

73. WIDE-RANGE APPLICATION OF *ALTEROMONAS* PROLIDASE FOR DECONTAMINATION OF G-TYPE CHEMICAL NERVE AGENTS

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ABSTRACT

Organophosphorus acid anhydrolases (OPAA: EC 3.1.8.2) are capable of catalytically hydrolyzing a wide range of organophosphorus compounds, including the fluoride containing chemical nerve agents such as soman and sarin. The genes encoding OPAA have been cloned and sequenced from two species, *Alteromonas sp.*JD6.5 and *A. haloplanktis*. Sequence and biochemical analysis of the cloned genes have established OPAA to be prolidases (E.C. 3.4.13.9), a type of dipeptidase hydrolyzing X-proline bonds. The development of recombinant clone XL1 (pTCJS-4) producing large quantities of active G-agent degrading enzyme, *A. sp.*JD6.5 prolidase, offers a real potential for large-scale production of the enzyme. With this enzyme, a simple and safe enzyme-based decontamination system that provides long-term stability and greatly reduced logistical burden was achieved. Formulation of the recombinant enzyme in different matrices provides opportunity for chemical agent decontamination, personnel protection, and detection. Such matrices include fire-fighting foams/sprays, environmentally safe detergents and degreasers. Retention of hydrolytic activity for extended period of time in lyophilized form allows enzyme use with existing spray or fire-fighting equipment. Appreciable retention of prolidase activity in various matrices, cross-linked prolidase enzyme crystals (CLEC), cellulose binding domain (CBD)-linked form, polyurethane foam conjugated form, offers wide-range applications. The prolidase enzyme is also stable in commercial laundry detergent "Tide-Free", and in "Protectall" skin lotion.

INTRODUCTION

Enzymes detoxifying the highly toxic acetylcholinesterase inhibitors are generally classified as organophosphorus acid anhydrolases (OPAA; EC 3.1.8.2), and have been found in both prokaryotes and eukaryotes (1,2). Of the microorganisms investigated thus far, several isolated halophilic bacterial strains including a number of *Alteromonas* species were found to contain high levels of OPAA activity (2-4). Diisopropyl fluorophosphate (DFP) which has been routinely used in research as a serine protease inhibitor, is a common substrate used to screen for OPAA activity. The OPAA enzymes are capable of catalytically hydrolyzing a wide variety of organophosphorus compounds (OPs), including the fluoride (F⁻) containing chemical warfare (CW) nerve agents such as soman (GD; *O*-pinacolyl methylphosphonofluoridate), sarin (GB; *O*-isopropyl methylphosphonofluoridate), GF (*O*-cyclohexyl methylphosphonofluoridate), and cyanide containing tabun (GA: ethyl *N,N*-dimethylphosphoramidocyanidate). Recently, the genes encoding OPAA have been cloned and sequenced from two species, *A. sp.*JD6.5 and *A. haloplanktis* (5,6). Sequence and biochemical analysis of the cloned enzymes have established these OPAA to be prolidases (E.C. 3.4.13.9), a type of dipeptidase hydrolyzing dipeptides with a prolyl residue at the carboxyl terminal position. The prolidase has been shown to play an important role in cellular dipeptide metabolism in humans.

Previously, a parathion-degrading enzyme, organophosphorus hydrolase (OPH) from *Pseudomonas diminuta* MG and *Flavobacterium* species ATCC 27551, was also found to have a significant G-type nerve agent hydrolyzing activity (7,8). OPH is the only well characterized enzyme known to catalytically hydrolyze VX. However, no sequence homology was found between *Alteromonas* prolidase and OPH.

The currently fielded decontamination solutions, DS2 and bleach, are corrosive in nature and result in hazardous waste. Because of their corrosivity, such solutions cannot be used on sensitive equipment or for personnel decontamination. The use of non-toxic enzymes has considerable potential for situations involving decontamination of sensitive equipment, vehicles, large fixed sites, cleanup operations resulting from a possible terrorist incident, and chemical agent handling during demilitarization and storage. Enzyme-mediated decontamination is non-toxic, non-corrosive, and environmentally compatible. The dry form of enzyme should also result in a significantly reduced logistic burden involved in storage and transportation, perhaps by as much as 50-fold. In this report, the inclusion of enzyme into different matrices and the feasibility of these matrices for decontamination, protection, and detection were evaluated.

