Biotechnology Journal

DOI 10.1002/biot.200600166

Biotechnol. J. 2006, 1, 1370–1380

Review

Enzymes fight chemical weapons

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Received 25 August 2006
Revised 18 October 2006
Accepted 9 November 2006

Keywords: Chemical weapons · Ecotoxicological effects · Enzymatic decontamination · Human health

1 History of chemical warfare

Chemical weapons have been used for millennia, with evidence found for the existence of advanced forms of chemical weapons in ancient and classical times (Fig. 1). Poisons were mainly derived from animal venom or poisonous plants at that period. The Chinese used irritant and poisonous smokes as early as 1000 B.C. Burning wax, pitch, and sulfur were used in wars between the Athenians and Spartans. During the 7th century A.D., Kallinikos, an architect from Heliopolis in Syria, invented Greek fire, probably a combination of rosin, sulfur, naphtha, quicklime and saltpeter. It could continue burning under almost any conditions, even floated on water, which was particularly effective in naval operations. During the Renaissance, people again considered using chemical warfare. Powder of sulfide and arsenic, verdigris and several toxic smokes and fumes were used for warfare in the 16th and 17th century. In the 19th century several proposals were made to initiate chemical warfare, most of them were never put into practice. Nevertheless, the Brussels Convention of 1874 attempted for the first time to prohibit the use of poisons in war. The modern chemical warfare era began during World War I when the first chemical agent to be used was chlorine gas, which was released into the wind by the Germans against the Allies on April 1915 near the Belgian village of Ypres. The attack produced heavy casualties, including 600 deaths. But this was not the only chemical weapons attack during World War I. Both the British and German forces used chlorine gas, mustard gas and phosgene heavily before the war ended. Overall about 51 000 tons of chemical weapons were used in World War I, killing around 85 000 and causing a total of 1.2 million casualties. After World War I, many governments wished to ban chemical weapons because of the horrendous means by which they killed and injured people. In 1925, the Geneva protocol for the prohibition of the use in war of asphyxiating, poisonous or other gases and of bacteriological methods of warfare was signed by the League of Nations, and has since been signed by over 130 nations. However, the protocol does not prohibit the manufacture and threat of use of chemical weapons, and the protocol has no provisions for the punishment of countries that use such chemical weapons. Between the wars, chemical agents were used by Spain in Spanish Morocco, by Italy in the invasion of Abyssinia, and by Japan in Manchuria and China. During World War II, even though no chemical weapons were used in battle, large amounts of a new chemical weapons developed by the Germans were later discovered. These new chemical weapons were known as nerve agents. During 1950s and 1960s, the United Kingdom and the United States developed and produced in large scale a chemical agent many times greater in lethal properties than any other known chemical agent known under the codename VX. Due to the secrecy of the Soviet Union's government, very little information was available about the direction and progress of the Soviet chemical weapons until relatively recently. Several highly toxic agents were developed during the mid 1980s by Soviet chemists. During the 1980s, many accounts of the use of chemical warfare agents occurred in Laos, Cambodia and Afghanistan. Iraq used chemical weapons against Iran and also against its own people and killed about 5000 Kurdish citizens in Halabja, in March 1988. In 1992, after a decade of long and painstaking negotiations, the Conference on Disarmament agreed to the text of the Chemical Weapons Convention.

The Chemical Weapons Convention was signed in 1993 and entered into force on April 1997, augmenting the Geneva Protocol for chemical weapons and includes extensive verification measures such as on-site inspections. Despite the numerous efforts to reduce or eliminate chemical weapons, some nations continue to research

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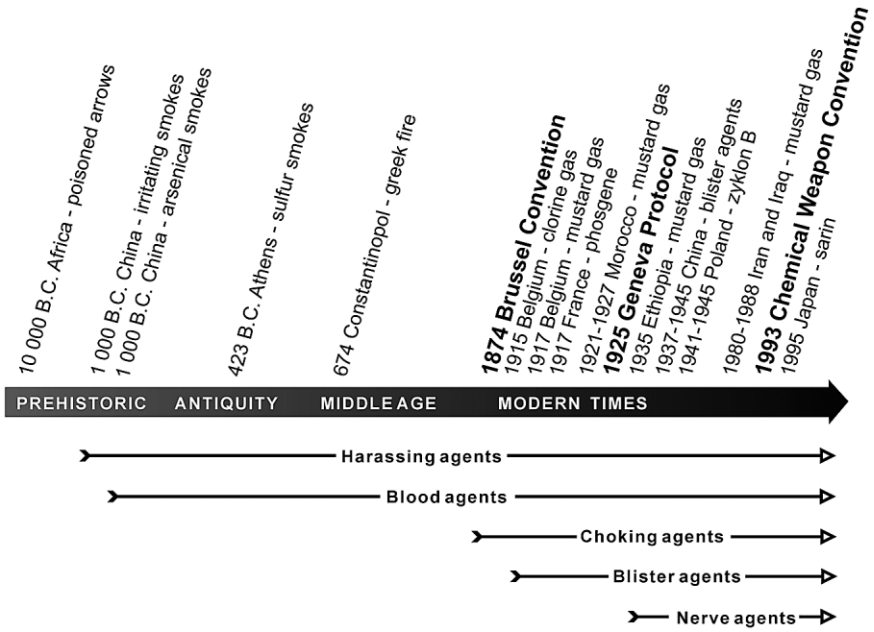

Figure 1. Historical development of chemical warfare agents.

and/or stockpile chemical weapon agents. Nevertheless, the major risk of use of chemical weapons at the current time is a terrorist attack. In 1994, a Japanese religious cult, Aum Shinrikyo, reportedly released a nerve agent in a residential area of Matsumoto, Japan, that killed 7 and injured 500 people. A second attack on March 1995 spread sarin through a crowded Tokyo subway. This act of terrorism killed 12 and injured more than 5500 civilians. In 2001, after carrying out the attacks in New York City on September 11, the organization al Qaeda announced that they were attempting to acquire radiological, biological and chemical weapons as well. For many terrorist organizations, chemical weapons might be considered an ideal choice for a mode of attack. They are cheap, relatively accessible, easy to transport and capable of generating significant panic among the general public. A skilled chemist can readily synthesize most chemical agents if the precursors are available. The threat of chemical weapons is now ubiquitous.

