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Production of human liver prolidase by *Saccharomyces cerevisiae* as host cells[1]

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ABSTRACT

AIM: To clone and express the recombinant human liver prolidase in yeast and explore the activities of both dipeptidase and organophosphoric acid anhydrolase (OPAA). **METHODS**: The cDNA encoding human liver prolidase derived from healthy adult liver was cloned into the pYES2, an expression vector of *S cerevisiae*, and then transformed into *S cerevisiae* INVSc1 by electroporation. The transformant with the highest enzymatic activity was induced by galactose for expression. The optimal induction conditions (temperature, induction time, and the initial amount of inoculation cells) were estimated by orthogonal experimental design. The recombinant prolidase and OPAA activities were assayed by spectrocolorimetric methods. **RESULTS**: The recombinant enzyme catalyzed the hydrolysis of organophosphorous compound soman as well as the hydrolysis of dipeptide Gly-Pro. Under the optimal induction conditions (20 h, 25 ºC, initial OD_{600}=0.4), the maximum activities of prolidase and OPAA came to 226.5 and 578 μmol·min^{-1}·g^{-1} protein in cell lysate supernatants, respectively. SDS-PAGE of the recombinant enzyme in disrupted cell supernatants showed a molecular weight of 56 kDa. Intensity scanning of the SDS-PAGE gel revealed that the enzyme accounted for 3.16 % of the total protein in the supernatant. One liter incubation medium produced 7 g of wet yeast cell containing 4.56 mg of the recombination protein. **CONCLUSION:** The recombinant human liver prolidase produced by yeast cell (*S cerevisiae*) exhibited both dipeptidase and OPAA activities.

INTRODUCTION

Mazur[1] first described the hydrolysis and detoxification of diisopropylfluorophosphate (DFP) using crude preparations from human and rabbit tissues in 1946. Organophosphoric acid anhydrolases (OPAA, EC 3.1.8.2) have been found in a wide variety of prokaryotes and eukaryotes such as bacteria, protozoa, squid, clams and mammals[2]. OPAA includes a group of enzymes hydrolyzing many highly toxic acetylcholinesterase-inhibiting compounds including DFP, the chemical warfare agents soman, sarin, tabun, and the pesticide paraoxon.

Use of the enzymes as prophylactics against organophosphorous compound intoxication and use in decontamination of hazardous chemical warfare agents and pesticides has been considered[3-5], since the enzyme-mediated decontamination is nontoxic, noncorrosive, and environmentally compatible.

The *Altermonas* OPAA sequence was found to exhibit homology with that of the human prolidase[6], and the OPAA from *Altermonas undina*[2] and

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Altermonas haloplanktis[7] showed prolidase activity, suggesting that the OPAA may be a prolidase[8]. These experimental observations prompted us to clone and express the human liver prolidase gene, characterize the recombinant prolidase, and find out whether it also exhibits OPAA activity besides its dipeptidase activity.

Prolidase (EC 3.4.13.9) is a widely distributed enzyme which splits dipeptides with proline or hydroxyproline as the C-terminal amino acid. It presents in microorganisms and most mammalian tissues and plays an important role in the recycling of proline for collagen synthesis and cell growth[9]. If prolidase would be proven to have OPAA activity as well, it should be recloned as a new member of the OPAA family.

MATERIALS AND METHODS

Liver tissue Human normal liver tissue encompassing a hepatic angioma was dissected immediately after the surgical operation from a female adult, and stored at once in liquid nitrogen before use.

Strains and plasmids *S cerevisiae* INVSc1 (MATa/a, his3-1/his-1, leu2/leu2, trp1-289/rep1-289, ura3-25/ura3-25), with a phenotype of His^-, Leu^-, Trp^-, and Ura^-, was used as the host for plasmid transformation. Plasmid pYES2 was purchased from Invitrogen amplified in small-scale in *E coli* TOP10F'. Plasmid PinPoint X_{a-1}-T was bought from Promega.

Media Yeast strains were grown on the synthetic complete (SC) medium[10] (Difco yeast nitrogen base without amino acids) supplemented with the appropriate amino acids, 15 g agar per liter, and 20 g glucose as the carbon source. For selection of recombinants, uracil was omitted from the medium (SC-Ura). All reagents were of analytical grade.

