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Phospholipase D from Savoy Cabbage: Purification and Preliminary Kinetic Characterization†

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ABSTRACT: Phospholipase D has been purified 680-fold from an acetone powder of savoy cabbage in an overall yield of 30%. The purification involves solubilization of the acetone powder in a Ca^{2+}-containing buffer and subsequent ammonium sulfate fractionation. Gel filtration on Sephadex G-200 and hydrophobic affinity chromatography using a γ-aminopropane–agarose gel complete the purification. The two chromatographic steps were conducted in buffers containing 50% ethylene glycol, which was necessary in order to maintain stability of the enzyme. Purity was established on the basis of gel electrophoresis and ultracentrifugation. A preliminary kinetic characterization of the enzyme was carried out by using lecithins with short-chain fatty acids below the critical micelle concentration. A complex series of results were obtained which demonstrated the following. (1) The enzyme is quite sensitive to ionic strength, being inhibited at high ionic strength. (2) The pH optimum depends on the concentration of Ca^{2+} used in the assay. At 0.5 mM Ca^{2+} the pH optimum is 7.25, but it is 6.0 at 50 mM Ca^{2+}. (3) The effect of substrate concentration at a given pH and ionic strength did not show simple hyperbolic kinetics but rather regions of parabolic and hyperbolic kinetics.

Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) catalyzes the hydrolysis of the ester linkage between the phosphatidic acid and alcohol moieties of phospholipids; additionally, this enzyme may catalyze a transferase reaction by which the phosphatidic acid moiety of the phospholipid substrate is transferred to an acceptor alcohol. This transphosphatidylation may be considered the general reaction, with hydrolysis repesenting a specific case in which the acceptor alcohol is water.

A partial purification of the cabbage enzyme was reported by Davidson & Long (1958) and Dawson & Hemington (1967). Yang et al. (1967) extended the original procedure of Davidson & Long to achieve a 110-fold purification with 20% recovery of activity.

Dawson & Hemington (1967) have reported several characteristics of the hydrolytic activity of cabbage phospholipase D by using egg lecithin dispersions. Ca^{2+} was found to be essential for hydrolysis with an optimum concentration of ~40 mM. The reaction had a sharp pH optimum at pH 5.4. Hydrolysis was stimulated by diethyl ether and by anionic amphipathic substances. Quarles & Dawson (1969), also utilizing egg lecithin as substrate, found that the pH optimum of the reaction was shifted with the addition of anionic amphipathic activators. Long et al. (1967) found that the action of phospholipase D upon lysolecithin required the presence of Ca^{2+}, with an optimum around 25 mM. A broad pH optimum at about pH 5.8 was observed.

The first observations of transferase activity by phospholipase D were reported by Yang et al. (1967) and Dawson & Hemington (1967). A variety of water-soluble primary alcohols were active as acceptors for the phosphatidyl group transfer. When both acceptor alcohol and water were present, the transphosphatidylation was usually the preferred reaction [a recent review has appeared by Heller (1978)].

Materials and Methods

Materials. Phospholipase D (grade B), lyophilized, salt-free, and free of choline-destroying activity, was purchased from Calbiochem (San Diego, CA). It was isolated, according to the manufacturer, from cabbage by the heat coagulation and acetone precipitation procedures of Yang et al. (1969). Different lots gave essentially identical results. Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], buffer grade, was from Pierce Chemicals (Rockford, IL). Ethylene glycol, analytical grade, was from Mallinckrodt (St. Louis, MO). 1,3-Diaminopropane was from Aldrich Chemical Co. (Milwaukee, WI). Sephadex G-200 was from Pharmacia Fine Chemicals (Piscataway, NJ). Agarose Bio-Gel A-5m was from Bio-Rad Laboratories (Richmond, CA). Acrylamide and *N*,*N*′-methylenebis(acrylamide) were recrystallized from chloroform and acetone, respectively. Dialysis tubing was prepared by boiling in a 50% ethanol solution and then a 1 mM EDTA solution followed by rinsing in distilled water. Short-chain lecithins were prepared as described by Wells (1972) and Yabusaki (1975). 1,2-Dihexanoylphosphatidic acid was prepared from *sn*-3-glycerobromohydrin (Bird & Chadha, 1966) and dibenzyl phosphate by the method of Hessel et al. (1954). It was isolated as the barium salt and gave analyses of P, 6.19%, and Ba, 26.7% (theoretical: P, 6.15%; Ba, 27.3%), $[\alpha]^{25}_{546} = +9.3°$ (*c* 8.5, chloroform; as free acid). Distilled water was further cleansed by passage through an organic filter and an inorganic ion-exchange resin from Continental Deionized Water Service (Tucson, AZ). This highly purified water

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was used in all instances. All other chemicals were of reagent grade and used without further purification.