2 Decontamination of warfare agents

Parallel to the development and use of chemical weapons, there was always interest in the methods to decontaminate these toxic chemicals. The aim of decontamination is to rapidly and effectively render harmless or remove poisonous substances from both personnel and equipment. The most important decontamination measure naturally concerns the individual. There are several ways that decontamination of chemical weapons can be achieved. Many of them are available in a standard chemical weapons decontamination kit, usually based on powder decontaminates that absorb liquid chemical weapon agents, thus removing them, or liquid decontaminates that dissolve the chemical weapons agent. Washing off or adsorbing the chemical agent may leave residual substance that is still poisonous. Most chemical agents can be destroyed by means of suitable chemicals. The chemical decontaminants bleach or strong oxidants, are effective against practically all types of warfare substances (Table 1). However, these decontaminants, some of which are toxic themselves, can generate toxic byproducts, adversely affecting the environment and potentially harming the user. Their chemically active components are stoichiometric materials that are gradually consumed during their reaction, exhibiting high unit consumption and if these compositions get into soil or water, they endanger the environment. Chemical decontaminants often exhibit undesirable aggressiveness on materials. Their application on instrumentation leads to depreciation of decontaminated material or surfaces by corrosion. Moreover, such chemicals should not be used in decontaminating skin, and are also difficult to use in enclosed spaces. These imperfections of chemical decontamination compositions can be overcame to a great extent by preparations consisting of enzyme catalysts. Enzymes are environmentally benign, highly efficient biological catalysts capable of detoxifying many times their own weight of agent. The enzymes are capable of increasing reaction rates up to 10^{12} times compared to uncatalyzed reactions [1]. In addition, enzymes are non-toxic, non-corrosive and non-flammable. When released to environment enzymes are easily biologically degradable. Another attractive feature of enzymes is that they can function effectively under mild ambient conditions and neutral pH, decreasing

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Table 1. Comparison of chemical and enzymatic decontaminants of warfare agents

Decontaminant	Advantages	Disadvantages
Chemical	Broad range effectiveness	Toxic
	Good stability	Corrosive
	Easy preparation	Harmful to environment
		Aggressive to skin
		High unit consumption
		Sometimes toxic byproducts
		Not compatible with other decontaminants
Enzymatic	Non-toxic	Less stable
	Non-corrosive	Laborious isolation
	Environmentally acceptable	Highest activity in water
	Highly efficient	Sometimes narrow specificity
	Operating at mild conditions	Narrow operation parameters
	Low unit consumption	Sometimes cofactor requirement
	Compatible with other agents	Prone to inhibitions
	Broadly applicable	Allergenic

energy costs, increasing safety and reducing damage to equipment, facilities and the environment. The obvious advantage of working under mild conditions can sometimes turn into drawback. If a reaction proceeds only slowly under given parameters of temperature or pH, there is only a narrow operational window for alternation. Low temperature can reduce the enzyme activity, elevated temperatures as well as extreme pH lead to deactivation of the protein [1].

Unlike most chemical catalysts, enzymes with different properties and specificities can be mixed together in a single formulation since enzymes generally function under the same or similar condition. There are also some drawbacks such as unsatisfactory stability of the enzymes or their laborious isolation. However, the improvement of purification procedures and the use of immobilized or engineered enzymes are progressively reducing these limitations. Another limitation can be caused by the low water solubility of a targeted substrate making it less available to aqueous hydrolytic enzymes. Some enzymes require natural expensive cofactors to be used in stoichiometric amounts. They can not be replaced by more economical man-made substituents and their recycling is still not a trivial task. As with all foreign proteins when inhaled or ingested, enzymes are also potential allergens [2]. Many enzymatic reactions are prone to substrate or product inhibition, which causes the enzyme to cease to work at higher substrate and/or product concentration.

In summary, the utilization of enzyme catalysts for decontamination of and protection against warfare agent offers an easy to use but at least equally effective decontamination alternative that is safer and more friendly to the environment.

The following sections give an overview of enzymes with significant capacity to neutralize particular warfare chemical agents (Table 2).

2.1 Nerve agents

During the early part of the 20th century, the interest in organophosphorus compounds was minimal. The situation has changed after discovery made by Lange in the late 1920s and early 1930s that some of these compounds were toxic [3]. These compounds block the active site of acetylcholinesterase, leading to accumulation of acetylcholine in the synapse and continued stimulation of neuro-neuro or neuro-muscular junction until the neuron is depolarized or the muscle becomes inactivated. Symptoms from the skeletal muscles are very typical. If the poisoning is moderate, this may express itself as muscular weakness, local tremors or convulsions. When exposed to a high dose of nerve agent, the muscular symptoms are more pronounced. The victim may suffer convulsions and lose consciousness. Muscular paralysis caused by nerve agents also affects the respiratory muscles. Nerve agents also affect the respiratory center of the central nervous system. The combination of these two effects is the direct cause of death by suffocation. The chemical industry has exploited the inhibition of acetylcholinesterase by developing several carbamates and phosphates for use as insecticides. The most serious application of organophosphorus fluoridates that inhibit acetylcholinesterase is for chemical warfare. These compounds have the same mechanism of action as the insecticides; however, they are orders of magnitude more toxic to humans. In 1937, Gerhard Schrader and associates in the pesticides development program in Germany discovered the high mammalian toxicity of a compound called tabun [4]. This compound was an organophosphate having a cyano leaving group and ethoxy and dimethylamino groups as modifiers.

