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IDENTIFICATION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF THE GV-DEGRADING ENZYME FROM ATCC # 29660 *ALTEROMONAS UNDINA*

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The GV (2-dialkylaminoalkyl N,N-dialkylphosphonamidofluoridate) nerve agent has a toxicity intermediate to G and V-type nerve agents and is not catalyzed by either organophosphorus acid anhydrolases (OPAA) or organophosphorus hydrolase (OPH) enzymes. We have screened and identified a number of *Alteromonas* strains possessing catalytic activity using a GV compound as substrate. The enzyme from one of these strains, *A. undina,* has been purified to homogeneity by ammonium sulfate fractionation and Q Sepharose anion exchange chromatography. The activity of GV-hydrolyzing enzyme peak is distinct from that of *A. undina* OPAA following the Q Sepharose column chromatography. The SDS-polyacrylamide gel electrophoresis of the GV-hydrolyzing enzyme fraction revealed a single polypeptide of ~20kDa. To our knowledge, this is the first report of enzymatic detoxification of GV.

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PREFACE

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IDENTIFICATION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF THE GV-DEGRADING ENZYME FROM ATCC # 29660 *ALTEROMONAS UNDINA*

1. INTRODUCTION

The enzymes organophoshorus acid anhydrolase (OPAA) from *Alteromonas sp.* JD6.5 and organophosphorus hydrolase (OPH) from *Pseudomonas diminita* have been shown to catalyze the hydrolysis of a number of toxic organophosphorus (OP) compounds including several G-type chemical nerve agents.[1-12] The OPH also has catalytic activity against V-type nerve agents.[13] Both these enzymes have been cloned into *Escherichia coli* and can be produced in large quantities. The OPH enzymatic activity for specific substrates has also been increased by genetic manipulation of the cloned gene.[14] The catalytic activity of these enzymes against nerve agents provides considerable potential for decontamination and detoxification of toxic OPs, and/or *in vivo* prophylaxis.

There is another important class of potential threat agents for which catalytic decontamination has not previously been demonstrated. The GV compounds have the generalized structure of 2-dialkylaminoalkyl N,N-dialkylphosphonamidofluoridate. An example is 2-dimethylaminoethyl N,N-dimethylphosphonamidofluoridate(Figure 1). The GV compounds are generally much more toxic than G agents and are not included in the Chemical Weapons Convention schedules of compounds.

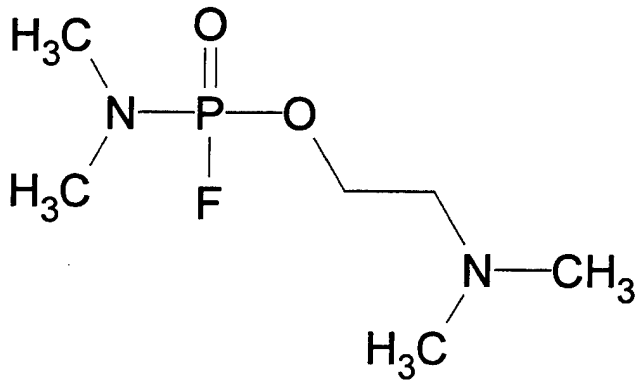

Figure 1. Structure of 2-dimethylaminoethyl N, N-dimethylphosphonamidofluoridate

Neither OPAAs from *A. sp.* JD6.5 or *A. haloplanktis* , nor OPH have any detectable catalytic activity against compound 1 (unpublished data). To date, there have been no reports on the enzymatic detoxification of GV compounds.

The GV poisoning is also refractory to traditional atropine/ reactivator treatments.[15] Enzymes, however, may offer a successful treatment for GV poisoning. *In vivo* circulating enzymes have already been demonstrated to confer protection against toxic OP agents in mice. The LD_{50} levels of protection exceed those obtained with atropine/reactivator.[16]

Finally, the gene encoding GV enzymes may offer a good basis for protein engineering for improving VX and G-agent activity due to the similarity of GV and VX structures.