MATERIALS AND METHODS

Materials

Alteromonas sp. JD6.5 was isolated from Grantsville Warm Springs, Utah, as described previously (3). *A. haloplanktis* (ATCC 23821) and *A. undina* was obtained from American Type Culture Collection (ATCC), Rockville, Maryland. The purification of prolidases from native *A. undina* (ATCC 29660) cells was performed by procedure similar to those described earlier (4). Organophosphorus hydrolase was prepared from XL1 (pVSEOP7) (unpublished work, Rastogi, Cheng, and DeFrank). The construct was prepared by first amplifying the OPH gene using pJK33 (courtesy Walter Mulbry, USDA, Beltsville, MD) as target DNA, and then sub-cloning it into pSE420 cloning vector (Invitrogen, CA). Most of the routine chemicals used in the report were purchased from either Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA).

Enzymatic Assays

Enzyme activity measurements, using DFP as a substrate, were determined by monitoring F⁻ release with an ion-specific electrode as described previously (3). The reaction mixture consisted of 50 mM (NH₄)₂CO₃, 0.1 mM MnCl₂, 3 mM DFP, and 0.3-0.4 U enzyme or enzyme matrices in a total volume of 2.5 ml. One unit (U) of enzyme catalyzes the release of 1.0 μmole of F⁻ per minute at 25°C. Specific activity is expressed as U per mg of protein. Protein concentrations were determined using Coomassie protein assay reagent (Pierce, IL) with bovine serum albumin to generate a standard curve.

Growth of Recombinant Cell Lines and Analysis of the Expressed Enzyme

The plasmid (pTCJS4) carrying *A. sp.* JD6.5 prolidase gene (9,10) was transformed into different *E. coli* strains. The recombinant cells were grown at 30°C in flask using Luria-Bertani (LB) complex media. The media was supplemented with 0.1 mM MnCl₂. The cells were induced with 0.6 mM isopropyl-β-D-thiogalactoside (IPTG) at early-mid-log phase ($A_{600nm} = \sim 0.5$).

For analysis of the expressed enzyme, 1-ml cells was collected at the time of harvest by centrifugation and resuspended in 2x SDS-PAGE sample buffer [0.125 M Tris, pH 6.8, 4% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), 10% glycerol and 0.025% bromophenol blue]. The samples were lysed by incubating at 100°C for 5 minutes, and then electrophoresced through a 10% SDS-polyacrylamide gel at 100 mA.

Preparation of Freeze-dried Recombinant Enzyme

The recombinant cell lines (*Alteromonas sp.* JD6.5 and *A. haloplanktis*) were grown as described above. After IPTG induction for 5-6 hours, cells were harvested, resuspended in 10 BM buffer (10 mM Bis-tris propane, pH 7.2, containing 0.1 mM MnCl₂ and 0.1 mM DTT), and lysed by passing through French Press cell. After removal of the cell debris by centrifugation, the cell-free lysate was subjected to (NH₄)₂SO₄ fractionation, and Q-Sepharose chromatography as described previously (9,10). The purified enzyme sample was then mixed with trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) to a final concentration of 250 mM and lyophilized to dryness (11).

Subcloning and Preparation of Prolidase-Cellulose Binding Domain (CBD) Fusion Protein

The *A. sp.* JD6.5 prolidase gene with N-terminal Met codon and the translation termination codon was obtained from pTCJS4 (9) by PCR subcloning using two synthetic primers; 5'-primer (5'-GGGTGATTCATGAATAAATTAGCGG-3') and 3' primer (5'-GGAATTCTACATGAGCCCAGCAG-3'). The amplified gene fragment cleaved with *RcaI* and *EcoRI* was ligated to the *NcoI* and *EcoRI* sites of the pET34DNA, the CBD expression vector from Novagen (Madison, WI). After ligation and transformation into *E. coli* BL21, the recombinant cell line induced by IPTG, was grown to mid-log phase at 30°C. Batch purification of enzyme-CBD fusion protein from cell lysate was performed with Novagen's CBind Resins following manufacturer's procedures. The cotton fabrics, cotton ball (ACME Cotton Products, Co., Inc., Valley Stream, NY.) and cheesecloth (American Fiber and Finishing Inc., Colrain, MA), were soaked in prolidase-CBD fusion protein for 10 min at room temperature and then rinsed with 1% ammonium carbonate solution. After air drying at room temperature, a piece of the fabric was tested for DFP hydrolyzing activity.