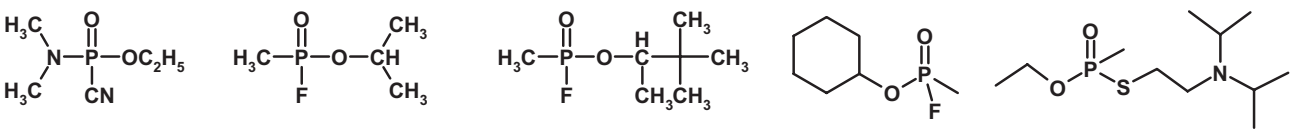
Continued experimentation under direction of the German military led to the discovery of compounds with

Table 2. Warfare chemical agents and their enzymatic decontaminants

Class	Mode of action	Symptoms	Name	Enzymatic decontaminants
Nerve	Inactivates enzyme acetylcholinesterase, prevents the breakdown of the neurotransmitter acetylcholine in the victim's synapses and causing both muscarinic and nicotinic effects	Miosis, headache, nausea, vomiting, diarrhea, muscle paralysis, loss of consciousness, death by suffocation	Tabun Sarin Soman Cyclosarin VX	organophosphate hydrolases (EC 3.1.8.1), diisopropylfluorophosphatases (EC 3.1.8.2), butyrylcholinesterase (EC 3.1.1.8)
Blister	Agents are acid-forming or alkylating compounds that damages skin and respiratory system, resulting burns and respiratory problems	Large fluid blisters, severe respiration difficulty, vomiting and diarrhea, death by pulmonary edema	Sulfur mustard Nitrogen mustard Lewisite Phosgene oxime	haloalkane dehalogenses (EC 3.8.1.5) no enzyme known no enzyme known
Choking	Action of this acids or acid-forming compounds is pronounced in respiratory system, flooding it and resulting in suffocation, survivors often suffer chronic breathing problems	Eye and skin irritation, airway irritation, dyspnea, cough, hypoxia, pulmonary edema, suffocation	Chlorine Hydrogen chlorine Phosgene Diphosgene Triphosgene	no enzyme known
Blood	Causes intravascular hemolysis that may lead to renal failure or directly prevents cells from utilizing oxygen	Cyanosis, nausea, seizures prior to death	Arsine Hydrogen cyanide Cyanogen chloride	no enzyme known cyanide sulfurtransferases (EC 2.8.1.1), cyano-L-alanine synthases (EC 4.4.1.9), cyanide oxygenases
Lachrymatory	Causes severe stinging of the eyes and temporary blindness	Irritation of eye, throat, trachea and lungs, coughing, vomiting	2-Chloroacetophenone Chlorobenzylidenemalononitrile	glutathione S-alkene transferases (EC 2.5.1.18)

P-C bond, which have toxicity exceeding that of widely used fluorophosphates. The first phosphofluoridate having enormous military potential was the isopropyl ester called sarin. Further work led to a less volatile analog, soman. Although the German military produced large quantities of tabun and sarin during World War II, they never used them against Allied forces. After the revelation of the toxicity of phosphofluoridates at the end of World War II, other countries began research to discover new agents that would be more effective than those synthesized in Germany. One of those was the cyclohexyl ester that is sometimes designated GF or cyclosarin.

As soon as highly toxic phosphorus fluoridates were developed, there was interest in finding enzymes that could hydrolyze and detoxify them. The earliest work dealing with enzymes capable of catalytically hydrolyzing organophosphorus esters was reported shortly after World War II by Mazur [5]. During the 1950s, Mounter was attempting to purify further and characterize the mammalian enzymes originally reported by Mazur. He referred to these enzymes as dialkylfluorophosphatases (DFPases), which are activated by Co^{2+} and Mn^{2+} ions [6]. In addition, Mounter and co-workers [7] were the first to report on the DFPases from microorganisms. During the late 1950s and 1960s, a number of additional groups became involved in the investigation of the enzymatic hydrolysis of diisopropylfluorophosphate, paraoxon, sarin and tabun [4]. One of the most significant events was the purification and characterization of DFPase from squid by Hoskin [8]. The significance of the squid enzyme lies in the fact that its chemical and biological properties are completely different from all other types of DFPases. The squid DFPase is characterized by its high stability and broad substrate specificity. The enzyme has molecular mass of approxi-