2. MATERIALS AND METHODS

2.1. Growth of Bacterial Strains.

Bacteria strains were grown in Instant Ocean Medium consisting of 38 g of Instant Ocean (Aquarium Systems, Mentor, OH), 5 g of proteose peptone, and 1 g of yeast extract per liter at 30 °C for 16-20 hr.

2.2. Enzyme Assays.

Enzyme assays were conducted with a fluoride electrode attached to a Corning 355 pH/ion analyzer. Fluoride release rates were followed until a stable release rate was observed over a period of several minutes. Reactions were conducted in a temperature-controlled vessel in a total volume of 2.5 mL. Background hydrolysis rates (Table 1) were measured to provide a comparison of the relative stability of the different substrates in an aqueous matrix. Background hydrolysis rates were measured by adding neat substrate directly to 2.5 mL buffer solution to a final concentration of 0.01 M. For assaying enzymatic activity, substrates were diluted into isopropanol for the OPAA assays and into methanol for the OPH assays. The reaction medium contained 50 mM Bis-tris-propane (BTP) pH 7.2, 0.1 mM $MnCl_2$ for OPAA assays and 0.05 mM $CoCl_2$ for OPH assays, 3 mM GV compound (1), and 1-5 μL of enzyme sample in a total volume of 2.5 mL. For each assay, the substrate was added last. After a 1-4 min equilibration period, a background hydrolysis rate was measured for 4 min. An identical reaction mixture was then monitored in the presence of enzyme and the background values were subtracted. Assays conditions were adjusted to ensure that <10% of the total substrate was consumed in the assay.

3. RESULTS AND DISCUSSION

3.1. Spontaneous Hydrolysis – Comparison to G-type Nerve Agents.

The GV is relatively stable in aqueous solution, as compared to GB and GD. Table 1 illustrates the spontaneous hydrolysis (defluorination) rate of 0.01 M GB, GD and GV at pH 7.2 and 25 °C.

Table 1. Spontaneous Hydrolysis Rate of 0.01 M Substrate in 50 mM Bis-tris-propane (pH 7.2) at 25 °C

Substrate	Rate (μmol/min)
GB	58
GD	35
GV	4.0

3.2. Screening of Bacterial Strains and Enzymes for GV Activity.

Because no enzymes have previously been reported to catalyze the hydrolysis of GV, a screening was conducted to search potential sources. The reaction scheme for GV detoxification is illustrated in Figure 2. Fluoride is the leading group (as HF) and defluorination yields a hydroxylated product (GV-OH). Reactions consisting of 3 mM GF, buffer, and crude cell lysate were monitored by fluoride electrode for the rate of increase in the fluoride ion concentration.

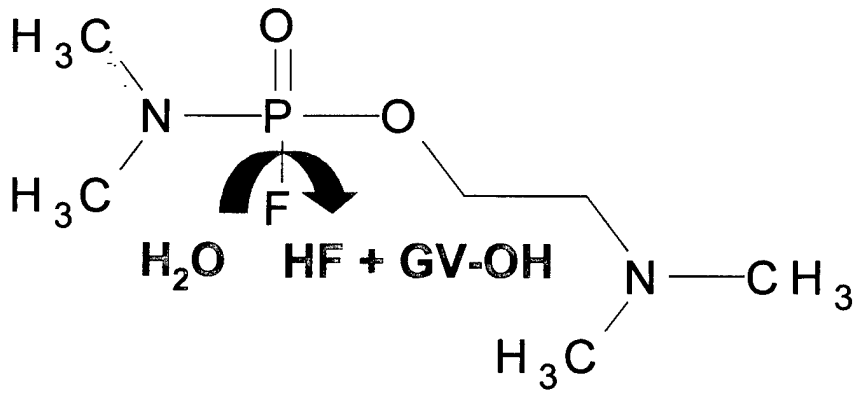

Figure 2. Reaction Scheme for the Hydrolytic Defluorination of GV

Twelve *Alteromonas* strains were used because they have been shown to possess activity against different OP neurotoxins. These *Alteromonas* strains were grown in Instant Ocean Medium, lysed twice in a French press, centrifuged and the lysates obtained were assayed for activity against GV compound (1). Assays were conducted in 50 mM Bis-tris-propane (pH 7.2) at 25 °C. Results are shown in Table 2.