Reverse transcription Total cellular RNA was isolated from the human liver tissue according to the protocol of the kit. Synthesis of cDNA was carried out in the reaction buffer (Tris-HCl 50 mmol/L, pH 8.3, KCl 75 mmol/L, $MgCl_2$ 3 mmol/L, dithiothreitol 10 mmol/L) using 2.5 μg of total cellular RNA (denatured at 65 ºC for 10 min), 20 pmol of oligo$(dT)_{15}$ (prolidase mRNA-specific downstream antisense oligonucleotide primer), 10 U of human placenta ribonuclease inhibitor, 200 U of M-MLV reverse-transcriptase, and 1 mmol/L dNTP in a final volume of 20 μL. The mixture was incubated at 42 ºC for 1 h, then the reverse transcriptase was inactivated in boiling water for 5 min. Restriction enzymes and other modifying enzymes were obtained from Promega.

Isolation of prolidase gene The human liver prolidase gene[6] was isolated by using the cloning kit. Two pairs of primers were designed as follows: P1 (Sense) 5'-AACATGGCAGCGGCAACCGGACCC-TCGTTTTGG-3' (3-30), P2 (Antisense) 3'-CGTTCA-AGTGACGTCTGGT-5' (887-905), P3 (Sense) 5'-CTC-CTTTGACGGCATCAGC-3' (483-501),P4 (Antisense) 3'-GGTTCATCTCGGTCGGTCTTTACCTAGGCGC-5' (1475-1505).

The full length of the prolidase gene was amplified in two steps. At first, a 0.9-kb fragment was obtained using primers P1 (sense) and P2 (antisense), and a 1.0-kb fragment using primers P3 (sense) and P4 (antisense). One tenth of the total cDNA product and 40 pmol of each primer [(P1+P2) or (P3+P4)] were added to 50 μL of reaction buffer (Tris-HCl 10 mmol/L, pH 8.3, KCl 25 mmol/L, $MgCl_2$ 1.5 mmol/L, dNTPs 200 mmol/L) containing 2.5 U of *Taq* DNA polymerase, layered with 2 drops of liquid paraffin, and incubated at 94 ºC for an initial 5 min denaturation. For PCR-amplification 30 cycles of denaturation, annealing, and polymerization were carried out for 1 min at 94 ºC, 1 min at 55 ºC and 1.5 min at 72 ºC, and then elongated at 72 ºC for 5 min. The PCR products (0.9 kb and 1.0 kb fragments) were isolated by agarose gel electrophoresis and purified by gel extraction kit according to the instruction. Then the mixture of 0.9 kb and 1.0 kb fragments was used as the templates for amplification of the 1.5 kb full length human prolidase gene using P1 and P4 as the primers. Thus a 1.5-kb prolidase gene was obtained (Fig 1).

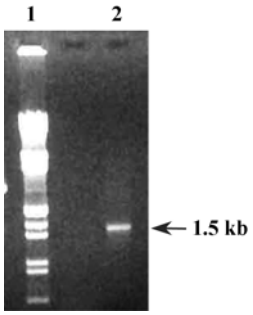

Fig 1. Electrophoresis indentification of cloned prolidase gene. Lane 1: DNA marker (*EcoR* I+*Hind* III); Lane 2: human prolidase gene.

Construction of yeast expression vector containing prolidase gene Human prolidase gene was ligased to PinPiont$^{TM}X_{a-1}$ T vector, and then inserted into pYES2 plasmid to construct the expression vector

pYES2-P (Fig 2).

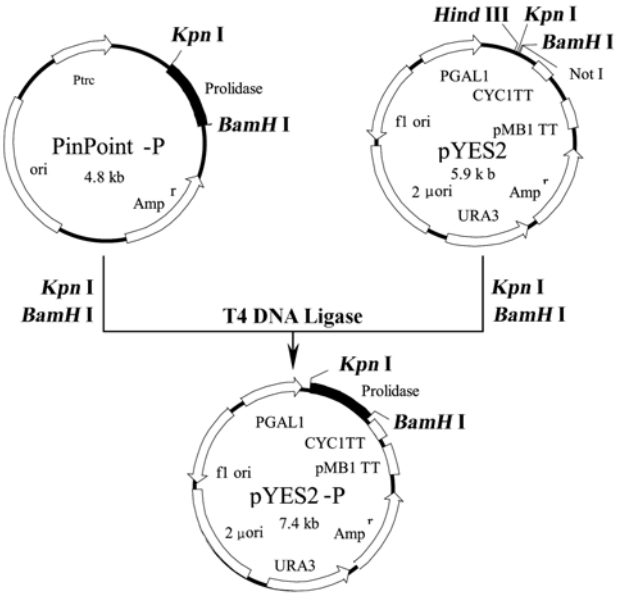

Fig 2. Construction of expression vector containing prolidase gene.