RESULTS AND DISCUSSION

The enzymes from different *Alteromonas* species are similar in their biochemical and catalytic properties, and functionally related to X-Pro dipeptidases with a broad range of substrate specificity (5,6). Besides cleaving the C-N bond of X-Pro dipeptides, *A. prolidases* catalyze hydrolysis of a wide range of OPs with P-F, P-O, and P-CN bonds (6), including DFP, soman (GD), sarin (GB), tabun (GA), and paraoxon. As shown in Table 1, the *A. prolidases* from various species exhibit various degrees of enzyme activity against GB, GD and GF, the highly toxic chemical nerve agents. In contrast to OPH, no VX hydrolyzing activity was observed for *Alteromonas prolidases*. Activity of the enzyme on these OPs may be due to the fortuitous similarity of these compounds on size, shape and surface charge to the X-Pro dipeptides.

As reported previously, the DH5α cell line carrying pTCJS4 was shown to produce high levels of *A. sp.* JD6.5 prolidase enzyme (9,10). Recently, we have compared the growth condition and expression level of the enzyme in

other *E. coli* strains, BL21 and XL1, containing pTCJS4. The specific activity of these recombinant cells increased sharply 2 hours after IPTG induction and reached maximum around 5-6 hours after induction. The highest specific activity was observed in lysate from XL1 recombinant cells. Total enzyme recovered from XL1 cells is 32 and 49% higher than that recovered from DH5 α and BL21 cells, respectively. After 5 h IPTG induction, the prolidase enzyme in XL1 cells was ~50% of total cell protein (corresponding to ~250 mg/L of culture). The recombinant enzyme has the same molecular weight as the purified enzyme from native cells. The results from the expression level of the enzyme suggest that XL1 is the most suitable host strain for optimum production of the *Altermonas* prolidase.

The full potential of enzyme decontamination technology can only be recognized if large quantity of enzyme can only be produced cheaply. On-going bioreactor fermentations with recombinant cells (XL1/pTCJS4) in our laboratory have resulted in higher recovery and yield (1 g/L) of prolidase enzyme (unpublished results, Kim, Cheng, Rastogi, DeFrank). Compared to shaker flask, the increase in enzyme yield was brought about by corresponding increase in cell mass recovery. Current fermentation protocol has resulted in a high level of enzyme production corresponding to 30-40% of the total cell protein, confirmed both by SDS-PAGE analysis and activity determination of the enzyme (unpublished results, Kim, Cheng, Rastogi, DeFrank). With the availability of fermentors ranging from 30L to 1,500L capacity in Bioprocess Engineering Facility at the US Army Edgewood Chemical Biological Center, large-scale production of *Altermonas* prolidase is now achievable with this recombinant clone. Availability of *Altermonas* prolidase in kilogram quantity is a key pre-requisite for development of a safe and non-corrosive decontamination system.

As reported previously (11), the enzyme can be packaged in a lyophilized form (Figure 1) and can be reconstituted as needed with any available liquid. A number of matrices such as water-based foams and wetting agents for use as enzyme-based decontaminants were examined (9,10). Several biodegradable wetting agents such as “Cold Fire” and “Odor Seal” (FireFreeze Worldwide Inc., NJ), were found to enhance or stabilize enzyme activity. “Cold Fire” effectively and safely suppresses fire, where “Odor Seal” is an environmentally safe odor eliminator. All these agents are biosurfactant-based and biodegradable in nature. The incorporation of such matrices in the enzyme decontamination system not only provides a medium to encapsulate the CW agents but also assist in the solubilization of the hydrophobic CW agents for enzyme action. Foam systems offer many advantages for decontamination of nerve agents in large areas. Interestingly, the enzyme also retained full catalytic activity in commercial laundry detergent “Tide Free” (Procter & Gamble, OH) and “Protectall” skin cream (J.G. Worldwide Medical, NJ). Such active enzyme matrices provide a useful means for protective clothing clean up and wound healing.