Table 2. GV Activity (nmol/min/mL) of Lysates

ATCC #	Strain	GV Activity	ATCC #	Strain	GV Activity
27127	*A. haloplanktis B*	169	14393	*A. haloplanktis Z*	77
23821	*A. haloplanktis C*	24		*A. espejiana*	132
19648	*A. haloplanktis J*	186	33043	*A. sp. G*	192
	A. haloplanktis M	444	33524	*A. sp. M*	238
33492	*A. luteoviolaceans*	5	29332	A. sp. P	40
35358	*A. undina*	155	19375	A. niger	29

In addition to the lysates shown in Table 2, three different purified enzymes (OPAA cloned from *A. sp.* JD6.5 and *A. haloplanktis*, and OPH from *P. diminita*) were also tested. Previously, all three of these enzymes had been demonstrated to possess significant activity against several G-type agents.[7,9,10,11] However, none of the three had any detectable GV activity under the conditions used for the assays (MATERIALS AND METHODS). Limits of detection for the assays were at least in the low nmol/min/mg range.

It should be noted that enzymes with higher specific activity are sometimes obtained from strains with lower crude lysate activity. In addition, the crude lysate enzymatic activity is usually dependent on growth conditions. Based on previous enzyme purification experience with *A. undina* # 35358 strain, it was selected for further purification of its GV-hydrolyzing enzyme(s), designated as GVH.

3.3. Purification of GVH.

A single colony was grown overnight in Instant Ocean Medium (pH 7.0) at room temperature in a 100 mL shake flask. The overnight cultures were transferred to a BioFlow 3000 bioreactor (New Brunswick Scientific Co) with a 6 L working volume of the same medium and cultivated for 7.5 hr at a pH of 7. A total of 53 g wet cells were harvested and resuspended in 200 mL 10 mM bis-tris-propane pH 7.2. After cells were broken in a French pressure cell (SLM Aminco), cellular debris was removed by centrifugation at 5,000 xg. The cell lysate, which contained the GVH activity, was further purified by 40-65% ammonium sulfate fractionation. The pellet was re-suspended in a minimal volume of 10 mM bis-tris-propane pH 7.2 and dialyzed against several changes of the same buffer. The dialyzed sample was then applied to a Q Sepharose (Pharmacia) column previously equilibrated with 10 mM bis-tris-propane

pH 7.2, washed with 75 mL of the same buffer, and eluted stepwise with 0.1 M and 1 M NaCl.

Table 3. Stepwise Elution of GVH Activity

Fraction	OD_{280}	GVH Activity (uM F-/min/50 uL)	Specific Acivity (uM F-/min/50 uL/OD_{280})
10 mM BTP Wash	2.82	1.93	0.687
0.1 M NaCl Eluate	0.365	10.8	29.6
1 M NaCl Eluate	1.39	6.76	4.87

The 0.1 M NaCl eluate with the highest enzymatic activity was dialyzed overnight against 10 mM BTP pH 7.2 and again loaded on a second Q Sepharose column. After the nonbinding protein was removed by washing, elution was carried out with a linear gradient of 0 - 0.6 M NaCl in bis-tris propane buffer and analyzed for GVH activity. Figure 3 shows the GVH activity, DFP activity (OPAA) and OD_{280} profile. The OPAA (DFP) activity peak is clearly distinct from the GVH activity peak, indicating these enzymes are not the same.