DNA sequence and deduced amino acid sequence analysis The nucleotide sequence of prolidase gene was determined using a sequencer. The DNASIS and PC/Gene sequence analysis software systems were employed to analyze DNA sequences. The nucleotide sequence and the deduced amino acid sequence were analyzed with the databases by using BLAST programs.

Transformation of yeast Gene Pulser II Electroporation System (Bio-Rad) was applied to transform vectors into yeast under the condition of 1.5 kV, 25 mF, 200 W. Yeast cells transformed with pYES2 plasmid without prolidase gene were performed in the same way as a control.

Orthogonal experimental design for optimizing induction conditions In order to investigate the influence of the induction conditions on the recombinant enzyme activity, experiments were designed and carried out based on the orthogonal design $L_9(3^4)$[11] setting three factors (A: temperature, B: induced time, C: initial OD_{600}) with three different levels of each (Tab 1). Generally, 3^3 experiments are sufficient to obtain the optimum conditions.

Culture conditions and preparation of crude cell extracts Yeast transformants were inoculated into SC-Ura (containing 2 % glucose) medium in a flask shaking at 28 ºC for appropriate hours, harvested by centrifugation at 4000×*g* for 6 min, washed twice in sterile distilled water, and resuspended in the induction medium SC-Ura (containing 2 % galactose). The cells were pelleted by centrifugation, washed in sterile distilled water twice, weighed up and resuspended in Tris-HCl 0.05 mol/L, pH 7.8 (3 mL/g wet cell pellet). The harvested cells were subjected to three cycles of freezing in liquid nitrogen and thawing, and then disrupted by ultrasound for 15 min.

Tab 1. Factors and levels of orthogonal design L_9 (3^4).

Level	A Temperature/ºC	B Induced time/h	C Initial OD_{600}
1	29	8	2.0
2	25	20	0.4
3	21	14	1.2

The cells produced under optimal conditions (initial OD_{600} 0.4, 20 h, 25 ºC) were ruptured at a rate of 90 % under 20 000 psi (138 MPa) in a high-pressure homogenizer EmulsiFlex-C5 (Utek Co, Canada). The cell lysates were then centrifuged at 4 ºC 14 000×*g* for 20 min. The supernatant containing soluble recombination proteins was collected, and kept frozen at -80 ºC ready for assay. Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as the standard.

Assay of prolidase activity Prolidase activity was determined according to Myara *et al*[12] based on measurement of the splited proline by Chinard's reagent with modification. Activation of prolidase required preincubation with manganese: 50 μL of enzyme extract at different concentrations were incubated with 50 μL of Tris-HCl 0.05 mol/L (pH 7.8) containing $MnC1_2$ 2 mmol/L at 37 ºC for 1 h. After the preincubation, the prolidase reaction was initiated by adding 100 μL of glycyl-proline (Gly-Pro) 94 mmol/L at a final concentration of 47 mmol/L, incubated at 37 ºC for 30 min, and the reaction was terminated with 1 mL of trichloroacetic acid 0.45 mol/L. Then the supernatant was subjected to proline estimation. A blank without substrate ran under the same conditions. Glacial acetic acid (0.5 mL) and 0.5 mL of Chinard's reagent (25 g ninhydrin dissolved at 70 ºC in 600 mL of glacial acetic and 400 mL of orthophosphoric acid 6 mol/L) were added to 250 μL of the supernatant, and incubated for 10 min at 90 ºC. The amount of the released proline was determined

spectrocolorimetrically at 515 nm and calculated using the proline calibration curve. Enzyme activity was reported as nanomoles per min per mg of supernatant total protein.