In collaboration with researchers in other government agency, academia, and industry, the recombinant *A. sp.*JD6.5 prolidase has been incorporated into various matrices for protection against and detection of chemical G-agents (Table 2). The application of immobilized, catalytically active enzyme to detoxify nerve agents on a variety of surfaces including protective clothing, filters in gas masks, or skin, is being investigated in our laboratory. Since cellulose is an integral component of various cotton fabrics including filters, sponges, cotton, and gauze, the enzyme could be immobilized via coupling of the enzyme to cellulose binding domain (CBD) protein. *A. sp.*JD6.5 prolidase gene was cloned in CBD expression vector, pET34DNA. The recombinant construct generated a recombinant fusion protein (see “Materials and Methods”) which is 204 amino acid longer than *A. prolidase* (Figure 2). Based on K_{cat} , approximately 60% of the enzyme activity (~650 U/mg) in the CBD-enzyme fusion protein was retained (Table 2). Loss of activity could be attributed to improper folding of the fusion enzyme. The enzyme activity of fusion protein is quite stable over 3 months period. In addition, the specific activity of cellulose bound CBD-enzyme has been determined (Figure 3). The CBD-enzyme appears to bind to cheesecloth better than cotton. After repeated use, the CBD-prolidase retained ~70% enzyme activity, compared to only ~14% activity in cotton ball. The inability of the CBD-enzyme to bind to cotton may be due to pretreatment of cotton with either dyes or fire retardant chemicals during manufacturer’s processing.

Another novel approach for enhancing enzyme activity and stability is by generation of a highly efficient enzyme nanoencapsulation. Through Dr. Ray Yin, US Army Research Laboratory, a number of well-defined, stable, water soluble, non-toxic dendritic polymers was synthesized. The three-dimensional and spherical tree-like dendrimers are capable of nanoencapsulating *A. prolidase* inside their structure. Testing the DFP hydrolyzing activity of the encapsulated enzymes in one of the dendrimers demonstrated that the encapsulated enzymes are not only stable but also improve the catalytic performance of the enzyme (Table 2). Compared to enzyme alone, the activity of encapsulated enzymes was increased by 10-15% at various temperature and pH for a long period of time. Most interestingly, the stability of enzyme activity in dendrimer-enzyme conjugate increased over 100% in organic solvents, such as 20% of methanol or acetone, but not in chloroform. This opens a wide variety of application possibilities for use of immobilized enzyme in areas such as sensitive equipment decontamination, coating protective clothing against agents, and incorporation into skin cream/lotion for wound healing.

The linkage of various enzymes as cross-linked enzyme crystals (CLECs) and polyurethane foam has been shown to provide enzymes with remarkable temperature, pH, and organic solvent stability (12). Formation of enzyme CLECs was achieved following reaction with a bifunctional agent such as glutaraldehyde. The immobilization of enzyme within polyurethane sponges was prepared by chemically incorporated enzyme within polyurethane matrices during synthesis. Through the research collaboration (Table 2), the *A. sp*JD6.5 prolidase in CLEC and polyurethane foam have been prepared and tested against DFP hydrolyzing activity. Preliminary results illustrated that the specific activity of the enzyme were greatly reduced in both CLEC (150-200 U/mg) and polyurethane foam (100-150 U/mg). Nonetheless, considerable enzyme activity was retained in these matrices. The enzyme was stable for over six-month period. The stability and re-usability of immobilized enzyme in such matrices provide a valuable tool for equipment decontamination and personnel protection.

Alteromonas prolidase can also prophylactically protect the mice to the same degree as 2-PAM or atropine, the commonly use antidote, administrated by the intraperitoneal route. When the liposome encapsulated enzyme was combined with pyridinium-2-aldoxime-N-methyl iodide (2-PAM) or atropine, almost ten-fold increase in the protection against lethal effects of DFP was observed (Petrikovoics *et al*; see this Proceedings). In addition, effort is now underway using silica gel beads coated with *A. sp* prolidase in development of biosensor for detection of DFP and other simulants.