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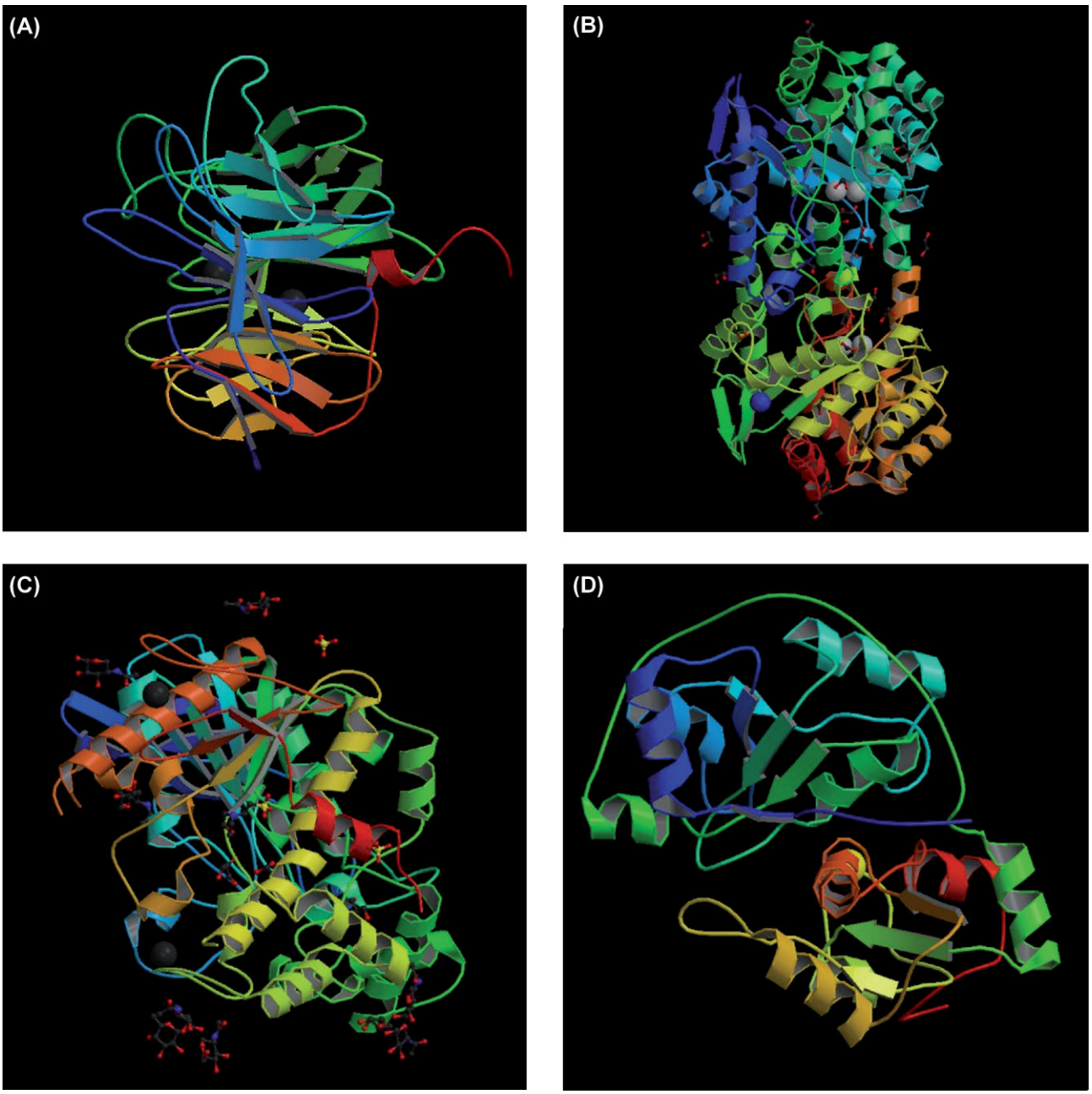


Figure 2. (A) Ribbon model of the crystal structure of diisopropylfluorophosphatase from *Loligo Vulgaris*, PDB ID 1E1A. (B) Ribbon model of the crystal structure of phosphotriesterase from *Brevundimonas* (formerly *Pseudomonas*) *diminuta*, PDB ID 1HZY. (C) Ribbon model of the crystal structure of butyrylcholinesterase from human, PDB ID 1POI. (D) Ribbon model of the crystal structure of liver rhodanese from *Bos taurus*, PDB ID 1RHD.

mately 30 kDa, requires Ca^{2+} for its activity and hydrolyzes diisopropylfluorophosphate five times faster than soman [4]. The large-scale production of recombinant protein has been developed [9]. The structure of DFPase (Fig. 2A) was solved by X-ray crystallography [10].

The interest in microbial enzymes for the degradation of organophosphorus compounds received a boost in the early 1970s by isolation of bacteria capable of growing on variety of pesticides. In the search for nerve agent degrading enzymes, investigations have gone in a number of directions. The nomenclature of these enzymes has been un-systematic and confusing. The literature is filled with references to enzymes such as phosphorylphosphatase, fluorophosphatase, somanase, sarinase and tabunase. According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Bi-

ology the general category of the phosphoric triester hydrolases (EC 3.1.8) are now divided into two subgroups. The first, organophosphate hydrolases (EC 3.1.8.1) are these enzymes with paraoxon and other P-O bonds as preferred substrate (also called A-esterases; aryldialkylphosphatases; aryltriphosphatases; organophosphate esterases, paraoxon esterases, pirimiphos-methyloxon esterases; OPA anhydrases; OPHs; organophosphorus acid anhydrases; organophosphorus hydrolases; paraoxon hydrolases; paraoxonases; phosphotriesterases; pirimiphos-methyloxon esterases; PTEs). The second group, EC 3.1.8.2, diisopropylfluorophosphatases (also called diisopropylphosphorofluoridate fluorohydrolases; dialkylfluorophosphatases; diisopropyl phosphorofluoridate hydrolases; diisopropylfluorophosphatases; diisopropylfluorophosphonate dehalogenases; diisopropylphosphofluoridases; isopropylphosphorofluoridases; somanases; tabunases) are the enzymes with preference for organophosphorus compounds with P-F or P-CN bonds [4].

The organophosphate hydrolase from *Pseudomonas diminuta* or *Flavobacterium* has been one of the most studied enzymes in regard to its activity on nerve agents and pesticides. The gene for the enzyme has been cloned and further modified by removing 29 amino acids from its sequence to get mature enzyme. The recombinant enzyme has been expressed in a variety of host cells. The mature enzyme is a metalloprotein with two Zn^{2+} ions present in the native structure (Fig. 2B) [11].