Assay of OPAA activity OPAA activity was assayed by the spectrocolorimetric method[13,14] using soman as the substrate. Soman (25 μL, 60 mmol/L) was added to 25 mL of the enzyme supernatant and a total volume of 200 μL was made with 0.05 mol/L Tris-HCl (pH 7.8). The mixture was incubated at 37 ºC for 30 min and immediately stopped by adding 400 μL of acetone. The reaction mixture was centrifuged (7000 ×*g*, 2 min) and the supernatant was diluted for 10 times with acetone-Tris-HCl 0.05 mol/L, pH 7.8, (2:1, v/v). To aliquots (500 μL) of the diluted supernatants, 100 μL of 0.5 % benzidine and 400 μL of 0.25 % sodium perborate solution were added in proper order, mixed and incubated at 37 ºC for 7 min. The absorbance was read at 414 nm by a spectrophotometer (UV-250, Shimadzu, Japan). The unhydrolyzed soman was calculated according to the soman calibration curve. In the blank control, 25 μL of soman was substituted with 25 μL of Tris-HCl 0.05 mol/L (pH 7.8). Enzyme activity was reported as the same above-mentioned.

SDS-PAGE analysis SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done in 12 % acrylamide gels by the method of Laemmli under denaturing condition with a Mini-Protein III system (Bio-Rad). Supernatant 10 μL was loaded on the gel together with 10 μL of the sample buffer. The proteins in the gels were stained with 0.1 % Coomassie brilliant blue G-250. The molecular weight of the enzyme was estimated using low molecular weight markers.

RESULTS

Construction of expression vector containing prolidase gene The prolidase cDNA (1.5 kb) was excised from the plasmid pGEM-P by *Kpn* I and *BamH* I and inserted into the pYES2 vector. The correctness of the constructed pYES2-P was proven by electrophoresis (Fig 3). The recombinant plasmid pYES2-P in which the prolidase gene was oriented from the 5' to 3' terminus was transformed by electroporation. Over 100 transformants were obtained, among which 8 were randomly chosen for further enzyme assay experiment. Transformant No 3 with the highest enzymatic activity was applied for expression induced by galactose.

Optimal induction conditions from the orthogonal experimental design The orthogonal test was designed (Tab 2) according to 3 factors and 3 different levels of each. The variance analysis showed that the prolidase and OPAA shared the same optimum induction conditions: induction time 20 h, initial OD_{600} 0.4, temperature 25 ºC (Tab 3).

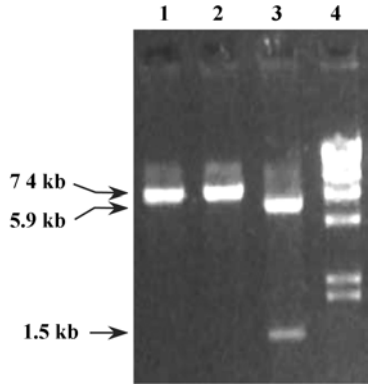

Fig 3. Identification of recombinant plasmid pYES2-P. Lane 1: plasmid pYES2-P digested with *Kpn* I. Lane 2: plasmid pYES2-P digested with *BamH* I. Lane 3: plasmid pYES2-P digested with *Kpn* I and *BamH* I. Lane 4: DNA marker (lDNA/*Hind* III).

Tab 2. Orthogonal test.

Tube No	Level A	B	C	Null	Enzyme activities Prolidase	OPAA
					μmol·min^{-1}·g^{-1} protein	
1st	1	1	1	1	69.05	149.2
2nd	1	2	2	2	211.7	549
3rd	1	3	3	3	146.6	416.2
4th	2	1	2	3	88.35	266.8
5th	2	2	3	1	211.85	491.8
6th	2	3	1	2	74.15	224.2
7th	3	1	3	2	78.7	251.6
8th	3	2	1	3	129.8	472
9th	3	3	2	1	160.7	509.8

Expression curves of recombinant plasmid pYES2-P The above-mentioned results showed that induction time and OD_{600} were two critical factors contributing to the enzyme activities, however the option of induction temperature ranged from 20-29 ºC (Fig 4).

Additional experiments showed that when the initial OD_{600} was lower than 0.4, both enzyme activities of the collected cells hardly increased any more (data not shown). An induction time of 20 h was the best choice

Tab 3. Variance analysis.

