Purification of Phospholipase D from *Dacus carota* by Three-Phase Partitioning and Its Characterization

Shweta Sharma and M. N. Gupta

Chemistry Department, Indian Institute of Technology, Hauz Khas, New Delhi 110 016, India

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Phospholipase D from *Dacus carota* (carrot) was purified by subjecting it to three-phase partitioning. The single step of three phase partitioning led to 13-fold purification with an activity recovery of 72%. SDS-PAGE analysis showed a single band with minimum molecular weight corresponding to nearly 80 kDa. The purified enzyme had a pH optimum in the range of 6.0–6.5 and was unstable above 30°C. Kinetic studies showed a *K_m* value of 9.5 mM and a *V_{max}* of 0.35 mL min⁻¹. The enzyme purified by three-phase partitioning was found to resolve into two isoenzymes on a DEAE-cellulose column.

Key Words: three-phase partitioning; phospholipase D; t-butanol; *Dacus carota*.

Phospholipase D is an important signaling phospholipase in higher systems (1, 2). In recent years, considerable work has been carried out regarding its involvement in mammalian (2, 3) and plant systems (4–7). Recently, we have standardized a convenient titrimetric assay for screening phospholipase D and found significant activity in carrot (*Dacus carota*) (8). In this paper, we describe the purification of this activity in carrot by a single step protocol using three-phase partitioning (TPP).² The extractive and partitioning components of TPP using C₄ and C₅ organic cosolvents tend to remove pigments and many other low-molecular-weight impurities from the proteins (9). It is believed that the cosolvents bind to the hydrophobic parts of the protein effectively to reduce the density of the protein(s) which float at the interface of the aqueous and organic solvent phases. The organic cosolvents are used in combination with salting out agents such as ammonium sulfate (9).

TPP is generally regarded as a nonspecific separation method and primarily an upstream technique. This work shows that, at least in fortuitous cases, it may work equally well as a downstream processing step and can, in fact, alone, yield a fairly homogenous protein/ enzyme preparation.

MATERIALS

Soybean lecithin (phosphatidyl choline) was obtained from BDH, Merck (India). Acid phosphatase (wheat germ) and *p*-nitrophenylphosphoryl choline were purchased from Sigma Chemical Co. (St. Louis, MO). Carrot was purchased from the local market. All solvents used were of HPLC grade and all other reagents were of analytical grade.

METHODS

Enzyme Assay

Phospholipase D activity was measured titrimetrically according to Sharma *et al.* (8). The reaction velocity was measured titrimetrically using 0.02 N NaOH by measuring the rate of proton liberation during hydrolysis of soybean lecithin. A Metrohm pH stat titrator (Model 718 STAT Titino) connected to an exchange unit (EXCH. UNIT 739) and a magnetic stirrer (Model 728 MAGN. STIRRER F. TITR/DOS) was used. One unit of enzyme activity was defined as the amount of
enzyme that liberated 1 μmol of acid from soybean lecithin per minute at 25°C at assay pH of 7.0.

The purified enzyme preparation was also assayed spectrophotometrically, using a synthetic substrate p-nitrophenylphosphoryl choline. The extent of hydrolysis was monitored by measuring the absorption of liberated p-nitrophenol at 400 nm. Substrate solution (25 μmol of p-nitrophenylphosphoryl choline in 50 mM acetate buffer, pH 5.6, containing 50 mM CaCl₂) was mixed with 100 μL of acid phosphatase (1 mg/mL in the same buffer) and phospholipase D was added such that the final volume was 0.9 mL. After incubation at 37°C for 1 h, 100 μL of 0.05 N NaOH was added to stop the reaction, and absorbance was read at 400 nm against blank from which phospholipase D was omitted (10). Blank containing only acid phosphatase and no phospholipase D was also taken to rule out the presence of wheat germ phospholipase D activity as the source of acid phosphatase was wheat germ:

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\text{p-nitrophenylphosphoryl choline} \xrightarrow{\text{phospholipase D}} \text{p-nitrophenylphosphate} + \text{choline} \xrightarrow{\text{acid phosphatase}} \text{p-nitrophenol} + \text{phosphate.}
\]

Phospholipase C is well known to hydrolyze p-nitrophenylphosphoryl choline to liberate p-nitrophenol which can be measured spectrophotometrically. The same substrate hydrolyses phospholipase D to give p-nitrophenyl phosphate but on subsequent addition of acid phosphatase p-nitrophenol is liberated. The enzyme showed no activity without the addition of acid phosphatase which provides sufficient evidence of the enzyme in use being only phospholipase D.

**Glycoprotein Assay (Phenol-Sulfuric Acid Test)**

Twenty-five microliters of 80% phenol solution in distilled water was added to 1.0 mL of the sample. To the same mixture 2.5 mL of concentrated sulfuric acid was added and kept at 25°C for 10 min. Absorbance was read at 489 nm (11).