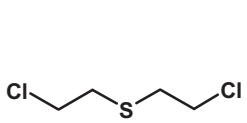
However, a variety of other divalent metal ions can be substituted. Organophosphate hydrolases have ability to hydrolyze a wide variety organophosphorus pesticides as well as sarin, soman, DFP and VX. Site-directed mutagenesis has resulted in a 20–40-fold increase in activity with soman and to lesser extent with DFP, VX and acephate [4]. In the late 1980s work on identifying diisopropylfluorophosphatase enzymes in halophilic bacteria resulted in isolation of an intracellular enzyme from a strain of *Alteromonas*. The activity towards soman corresponds to a 10^9-fold increase in the rate of reaction compared to its spontaneous hydrolysis rate [4]. Based first on amino acid sequence homology and then substrate analysis, this enzyme was determined to be an X-Pro dipeptidase (prolidase), an enzyme that is ubiquitous in nature. Its activity on organophosphorus compounds has nothing to do with its natural function and is strictly serendipitous. Other prolidases, which are highly conserved and activated by Co^{2+} and Mn^{2+} ions, have also been shown to have activity on DFP and the G-type nerve agents. In all likelihood, the enzymes studied by Mazur and Mounter were prolidases. Both the prolidase from *Alteromonas* and a organophosphorus hydrolase are now in large scale production by Genencor International, USA.

Through the use of modern molecular biology and protein engineering tools, new efficient enzymes with catalytic activity towards nerve agents and organophosphorus pesticides are also being constructed. Human butyrylcholinesterase (EC 3.1.1.8) naturally inactivates one molecule of organophosphorus compounds in a suicide reaction that irreversibly inhibits the enzyme. By contrast, the genetically engineered butyrylcholinesterase containing a single amino acid substitution, Gly117His, has the unusual property of being resistant to inhibition by virtue of its ability to hydrolyze organophosphorus compounds [12]. The hydrolysis of paraoxon by geneticallly engineered butyrylcholinesterase was 10^5 times faster than its spontaneous hydrolysis in water [13]. The structure of butyrylcholinesterase (Fig. 2C) is available from crystallographic analysis [14].

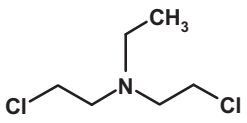
2.2 Blister agents

Blister agents, or mustard agents, are chemical substances that get their name from wounds caused by the agents, which resemble blisters or burns. Mustard gas was produced for the first time in 1822 but its harmful effects were not discovered until 1860. Mustard gas was first used as a chemical weapon during the latter phase of the World War I on July 1917 near Ypres in Belgium. The number of fatalities was relatively low, but many of the victims were badly hurt. All those affected lost large patches of skin, many of the men were blinded, and some died from the massive damage done to throat and lungs. During the war between Iran and Iraq in 1980s about 5000 Iranian soldiers were reportedly killed, 10–20% of whom by mustard agent. Mustard agent is very simple to manufacture and can therefore be a "first choice" when a country or organization decides to build up a capacity for chemical warfare. Apart from mustard agent, there are also several other closely related compounds that have been used as chemical weapons. The Germans introduced Phosgene in 1915 for use in World War I. Lewisite was discovered near the end of World War I by a team of Americans but was not used because of the armistice. During the 1930s, several reports were published on the synthesis of nitrogen mustard agent and its remarkable blistering effect [3]. The mechanism of action and symptoms largely agree with those described for mustard agent. The toxic effects of mustard agents depend on their ability to covalently bind to other substances. The chlorine atom is cleaved off the ethyl group and the mustard agent is transformed into a reactive sulfonium ion. This ion can bind to different biological molecules such as nucleic acids or proteins. Since mustard agent contains two reactive groups it can create cross-links between nucleotides of DNA and RNA, thus inhibiting replication. Mustard agent can also destroy a large number of different substances in the cell by means of alkylation, and thereby causes massive cellular mutations within an individual. The symptoms of a mild poisoning by sulfur mustard are aching eyes, a massive amount of tearing, inflammation of the skin, irritation of the mucus membrane, coughing, sneezing and hoarseness. Exposure to large

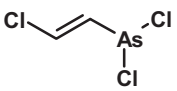
amounts of sulfur mustard causes loss of sight, nausea, severe respiration difficulty, vomiting, blistering of the skin and diarrhea. If the inhaled dose has been sufficiently high, the victim dies within a few days, either from pulmonary edema or mechanical asphyxia due to fragments of necrotic tissue obstructing the trachea or bronchi.