Prolidase	m_{1j}	142.45	78.7	91	147.2	T=1171
	m_{2j}	124.8	184.45	153.6	121.5	
	m_{3j}	123.05	127.15	145.7	121.6	y=130.1
	F_j	0.516	13.02	5.388		
	Notability	Unnotable (α=0.1)	Notable (α=0.1)	Notable (α=0.25)		
OPAA	m_{1j}	371.4	222.6	281.8	383.6	T=3330
	m_{2j}	327.6	504.2	441.8	341.6	
	m_{3j}	411.2	383.4	386.6	385	y=370
	F_j	2.87	32.84	10.87		
	Notability	Unnotable (α=0.1)	Notable (α=0.05)	Notable (α=0.1)		

Notes: m_j represents the mean value of the enzyme activity corresponding to the jth in the jth factor; T and y represent respectively the sum and the mean value of the enzyme activities of all 9 experiments; F_j denotes the variance; and α is the significance level.

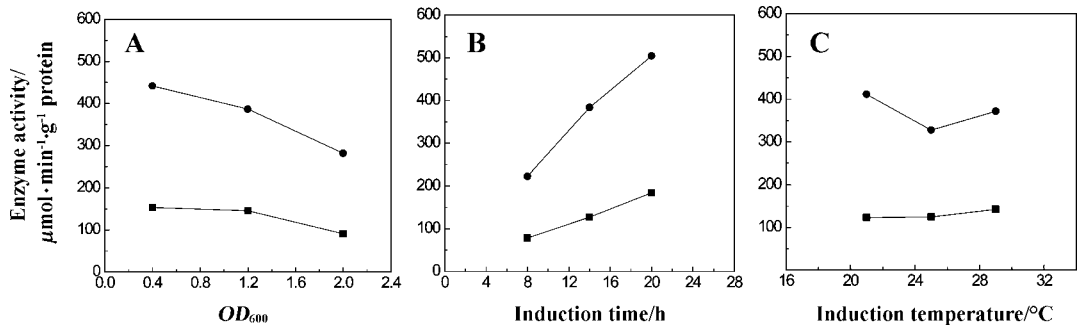

Fig 4. Effects of three factors on the enzyme activities. ■: Prolidase; ●: OPAA.

(Fig 5).

The recombinant plasmid pYES2-P expressed well in yeast cells. The recombinant human prolidase exhibited not only prolidase activity but also OPAA activity. The prolidase and OPAA activities increased along with the induction time and finally reached up to a maximum level of 226.5 and 578 μmol·min^{-1}·g^{-1} at 20 h. The prolidase activity was 4.27 times higher than that in the control cells, whereas the OPAA activity, 2.58 times higher. Both enzyme activities began to decline after 20 h (Fig 5).

SDS-PAGE analysis In comparison with the controls (Lane 1, 2 and 4), a protein band (56 kDa) appeared at lane 5 and 6. The recombinant protein band at lane 6 (transformant No 3 induced under optimal induction conditions and ruptured by high-pressure homogenization) showed higher intensity than that at lane 5 (transformant No 3 induced by galactose at 25 ºC for 12 h and OD_{600} of 1.2 and ruptured by ultrasound) with yields of 3.16 % and 1.97 % of the total protein, respectively. Under optimum conditions (lane 6), 7 g of wet yeast cells containing 4.56 mg of recombination protein were produced per liter induction medium.

Lots of native proteins existed in the *S cerevisiae* cell supernatants (Fig 6). Fortunately, there was not a native abundant cellular protein with the same molecular size (56 kDa) as the expressed recombinant protein in the cell lysate supernatants, so that the band of the recombinant protein could be clearly seen at lane 5 and lane 6 (Fig 6).