Purification of Phospholipase D. Protein concentration was determined by assuming $E_{280}^{1\%} = 10.0$. The crude enzyme (1 g) was suspended in 100 mL of diethyl ether, collected by filtration through sintered glass, resuspended, and collected again. The ether-insoluble residue was dried on the filter under an atmosphere of nitrogen. The dry powder was then dispersed in 40 mL of a solution of 50 mM sodium acetate and 25 mM calcium chloride (pH 5.0) in a Potter–Elvehjen homogenizing flask by hand movement of the Teflon pestle. The insoluble material was separated by centrifugation at 13000*g* for 10 min at 4 °C. The supernatant solution (I) contained essentially all the phospholipase D activity and was subjected to ammonium sulfate fractionation. Solid ammonium sulfate (20.64 g) was dissolved in 40 mL of the sodium acetate–calcium chloride buffer and chilled. Solution I was stirred on an ice bath while the ammonium sulfate–buffer mixture was added dropwise over a 10-min period. The final mixture [45% saturation in $(NH_4)_2SO_4$ at 0 °C] was stirred for an additional 15 min and then centrifuged at 13000*g* for 10 min. The pellet was collected and resuspended in 5.0 mL of 0.05 M Tes, 50% ethylene glycol (v/v), 0.001 M EDTA, and 0.04% NaN_3 (w/v) (pH 6.5) buffer (Tes–EG buffer). The suspension was allowed to stand at room temperature for 20 min and then centrifuged at 27000*g* for 10 min. The supernatant solution (II) was decanted and applied to a Sephadex G-200 column.

Gel Filtration. Sephadex G-200 was swollen in 0.02% NaN_3 as recommended by the manufacturer. The gel was fined by gravity sedimentation with decantation of the supernatant. Following each decantation, volume was restored by addition of Tes–EG buffer. After three decantation and replacement steps, the gel slurry was degassed and packed into a 2.5-cm diameter glass column to a height of ~90 cm while pumping out from the column bottom at a rate corresponding to a pressure head of 10 cm. The column was equilibrated by a downward flow of ~2 column volumes of Tes–EG buffer at a rate of 17 mL/h. Both downward and upward column flows were accomplished by pumping both ends of the column simultaneously with a peristaltic pump. This technique reduced the likelihood of gel collapse and was necessitated by the high viscosity of the buffer. Solution II was applied to the column by using upward flow at a rate of 16 mL/h, and elution with Tes–EG buffer was continued at this rate. The eluate was collected in 5-mL fractions, and the peak phospholipase D activity was found at a relative elution volume of ~1.57. The fractions containing phospholipase D activity were pooled and concentrated to ~3 mL with PM-30 Amicon ultrafiltration membranes (Amicon, Lexington, MA). This solution (III) was subjected to hydrophobic affinity chromatography.

Affinity Chromatography. A modification of the technique of Shaltiel and Er-el (1973) was used to synthesize a γ-aminopropane–agarose gel. One volume (120 mL) of Bio-Gel A-5m (100–200 mesh) in an equal volume of water was activated by the addition of 24 g of finely divided cyanogen bromide, while maintaining the temperature at 20 °C by the addition of ice and the pH near 10.5–11.0 by the addition of 6 N NaOH. The reaction was terminated after 10 min by filtration and washing of the gel with 8 volumes of ice-cold water. The activated gel was suspended in 1 volume of cold 0.1 M $NaHCO_3$ (pH 9.0) and combined with an equal volume of water containing 240 mmol of 1,3-diaminopropane (pH 9.0). The coupling mixture was stirred slowly at 4 °C for 18–20 h. The product was subsequently washed with 400 mL each of water, 0.1 M $NaHCO_3$, 0.05 M NaOH, water, 0.1 M acetic acid, and finally water. The γ-aminopropane–agarose was fined and degassed in 1.0 M NaCl and 0.04% NaN_3 and packed in a 1.5 × 60 cm glass column. The column was equilibrated with 3 column volumes of Tes–EG buffer at a flow rate of 39 mL/h. Solution III was applied to the column, and elution was initiated with Tes–EG buffer at the same flow rate. The eluate was collected in 6.5-mL fractions. When the eluate optical density dropped below 0.05, a linear salt gradient was started by using 350 mL of Tes–EG buffer and an equal volume of 0.05 M NaCl in Tes–EG. The phospholipase activity was eluted in a peak centered around an approximate conductivity of 580 μmhos. The active fractions were combined (solution IV), concentrated as described above, and stored at −18 °C.

Gel Electrophoresis. Analytical disc gel electrophoresis was conducted by the method of Ornstein (1964) and Davis (1964). Sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis was conducted by the method of Laemmli (1970).