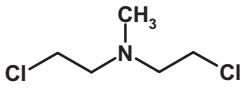
Sulfur mustard



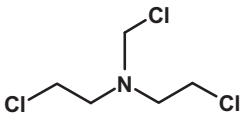
HN-1, Nitrogen mustard



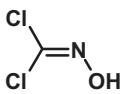
Lewisite



HN-2, Nitrogen mustard



HN-3, Nitrogen mustard



Phosgene oxime

Because of the initial lack of an enzyme neutralizing mustard agents, the first approach was to identify chemical additives that will enhance the solubilization of mustard but at the same time are compatible with the enzymes in the formulation. A variety of detergents, foams, phosphonium compounds, and quaternary ammonium compounds were examined to determine their efficacy [15]. Most of the materials had either little effect on the dechlorination rate or inhibited hydrolysis. The initial approach of an enzymatic decontamination of sulfur mustard used mild chemical oxidants (such as those used in laundry detergents with enzymes) to convert mustard to its sulfoxide, which is much more water soluble. A dehalogenase enzyme was then used to convert the mustard sulfoxide to thiodiglycol sulfoxide, which is known to be non-toxic. Such a dehalogenase was identified in γ-hexachlorocyclohexane-metabolizing bacteria *Sphingomonas paucimobilis* [16]. Search for enzymes that could directly attack mustard has continued, especially among those known to be able to dechlorinate chlorinated alkanes. A bacterial enzyme with this ability was identified and shown to significantly enhance the hydrolysis rate of mustard even in the absence of any solvent [17]. An enzyme, derived from a *Rhodococcus* bacterium, was found to have catalytic activity with sulfur mustard as the substrate. Recently, the hydrolytic enzymes were identified as bacterial haloalkane dehalogenases (EC 3.8.1.5) and are being further studied for their capacity to neutralize sulfur mustard at the Military Institute of Protection Brno and Loschmidt Laboratories at Masaryk University in the Czech Republic (Table 3), the Edgewood Chemical Biological Center in the United States, and by other groups.

Haloalkane dehalogenases are enzymes able to remove halogen from halogenated aliphatic compound by a hydrolytic replacement, forming the corresponding alcohols [18]. Structurally, haloalkane dehalogenases belong to the α/β-hydrolase fold superfamily [19]. The structure of dehalogenase LinB from *Sphingobium japonicum* UT26 [20] is depicted in figure 3B.

The benefit of these enzymes results not only from their catalytic power, but also from the mechanism of action. When solubilized, mustard gas hydrolyses to the relatively non-toxic thiodiglycol spontaneously. However, during the first step of this hydrolysis the horrific alkylation compound, a sulfonium ion intermediate, is formed (Fig. 3A). By contrast, the enzymatic hydrolysis of sulfur mustard is based on cleavage of halogen ion with parallel formation of non-toxic enzyme bound intermediate. The intermediate is subsequently hydrolyzed. The overall enzymatic reaction is buried deeply in the active site of enzyme. The resulting thiodiglycol, chloride ion and proton are released from the enzyme after the reaction, and the enzyme is ready to convert another molecule of sulfur mustard. A weak haloalkane dehalogenase activity in hydrolysis of nitrogen mustard was also observed (Prokop Z. *et al.*, unpublished).

Another example of biological detoxification of sulfur mustard uses bacterial species *Rhodococcus rhodochrous* IGTS8 (ATCC 53968), which has the ability to utilize a chemical analog of Yperite 2-chloroethyl-ethylsulfide as the only carbon and energy source for growth [21]. Detoxification activity of this species is based on splitting the C-S bond in the molecule. Furthermore, a mixture of chloroperoxidase isolated from fungus *Caldariomyces fumago* together with urea hydrogen peroxide and sodium chloride as co-substrate caused rapid degradation of sulfur mustard. Sulfur mustard incubated in the presence of chloroperoxidase, urea hydrogen peroxide and sodium

Table 3. Kinetics of spontaneous and enzymatic hydrolysis of sulfur mustard by haloalkane dehalogenases (EC 3.8.1.5) measured at 37°C and pH 7.5

Organism	Enzyme	Rate of conversion	Unit
Spontaneous hydrolysis	–	0.0046	/s
Sphingobium japonicum UT26	LinB	6.9	/s·mM
Rhodococcus rhodochrous NCIMB 13064	DhaA	6.0	/s·mM
Mycobacterium bovis 5033/66	DmbA	5.7	/s·mM