**Protein Estimation**

Protein concentration was determined by the dye binding assay (12) using BSA as the standard protein.

**TABLE 1**

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity units (U)</th>
<th>Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>78</td>
<td>1.1</td>
<td>70.9</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Phase 1 (ppt)</td>
<td>20</td>
<td>0.71</td>
<td>28.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Phase 2 (ppt)</td>
<td>56</td>
<td>0.06</td>
<td>933</td>
<td>13</td>
<td>72</td>
</tr>
</tbody>
</table>

Note: Crude extract of carrot phospholipase D (78 U) was saturated with 30% ammonium sulfate followed by addition of t-butanol. Three phases formed were collected separately. Activity was estimated in the interfacial precipitate. The lower aqueous phase was subjected to second phase of TPP. Interfacial precipitate was tested for enzyme activity. The activity initially added has been taken as 100%.
Preparation of the Phospholipase D Crude Extract from Carrot

The crude extract of phospholipase D was prepared according to Sharma et al. (8). Carrot roots (200 g) were washed, chopped, and then homogenized in 170 mL of distilled water at 4°C for 3 min in a prechilled Waring blender, filtered through four layers of cheese cloth, and centrifuged at 15,000g for 30 min. The pH of the supernatant was adjusted to 7.0 by adding microliter (<5 μL) amounts of 1 N sodium hydroxide.

Purification of Phospholipase D by Three-Phase Partitioning (TPP)

The crude extract of phospholipase D (78 U) was brought to 30% ammonium sulfate (wv⁻¹) saturation. The addition of ammonium sulfate resulted in the pH
**PURIFICATION OF PHOSPHOLIPASE D FROM *Daucus carota***

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**Effect of Temperature**

The purified enzyme was exposed to different temperatures (20–80°C) for 15 min. This was followed by adjusting the temperature to 25°C and assaying the residual enzyme activity.

**Effect of Calcium Ions on Enzyme Activity**

Different concentration of calcium chloride (10–70 mM) were added to the substrate solution to determine the enzyme activity in the presence of calcium ions.

**Effect of Surfactants on Enzyme Activity: Sodium Dodecyl Sulfate (SDS), Cetyl Trimethyl Ammonium Bromide (CTAB), and Triton X-100**

The ratio of phosphatidyl choline to the various surfactants (viz., SDS, CTAB, and Triton X-100) was varied in the substrate solution (keeping the substrate concentration constant in each case) and the enzyme activity was then assayed as above.

**Determination of $K_m$ and $V_{max}$**

Different concentrations of substrate were used to assay enzyme activity. Leomara software was used to determine $K_m$ and $V_{max}$ (14).

**Ion Exchange Chromatography**

The purified phospholipase D (in 10 mM Tris-HCl buffer, pH 7.0) was subjected to an ion exchange column (1.0 × 12) packed using DE-52 (DEAE–cellulose) which was preequilibrated with 10 mM Tris–HCl, pH 7.0. Elution was carried by salt gradient (0–1 M sodium chloride in 10 mM Tris-HCl, pH 7.0). The fractions of 1.0 mL each were collected and were monitored for protein and enzyme activity.

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**RESULTS AND DISCUSSION**

The various purification protocols described for phospholipase D generally consist of multistep procedures (15–19). Colored crude aqueous extracts of carrot contained about 15 U/mL of phospholipase D activity as estimated by the titrimetric assay. While using TPP for enzyme purification, t-butanol was chosen as the organic cosolvent as it has been generally reported to give the best results (9, 20, 21). Figures 1a and 1b show the effect of varying ammonium sulfate concentration, temperature, and ratio of t-butanol to crude (during TPP) on total units of activity and specific activity (fold purification) obtained in the interfacial precipitate, respectively, in all the three cases. It can be seen that the best results are obtained with 30% ammonium sulfate. The temperature for this operation is important since

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**SDS–Polyacrylamide Gel Electrophoresis**

SDS–PAGE of the samples using 12% gel was performed according to Hames (13) using Genei electrophoresis unit (Bangalore Genei Pvt. Ltd., Bangalore, India).

**Properties of the Purified Enzyme**

**pH stability.** The purified enzyme solution was kept in three different buffers (10 mM citrate–phosphate, pH 3.0–7.0; 10 mM phosphate, pH 7.0–8.0; 10 mM Tris-HCl, pH 8.0–9.0) at 4°C. After 30 min, the pH of the enzyme solution in different buffers was brought to assay pH (without any significant changes in the volumes) to estimate the residual enzyme activity.

**pH optimum.** The substrate and enzyme solutions were preadjusted to different pH values (3.0–9.0) using different buffer solutions as described above and the assay was carried out at these respective pH values.