CONCLUSIONS

The development of recombinant clone XL1 (pTCJS-4), offers a real potential for large-scale production of the *A. sp*.JD6.5 prolidase, an enzyme with high level of activity against chemical G-agents. Although the enzyme activity in different matrices varies considerably, results have been very promising. The inclusion of enzyme into different matrices that are simple and safe for users, equipment, and the environment, offers a valuable tool for wide-range application of *A. sp*.JD6.5 prolidase. In addition to decontamination applications, the enzyme has also been shown to be potentially useful for protection, surface clean up, and detection. Currently, further wide-range applications are being extensively explored.

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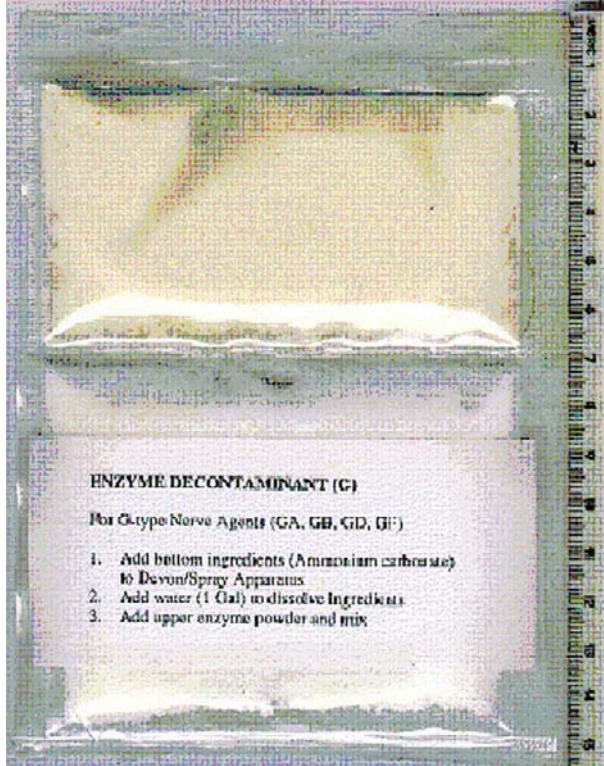
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FIGURES AND TABLES

Figure 1. A prototype of enzyme decontaminant. The package is for one gallon of decontaminant solution. The package contains dried enzyme (upper compartment) and reaction component, ammonium carbonate and $MnCl_2$ (lower compartment).

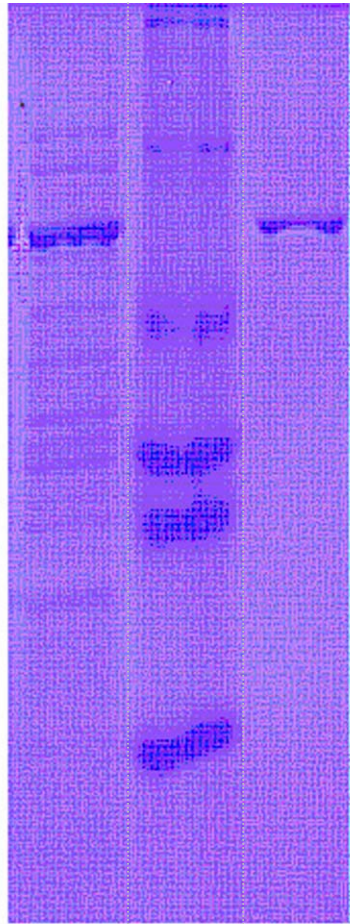


ENZYME DECONTAMINANT (G)

For G-type Nerve Agents (GA, GB, GD, GF)

1. Add bottom ingredients (Ammonium carbonate) to Decon/Spray Apparatus
2. Add water (1 Gal) to dissolve Ingredients
3. Add upper enzyme powder and mix

Figure 2. SDS-PAGE Analysis of *A. spJD6.5* prolidase-CBD fusion protein.