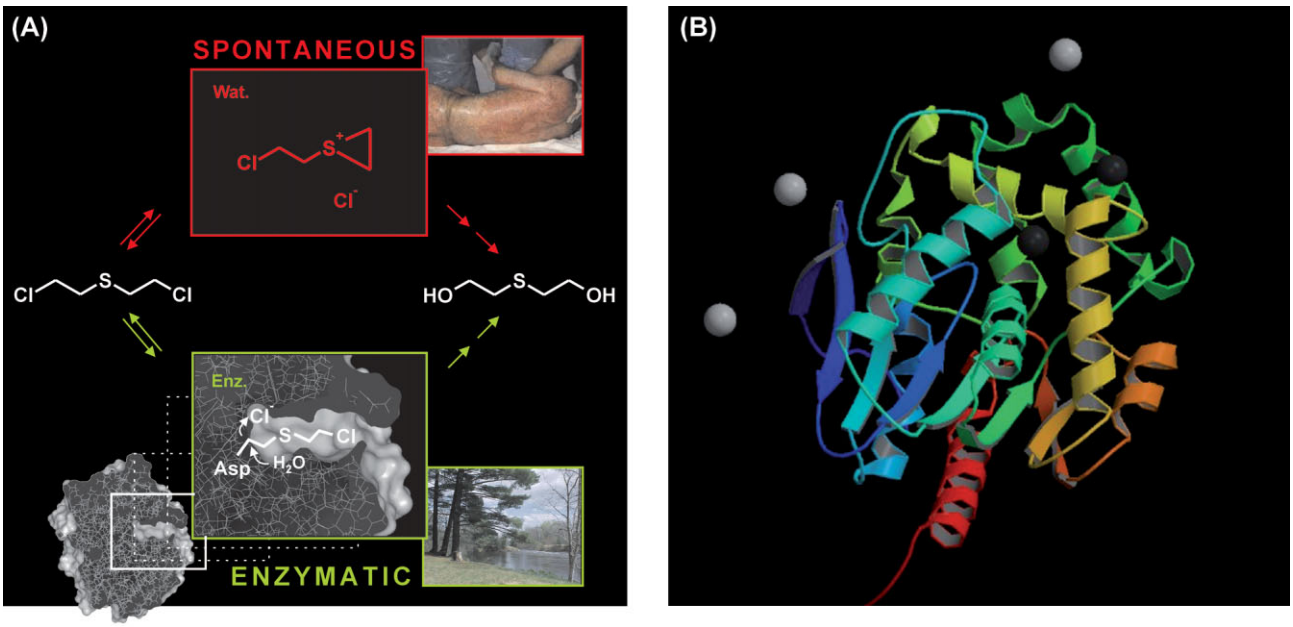


Figure 3. (A) Mechanism of spontaneous and enzymatic hydrolysis of sulfur mustard. Spontaneous hydrolysis proceeds through a sulfonium ion intermediate, which is an alkylation compound causing massive cellular damage. Enzymatic hydrolysis of sulfur mustard proceeds *via* non-toxic intermediate in deeply buried active site of the haloalkane dehalogenase. (B) Ribbon model of the crystal structure of haloalkane dehalogenase from *Sphingobium japonicum* UT26, PDB IV 1CV2.

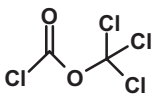
chloride was oxidized by 99% within 10 min. The oxidation products of sulfur mustard such as sulfoxide, sulfone and sulfoxidivinyl were identified in the enzymatic chloroperoxidation mixture [22].

Several enzymes were isolated exhibiting notable activity in neutralization of mustard agents, but the major limiting factor in the development of an enzyme-based decontaminant is low water solubility of the agents.

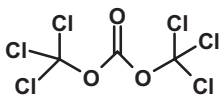
2.3 Choking agents

Chemical agents that attack lung tissue, primarily causing extensive damage to alveolar tissue and thus resulting in severe pulmonary edema, are classed as choking or lung damaging agents. To this group belong chloropicrin, chlorine, hydrogen chloride, phosgene, diphosgene and triphosgene. Phosgene is the most dangerous member of this group and the only one considered likely to be used in the future. Phosgene was first synthesized by British chemist John Davy in 1812. Subsequent development as a potential chemical warfare agent led to the first battlefield use of phosgene at Verdun in 1917 by Germany. Later, both sides in the conflict employed phosgene either alone or in mixed-substance shells, usually in combination with chlorine. Phosgene accounted for 80% of all chemical fatalities during World War I. The toxic action of phosgene is typical of a certain group of lung damaging agents. Overall, choking agents increase the permeability of the alveolar capillaries, thus causing pulmonary edema. The edema fluid pours from the bronchi into the lungs causing suffocation from what can be seen as drowning in a frothy black liquid.

Phosgene



Diphosgene



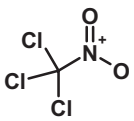
Triphosgene

Cl_2

Chlorine

HCl

Hydrogen chloride



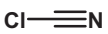
Chloropicrine

Chlorine, hydrogen chloride, phosgene, diphosgene and triphosgene all affect the lungs directly, and have no specific antidotes. There is also no report about an enzyme applicable for decontamination of these agents.

2.4 Blood agents

Chemicals that interfere with the ability of blood to transport oxygen and causes asphyxiation are known as blood agents. These substances injure a person by interfering with cellular respiration and the exchange of oxygen and carbon dioxide between blood and tissues. Common examples are hydrogen cyanide or hydrocyanic acid, cyanogen chloride and arsine. Hydrocyanic acid is the basic composition of insecticide Zyklon B notorious for its use by Nazis to kill over one million people in the gas chambers of Auschwitz and Majdanek during World War II. Hydrogen cyanide is noteworthy in recent world history, as it was used by Iraq in 1988 on an attack on the Kurdish town of Halabja. What makes hydrogen cyanide so lethal is the fact that it readily binds with metal-containing enzymes, such as the cytochrome oxidase, an enzyme system that is essential for oxidative processes within the cell. When a cyanide ion binds with such an enzyme, cellular respiration is stopped. When cellular respiration ceases, bodily functions no longer receive the necessary oxygen and nutrients needed to survive, and therefore shutdown. Overall, the cause of death due to hydrogen cyanide exposure is suffocation. Cyanogen halides do the same thing as hydrogen cyanide except that they irritate the mucus membranes and eyes. They also cause burning sensations in the throat and lungs of an exposed individual. Arsine differs from other blood agents in that it destroys the red blood cells and cause additional damage to the kidneys and liver.

Hydrogen cyanide



Cyanogen chloride

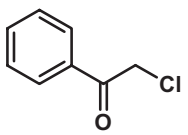
Arsine

The effects of hydrogen cyanide and cyanogen chloride can be treated using inhaled amyl nitrite, intravenous sodium nitrite and sodium thiosulfate, and inhaled oxygen. Several enzymes can be considered as promising in neutralizing these compounds. Cyanide can be enzymatically converted to the less toxic thiocyanate by rhodaneses (thiosulfate:cyanide sulfurtransferases, thiosulfate sulfurtransferases, thiosulfate thiotransferases; EC 2.8.1.1). A genetic system to express high levels of recombinant *Pseudomonas aeruginosa* rhodanese in *Escherichia coli* have been recently engineered and tested in cyanide detoxification [23]. The crystal structures from of rhodaneses are available from three different organisms *Bos taurus* (Fig. 3B) [24], *Azotobacter vinelandii* [25] and *Escherichia coli* [26]. Besides sulfurtransferases, β-cyano-L-alanine synthase (EC 4.4.1.9) identified in *Arabidopsis thaliana* is also suggested to be involved in cyanide detoxification [27]. Another enzyme, cyanide oxygenase from *Pseudomonas fluorescens* NCIMB 11764 has been found to catalyze the oxygenolytic cleavage of cyanide to formic acid and ammonia in the metabolic detoxification and utilization of cyanide as bacterial nitrogenous growth substrate [28].