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**FIG. 6.** Effect of calcium ions on enzyme activity. 50 µL of the purified enzyme (containing 1.4 U) was used for testing the enzyme activity using different concentrations of the Ca²⁺. Each set of experiment was carried out in duplicate which did not vary more than 5% decreasing to about 6.7. The pH was adjusted back to 7.0 by addition of 2 µL of 1 N sodium hydroxide. This was followed by addition of t-butanol (9) in the ratio of 1:1 (v/v) (crude extract to t-butanol). The solution was kept at 25°C for complete phase separation. After 30 min, the mixture was centrifuged at 2000g for 10 min and the three phases formed were collected separately. In the first stage the enzyme did not precipitate completely, so the lower aqueous layer was subjected to second stage of three-phase partitioning (similar to first stage) and the middle layer containing enzyme activity was collected.
FIG. 7. Effect of varied ratio of phosphatidyl choline to surfactants. 50 μL of the purified enzyme solution was used for the enzyme assay. Sodium dodecyl sulfate (SDS), cetyl trimethyl ammonium bromide (TAB), and Triton X-100 were added to the substrate solution keeping the substrate concentration constant. The starting activity was 91 U. Each set of experiment was carried out in duplicate which varied within ±5%. Effect on activity units on when the following surfactants were used: (a) SDS, (b) CTAB, (c) Triton X-100.
lowering the temperature from 25 to 4°C led to a decrease in the activity yield by 13% although there was no change in the specific activity of the enzyme in the precipitate. This shows that decrease in temperature affects partitioning of all proteins in a similar way. In this case (at least) 25°C seems to be a better option for carrying out TPP. Conditions for TPP may vary from enzyme to enzyme, so optimization is desirable in each individual case.

The relative amount of t-butanol is also rather important. The best results were obtained when the ratio of t-butanol to aqueous extract was 1:1. However, when the ratio of t-butanol to aqueous crude extract (v:v⁻¹) was 1.5:1, most of the enzyme and protein precipitated in the first stage only and the second stage was not carried out (Figs. 1a and 1b). Table 1 shows the results obtained by using these optimized conditions. In a single step, phospholipase D was purified 13-fold with 72% activity recovery. SDS-PAGE analysis of the purified preparation showed that the enzyme was homogenous with a minimum molecular weight corresponding to nearly 60 kDa (Fig. 2). The actual molecular weight of the subunit may be in fact greater. The purified preparation was found to be glycoprotein as tested by phenolsulfuric acid method (data not shown). SDS-PAGE is known to underestimate the molecular weight of glycoproteins since they show less binding for SDS (22). In case of phospholipase D from peanut, the enzyme have been reported to be oligomer with subunit weight of around 50,000 Da (15). Thus, the value of the molecular weight here is in the similar range. Purified preparation was found to be stable within the pH range of 7–8 (Fig. 3). The broad pH optimum was in the range of 6.0–6.5 (Fig. 4). An earlier report using a different and crude enzyme indicated an optimum pH range of 5.6–5.8 (23). The enzyme was not very stable even at moderately high temperature (Fig. 5) and started losing activity at 30°C. This agrees well with the reported thermostable nature of the enzyme (23). Also, the purified preparation was quite stable for at least a week when stored at 4°C.

Calcium chloride (30 mM) was found to be the optimum for maximum enzyme activity, as has been earlier
reported for peanut phospholipase D (Fig. 6) (8). Detergents were also found to effect the activity of enzyme. SDS, an anionic detergent in the molar ratio of 4:3:2:1 (phosphatidyl choline to SDS) triggered enzymatic hydrolysis by nearly 150% (Fig. 7a). Maximum activity of various phospholipases D has been reported when this molar ratio was between 2:1 and 5:1 (PC:SDS) (23). A neutral detergent, Triton X-100 increased the activity by nearly 120% when the ratio was 2:5:1 (Fig. 7b). CTAB did not cause any significant change in enzyme activity (Fig. 7c).

In order to check the homogeneity of the enzyme purified by TPP alone, the preparation was chromatographed on DEAE-cellulose using a salt gradient (Fig. 8). The enzyme activity of the fraction was estimated by a coupled assay wherein the hydrolysis of \( \rho \)-nitrophosphoryl choline in the presence of an acid phosphatase was estimated. The coinciding protein and activity profiles indicate two homogenous protein peaks.

Thus, TPP did not discriminate between these two isoenzymes which obviously differ in the charges present in the molecules.

\( K_m \) and \( V_{max} \) of the purified enzyme were found to be 9.5 \pm 1.4 mM and 0.35 \pm 0.039 ml mm \(^{-1}\) respectively, as determined using Leonara software. Eisent and Clark (24) have reported somewhat similar \( K_m \) value of 11 mM for carrot phospholipase D with a crude enzyme preparation.

In any case, these values for the kinetic parameters are average values since this preparation purified by TPP turned out to be a mixture of at least two major isoenzymes.

To conclude, this work shows that with necessary optimization, TPP may be a useful technique for purification of enzymes. It is a simple, quick and economical technique and scaling up is convenient. The target enzyme chosen here, phospholipase D from carrot, has been reported (24) but not purified.

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