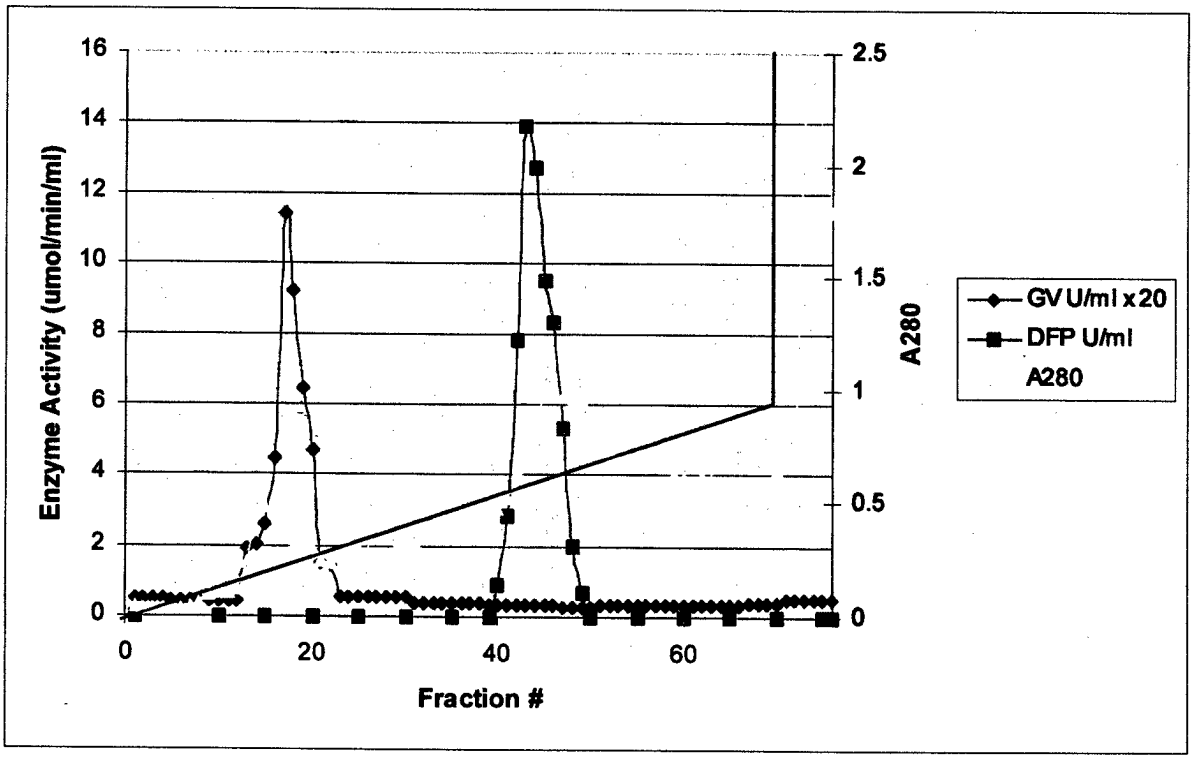

Figure 3. Purification of GVH on Q Sepharose Column with 0 - 1.0 M NaCl Gradient

The fractions with GVH activity (fraction number 16-20) were pooled and loaded on a second smaller (5 mL) Q Sepharose column and eluted with a 0-0.15 M NaCl gradient (Figure 4).