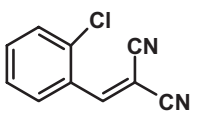
2.5 Lachrymatory agents

Agents that could temporarily incapacitate victims were among the first to be developed and were deemed »harassing agents«. Irritating gases have been used in war since ancient times. Harassing agents are capable of a number of immediately perceived effects such as intense irritation of the eyes, causing crying or temporary blindness, irritation of the mucous membranes of the nose, trachea, or lungs, causing coughing, irritation of the throat and stomach, with the induction of vomiting and possibly diarrhea and irritation of the skin. For many years, 2-chloroacetophenone was the most widely used agent by civil and military authorities. Dissatisfaction with its potency and chemical instability, however, led military scientists to search for alternative agents. In the 1950s, the Chemical Defence Experimental Establishment in Porton, England, developed *o*-chlorobenzylidenemalononitrile. Its useful form is intended to be a smoke or fog of suspended particles.

Chlorobenzylidenemalononitrile that has been micronized and mixed with an anti-agglomerant or treated with silicone water repellant can remain active for days to weeks when dusted on the ground.



2-chloroacetophenone



***o*-chlorobenzylidenemalononitrile**

During the Vietnam War, the United States developed an array of delivery vehicles for *o*-chlorobenzylidenemalononitrile, including small pocket grenades, continuous spray device used in caves and tunnel systems and cluster bombs dropped from helicopters and planes. The use of tear gas in recent times is mainly in the situations of civil unrest. Available toxicological data are lacking in details of the potential of tear gas agents to cause long-term pulmonary, carcinogenic, and reproductive effects. Severe traumatic injury from exploding tear gas bombs as

well as lethal toxic injury, most likely in the form of toxic pulmonary edema, have been also documented [29]. Oral toxicology studies have noted the ability of *o*-chlorobenzylidenemalononitrile to cause severe gastroenteritis with perforation. In 1969, 80 countries voted to include tear gas agents among chemical weapons banned under the Geneva Protocol.

There are very few studies dealing with enzymatic transformation of tear gas agents. The military riot control agent 1,4-dibenz-oxazepine (referred to as CR) was shown to be biodegradable, but the actual enzymatic pathways were not determined [30]. The enzymatic reduction of 2-chloroacetophenone has been observed in anoxic sediment slurries from both freshwater and marine sources. The reduction of 2-chloroacetophenone produces acetophenone via electron transfer and chlorophenylethanol via hydride transfer [31]. A study of the distribution of glutathione S-alkenetransferases in the livers of vertebrate species suggests that the enzyme in hamster liver was significantly active towards chlorobenzylidenemalononitrile [32]. The depleting of glutathione in freshly isolated rat hepatocytes was also observed in the presence of 2-chloroacetophenone [33]. However, pure solubility of the agents in water is a significant limiting factor in the development of an enzyme-based decontamination technology.

3 Conclusions and outlook

The chemical weapons exert their terrifying effects by interrupting biological processes. Fortunately, they can also be degraded by enzymes through fortuitous side reactions. The activity of these enzymes can be the ground of our defensive arsenal. Nowadays, the spectrum of such enzymatic tools are significantly widened and enriched due to the fast appearance of new biochemical methods utilizing natural catalysts, enzymes, for the transformation of non-natural man-made organic compounds. Enzymes provide high catalytic efficiency with minimal unit consumption. As natural proteins, enzymes contribute to the "green" solution of the decontamination problems. One of the greatest advantages of enzymatic decontaminants is their mutual compatibility. The enzymes evolved to act simultaneously within a single cell, tightly packed and acting specifically with their natural substrates. Preparation of multicomponent decontaminants against wide range of chemical weapons should be feasible, creating a number of benefits like easy logistic and handling, or applicability of the general preparation after attack by chemical weapons with unknown agents.

For a long time, dealing with the natural disadvantages of enzymatic decontaminants, such as low stability, requirements for water environment or narrow operation conditions, has been a challenge. Recent advances in molecular biology and protein engineering, *i.e.*, development of directed evolution techniques, have been extremely helpful in creating enzymes that are significantly less prone to these limitations. It is no longer a problem to enhance stability of natural enzyme or adapt it to the new operation conditions, including improved activities in non-aqueous solvent environments. Natural enzymes were not exposed to the selection that would let them to act under these conditions, but modern molecular techniques enables setting-up laboratory experiments with the right selection pressure, leading to evolution of tailor-made biocatalysts. We expect that many natural enzymes will be optimized for their application by this way, but also completely new enzymes will be isolated from extreme environments. These improved enzymes will create a basis of multicomponent decontaminants that will provide us with effective preparations against chemical weapons.

The use of catalytic enzymes could also play a role in the destruction of stocks of chemical agents as mandated by the Chemical Weapons Convention. However, with regard to this point, there have been no significant efforts in this area. The primary reason for this lack of use has been time and to a lesser degree cost. Facilities that were constructed and permitted for chemical agent destruction would require significant time delays in receiving authorization to utilize different technologies. Also, until recently, no industrial scale production of the enzymes was in place, thus resulting in very high costs. Enzyme-based decontaminants could be used to decontaminate leaks and spills that may occur during destruction operations, but so far have not reached commercial status.

A promising area of enzyme application in decontamination of chemical warfare substances is in protection or treatment of human casualties. A number of protective materials are being developed containing the enzymes for destruction of and protection against chemical warfare agents.

Z.P. and J.D. express their thanks to the Czech Ministry of Education for financial support (project INCHEMBIOL MSMT0021622412).

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