Crude Lysate	MW Marker	Pure Enzyme-CBD
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Figure 3. Performance of CBD-prolidase immobilized to fabrics

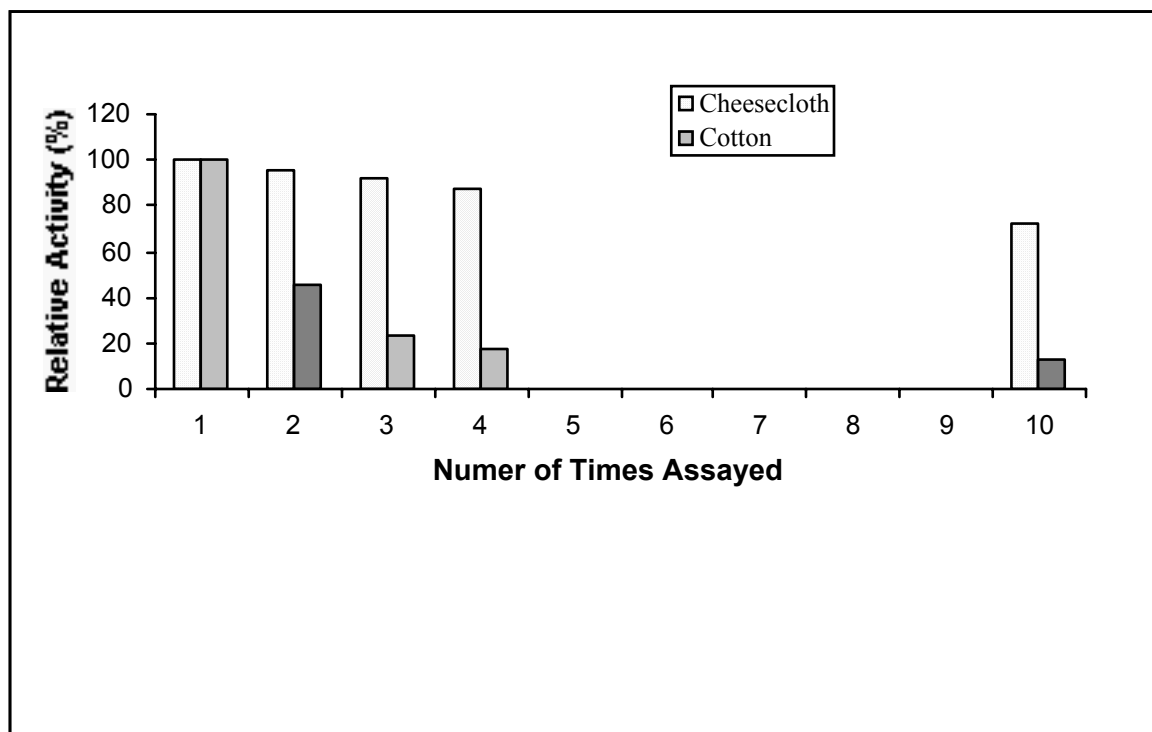


Table 1. Comparison of various enzyme activities against DFP and chemical nerve agents

Enzyme Source	K_{cat} (sec^{-1})					
	DFP	GA	GB	GD	GF	VX
<i>P. diminuta</i> (OPH)	465	N/A	56	5	N/A	0.3
<i>A. spJD6.5</i>	1650	85	611	3145	1650	0
<i>A. haloplanktis</i>	575	113	257	1389	269	0
<i>A. undina</i>	1239	292	376	2496	1586	0

N/A: not available.

Table 2. Applications of *A. sp.*JD6.5 prolidase in various formulation for protection and detection against chemical G-agents

Application	Matrics	Investigator/Collaborator	Specific Activity (U/mg)*
Protection	Cellulose binding domain (CBD)	Dr. Tu-chen Cheng US Army ECBC APG, MD	500-700
	Dendritic polymers	Drs. Ray Yin & Tu-chen Cheng US Army Army Research Lab. & US Army ECBC APG, MD	1700-2000
	Cross-linked enzyme crystals (CLECs)	Dr. Alexey Margolin Altus Biologics, Inc. Cambridge, MA	150-200
	Polyurethane Foam	Dr. Alan Russell University Of Pittsburgh Pittsburgh, PA	100-150
Personnel Protection	Prophylactic use	Drs. Ilona Petrikovoics & James Way Texax A & M College Station, TX	Enhance antidotal protection**
Detection	Biosensor	Drs. Aleksandria Simonian & James Wild TexaxA & M College Station, TX	on going

*Use DFP as substrate; ** see this proceeding.