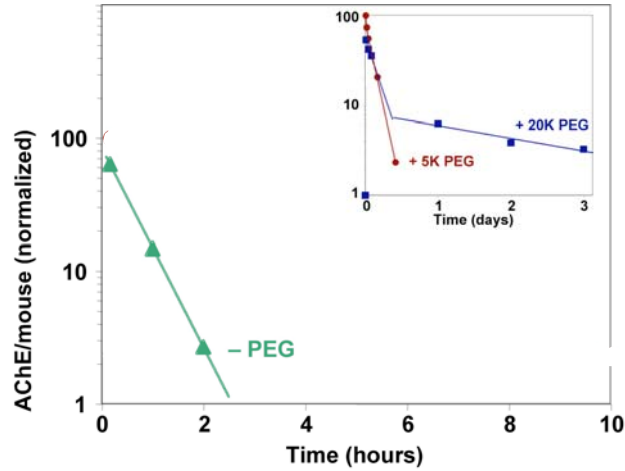
## Kinetic analysis of plant derived AChE-R<sub>ER</sub>



Human AChE in plants, like its native counterpart, is processed by cleavage of the N-terminal signal peptide.

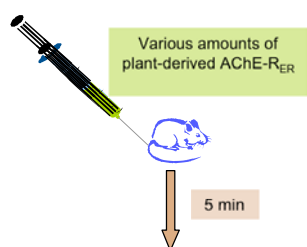
Activities of plant-derived human AChE-R<sub>ER</sub> and human AChE-S were compared to cell culture-derived human AChE-S.  $K_m$  values for all three variants were very similar to each other and to those reported in the literature. The enzymes exhibited characteristic inhibition by substrate conc. >2 mM, with no significant differences between the plant- and cell-derived AChE-S. Interestingly, substrate inhibition was more pronounced in the case of the R variant, compatible with C-terminus- active site intramolecular signaling. Only slight differences were observed in the ability of AChE-R<sub>ER</sub> to bind and be inhibited by paraoxon, the active OP metabolite of the pesticide parathion (Fig. 2E).

## Pharmacokinetics



The clearance of plant-derived AChE-R<sub>ER</sub> in mice was determined by i.v. injection of enzyme (either 400 U or 1000 U) and measuring its residual activity in plasma samples in the presence of iso-OMPA (Fig. 3A). The plant-derived human enzyme was cleared rapidly with circulatory  $t_{1/2} = 24$  min, similar to observations with AChE from other sources. *In vitro* conjugation of polyethyleneglycol (PEG) significantly prolongs the circulatory half life of the plant-derived enzyme.

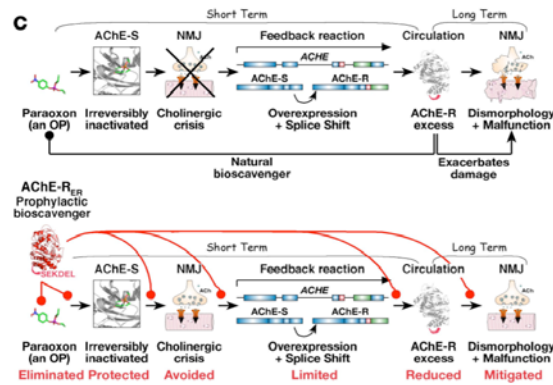
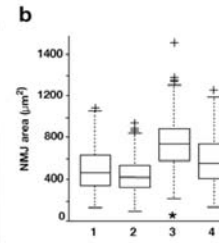
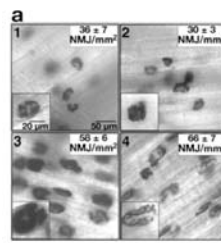
### Protection against organophosphate challenge



*In vivo* titration showed dose-dependent protection by intravenously injected AChE-R<sub>ER</sub> of FVB/N male mice challenged with a lethal dose of paraoxon, with complete elimination of short-term clinical symptoms at near molar equivalence.

## Mitigation of long-term effects of OP-poisoning

Group	Enzyme	OP
1	-	mock
2	400 U	Mock
3	-	0.8 x LD <sub>50</sub>
4	400 U	0.8 x LD <sub>50</sub>



Importantly, by 10 days post-exposure, AChE-R prophylaxis markedly limited post-exposure increases in plasma murine AChE-R levels while minimizing the organophosphate-induced neuromuscular junction dismorphology. Our findings present plant-produced AChE-R<sub>ER</sub> as a bimodal agent, conferring both short and long-term protection from organophosphate intoxication.

### **Mechanism of chronic toxicity and AChE-R treatment**

- Do alternative splicing events play a role in the toxicity of organophosphate toxins?
- Can we see evidence to such involvement in the RNA level?
- Are these events preventable with cholinesterase therapy?
- AChE-R, AChE-S, ARP, BChE?

### **AChE-R treatment to mitigate other neurodegenerative pathologies**

- Alzheimer's disease
- Parkinsonism

**A "cholinergic system" in plants?**

## A Plant Cholinesterase?



Figure 1. AChE activity staining in 12 day old peas roots. (A) With substrate. (B) ChE inhibited by 0.5 mM BW. (C) ChE inhibited by 0.5 mM neostigmine. (D) No substrate. (E) Tissue boiled prior to staining.

Although the best-defined role of AChE is to terminate cholinergic transmission signals, the presence of the enzyme and its substrate is not limited to the metazoan nervous system. AChE activity was visualized by histochemical staining of roots of 12-day old Alaskan pea plants (*Pisum sativum* L., A). Activity could be partially blocked by the bisquaternary AChE inhibitor BW (B), similar to our observations with the tomato enzyme, but was completely inhibited by the carbamate neostigmine (C). Very little rust colored copper deposits were visible in the absence of the acetylthiocholine substrate or upon denaturation of the proteins by boiling the root tissue prior to staining (D and E).

Many signal transduction pathways have been evolutionarily conserved and their roots can be traced back to progenitor unicellular organisms. While the pathways have acquired different roles in different organisms, much of their biochemistry has been conserved. Thus, acetylcholine (ACh), its receptors, and the enzymes involved in its metabolism, such as AChE, are ubiquitous in multicellular animals, however, there is evidence for their presence also in plants. Our lab is interested in identifying the components of such a putative plant “cholinergic” system and in elucidating their roles .

## Collaborations

### **ASU**

Charlie Arntzen (CIDV)  
Hugh Mason (CIDV)  
Steve Slater (CIDV)  
Qiang Chen (CIDV)  
Bert Jacobs (CIDV)  
Petra Fromme (Chemistry/Biochemistry)

### **Hebrew University**

Hermona Soreq

### **California DHHS**

Carl Hanson

### **Center for Catalytic Bioscavengers**

David Lenz (USAMRICD)  
Dan Tawfik (Weizman)  
John Cashman (HBRI)  
Christopher Hadad (OSU)

### **Institute Cochin (Paris V)**

Morgane Bomsel  
Annett Alfsen

### **Bioscavengers:**

(DARPA contract); USAMRICD; NIH U54 Cooperative Agreement

### **HIV:**

(NIH R21); NIH U19