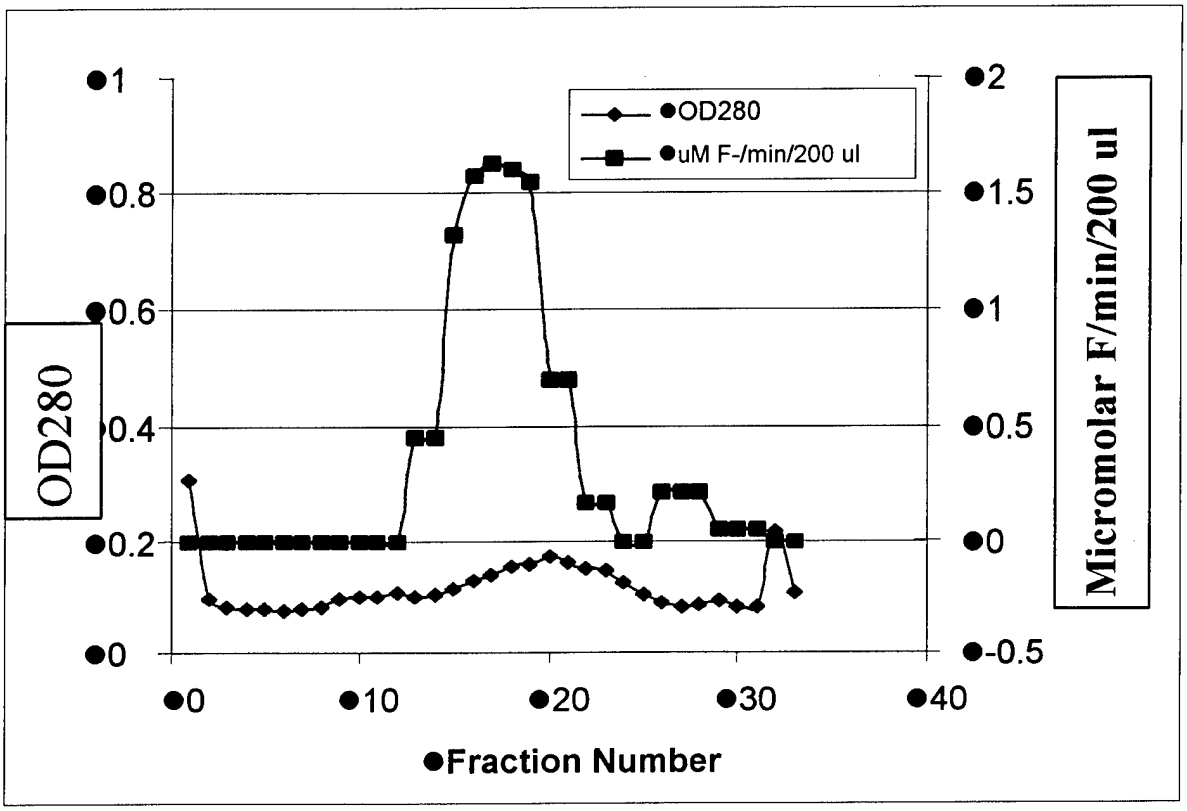

Figure 4. Purification of GVH on Second Q Sepharose Column

The enzyme fraction with highest activity (fraction #18) from this second Q Sepharose chromatogram was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Results (Figure 5) show a single band with a molecular weight of approximately 20 kilo Daltons (kDa).