DISCUSSION

Prolidases[4] from *Alteromonas undina* and *Alteromonas haloplanktis* catalyzes the hydrolysis of organophosphorous compounds including DFP, soman, sarin and tabun. Our results showed that the recombinant human liver prolidase expressed in COS-7 cells[15] and in yeast cells (this paper) also exhibited the OPAA activity. The similarity in biochemical properties of

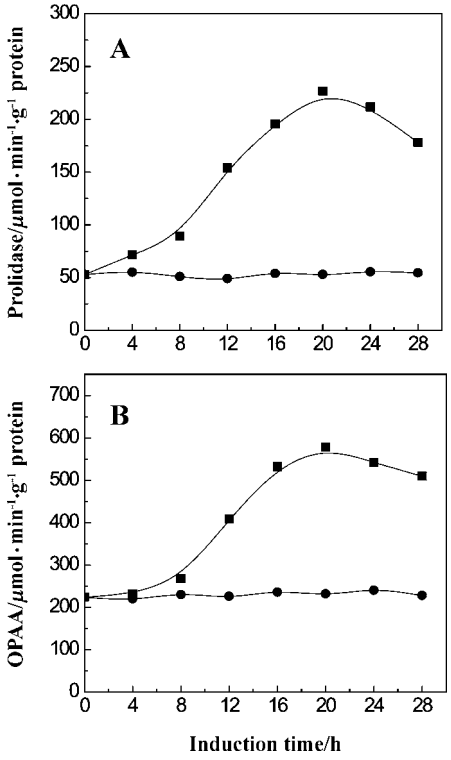

Fig 5. Induction of prolidase and OPAA at optimum conditions (25 ºC; OD_{600} 0.4). ■: Transformant; ●: Control. A: Prolidase; B: OPAA.

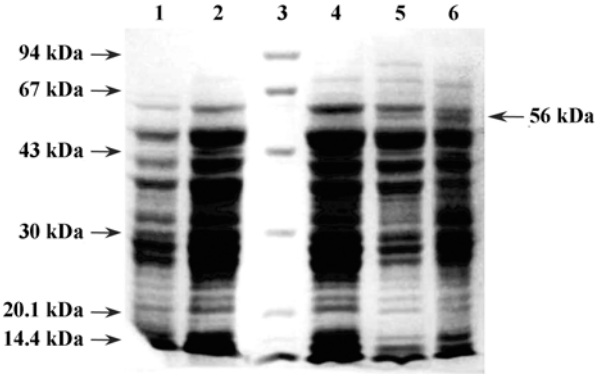

Fig 6. SDS-PAGE profile of the cell lysate supernatants containing recombinant protein of 56 kDa. Lane 1: Parent cell INVSc1. Lane 2: Transformant of empty vector pYES2. Lane 3: Protein marker. Lane 4: Transformant No 3 not induced by galactose. Lane 5: Transformant No 3 induced by galactose for 12 h at 25 ºC and OD_{600} of 1.2. Lane 6: Transformant No 3 under optimal induction conditions (20 h, 25 ºC, OD_{600} 0.4).

OPAA and prolidase suggests that human liver prolidase is one kind of OPAA.

Compared with other expression systems, *S cerevisiae*, a rapidly dividing unicellular eukaryote system, is cheaper, non-pathogenic, and easy for cultivation. The enzyme produced in yeast cells often shows a relative high specific activity. In our study, there were mainly two factors effecting the enzyme activity and production, namely the initial cell concentration and the induction time. The intrinsic proteases in yeast cells might be another factor influencing the enzyme activities. Since as soon as the cells were disrupted or lysed, the released proteases would partially hydrolyze the expressed proteins. Thus protease inhibitors were needed in the cell suspension throughout the duration of lysis.

The molecular weights of the purified human kidney prolidases, native and denatured, were estimated at 115 kDa (Sephacrgl S-200 chromatography) and 55 kDa (SDS-PAGE), respectively[16]. It indicates that the prolidase presented as a homodimer, the molecular weight of the monomer was similar to the human erythrocyte prolidase, 56 kDa[17] and 58 kDa[18], and in agreement with the calculated value derived from the human liver prolidase sequence (492 amino acid residues, M_r 54 305)[6], the estimated value (56 kDa) in the paper.

Only less than 5 % of the invaded nerve agents is able to reach the target organs, most of the poisons are eliminated by OPAA and others *in vivo*. Inhibition of OPAA enhances the toxicity, whereas induction of the production of OPAA fairly decreases the toxicity. It clearly indicates the important role of OPAA in degrading the toxins. OPAA has a considerable potential for use in detoxification of a broad range of highly toxic organophosphorous compounds including chemical nerve agents, and in the development of new generation decontamination systems for detoxification of these compounds.

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