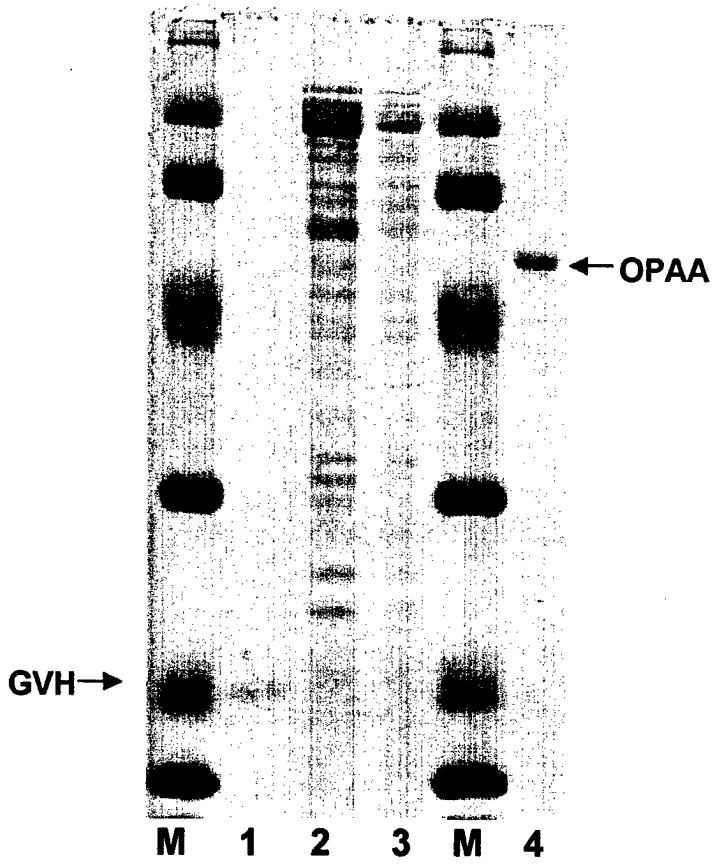

Figure 5. SDS-PAGE of Fractions from GV Degrading Enzyme Purification Lanes 1 to 3 contain purified enzyme, 40-65% ammonium sulfate ppt, and crude extract, respectively. M; molecular weight standards.

3.4. Enzyme Characterization.

3.4.1. pH Profile.

The most active fraction (partially purified enzyme) from the first Q Sepharose column was used to determine the optimal assays conditions for the enzyme. The profile of the enzyme activity versus pH (Figure 6) was obtained using 3 mM substrate at 25 °C in 50 mM bis-tris-propane buffer.

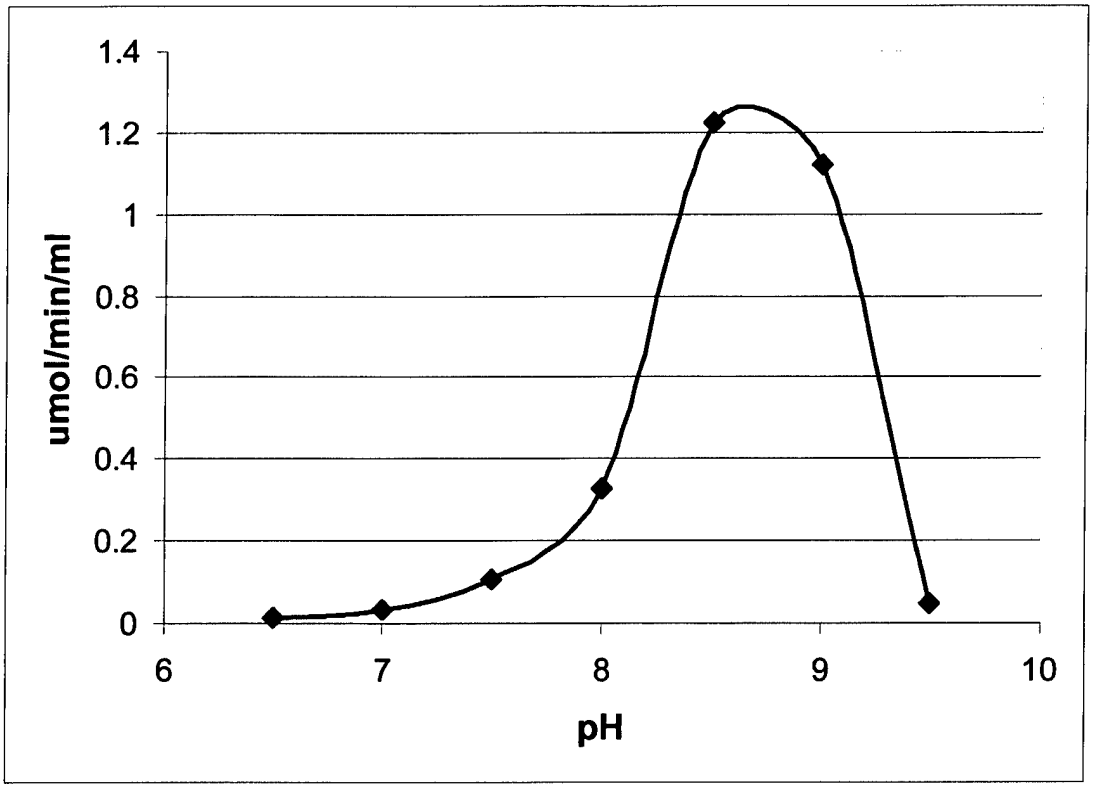

Figure 6. pH Profile of GVH

3.4.2. Temperature Profile.

Under the same condition used for the pH profile, a temperature profile was determined for the GVH enzyme at the optimal pH of 8.5 (Figure 7).

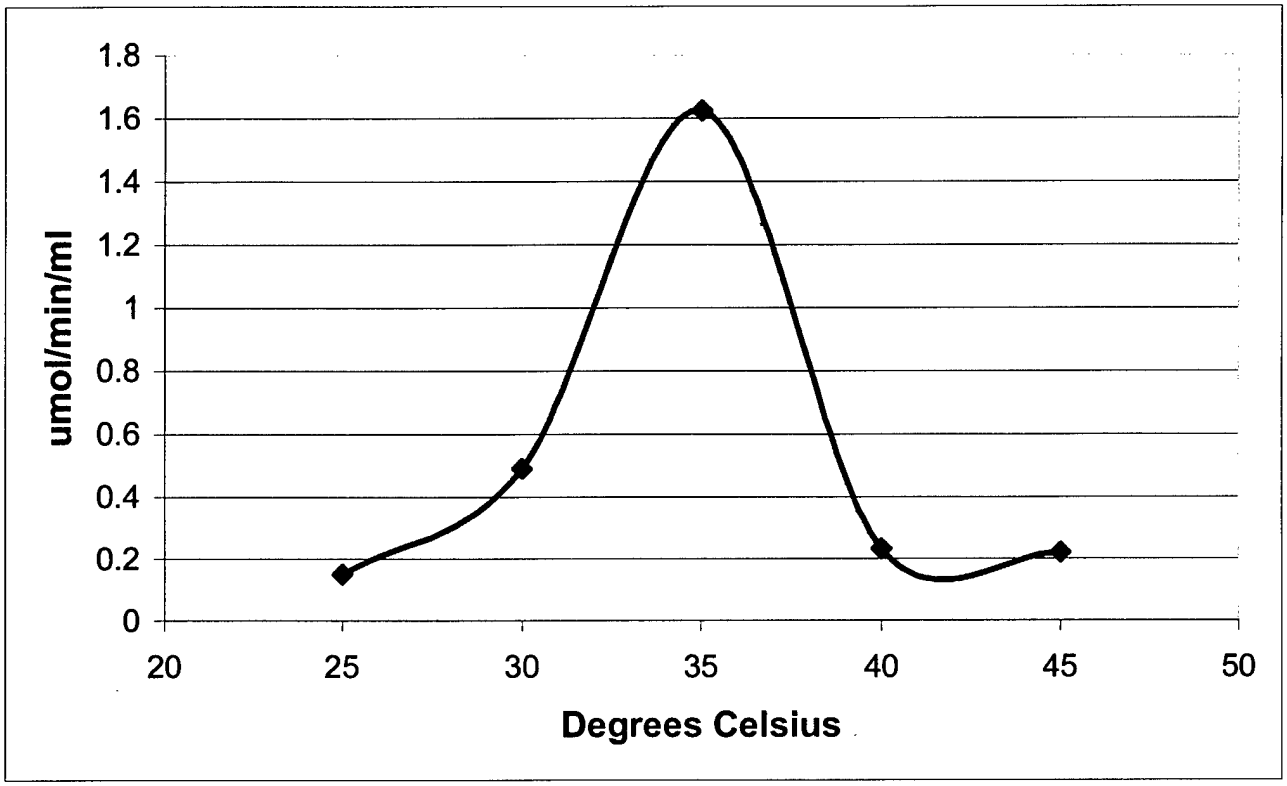

Figure 7. Temperature Profile of GVH

4. CONCLUSIONS

First report of enzymatic degradation of GV -demonstrated presence of GVH in several strains.

The *A. undina* GVH has been purified to apparent homogeneity (as determined by SDS-PAGE) following ammonium sulfate precipitation and two successive Q Sepharose chromatography steps.

The GVH enzyme has fairly narrow pH and temperature profiles with optima at approximately pH 8.5 and 35 °C, respectively.

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