Ultracentrifugation. Ultracentrifugation was conducted in a Spinco Model E analytical ultracentrifuge equipped with electronic speed control and a photoelectric scanner. All runs were performed in double-sector cells equipped with sapphire windows and a Kel F centerpiece at 20.0 °C. Solvent densities were determined in a 10-mL specific gravity pycnometer tube (Ace Glass, Vineland, NJ) calibrated with distilled water. The reproducibility of densities was ±0.0002 g/mL. Solvent viscosities were determined in a Cannon–Ubbelohde capillary viscometer, Type 50, L33 (Cannon Instrument) and were reproducible to ±0.28%. In all cases, the solution's density and viscosity were assumed to be identical with those of the solvent. A partial specific volume, $\bar{v}$, of 0.75 was assumed. Sedimentation equilibrium ultracentrifugation of bovine serum albumin in Tes–EG buffer was conducted to determine if the assumed value of $\bar{v}$ was to be a likely source of error as a result of the anomalous solution characteristics of ethylene glycol. The effect of ethylene glycol on the $\bar{v}$ of serum albumin was found to be negligible.

Enzyme Assays. Three types of phospholipase D assays were employed: a semiquantitative method for expeditiously assaying fractions of column eluate, a spectrophotometric method, and a pH stat technique. All methods assayed hydrolytic activity by quantitating the liberation of hydrogen ions from the phosphatidic acid product.

In the semiquantitative assay, an indicator–substrate solution (0.1 mM methyl red, 50 mM calcium chloride, 1 mM DiC_6,[1] and 0.5 mM dithiothreitol, pH 6.5) was dispensed in 0.1-mL aliquots into the wells of a Lucite microdilution plate, "U" type (Scientific Products, McGaw Park, IL). One-microliter aliquots from column fractions were serially injected into the wells, and the reaction was allowed to proceed at room temperature for ~15 min. Substrate hydrolysis demonstrating phospholipase D activity was indicated by the development of a red color. Activity was evaluated on a scale of 1, 2, 3, or 4, corresponding to increasing red-color intensity. This technique easily permitted assay of all fractions of a given column in 30 min.

In the spectrophotometric assay, a pH indicator (phenolsulfonephthalein) was used to buffer the reaction and the rate of disappearance of the basic form of the indicator was used to measure the rate of hydrolysis of the substrate. The reaction cuvette contained 1.0 mL of a solution of substrate, calcium chloride, dithiothreitol, and 1 mM phenolsulfonephthalein

[1] Abbreviations used: DiC_6, dihexanoylphosphatidylcholine; DiC_4, dibutyroylphosphatidylcholine; DiC_5, divaleroylphosphatidylcholine; DiC_7, diheptanoylphosphatidylcholine.

Table I

step	vol (mL)	total protein[a] (mg)	total units[b]	sp act.[c]	purification (x-fold)
crude suspension	40	2862.0	37.0	0.013	1.0
	40	2634.0	33.2	0.013	1.0
13000g supernatant (I)	40	1024.0	35.3	0.034	2.6
	40	824.0	30.6	0.037	2.8
resuspended ammonium sulfate precipitate (II)	5	89.0	15.1	0.17	13.1
	5	94.0	14.9	0.16	12.3
Sephadex G-200 (III)	59	7.0	13.8	1.97	152
	54	7.1	9.6	1.35	104
hydrophobic affinity (IV)	100	2.4	21.1	8.80	680

[a] Assuming $E_{280}^{1\%} = 10.0$. [b] One unit of enzyme activity equals hydrolysis of 1.0 μmol/min. [c] Units of specific activity are μmol/(min mg), using the spectrophotometric assay (conditions: 0.6 mM DiC_6, 0.4 mM $CaCl_2$, 10^{-5} M DTT, 30.0 °C).

adjusted to pH 7.5. The reference cuvette contained only 1 mM indicator, pH 7.5. Both cuvettes were stoppered. The reaction was initiated by addition of enzyme solution to the reaction cuvette. The absorbance at 595 nm, which is near the absorption maximum (558 nm) of the basic form of the indicator and in a region where absorption of the acidic form is negligible, was measured on the 0.1-*A* slide-wire of a Cary Model 15 spectrophotometer. A change in absorbance of 0.01 corresponded to the production of 1.16 nmol of phosphatidic acid. Although the spectrophotometric assay was more sensitive, the pH stat assay was employed for most kinetic determinations to eliminate possible ambiguity introduced by inclusion of the indicator dye.

In the pH stat assay, hydrolysis of substrate was measured using a Radiometer (Copenhagen) TTA31 microtitration assembly as described by Wells (1972) for the measurement of phospholipase A_2 activity. The reaction was contained in a volume of 1.0 mL at 30 °C under an atmosphere of nitrogen. Plastic reaction vessels were used in place of the standard glass vessels. The glass electrode was siliconized prior to use, and the stems of both the glass and calomel electrodes were masked with Teflon tape. The pH was maintained by addition of 0.025 M NaOH. Unless otherwise stated, all data were obtained at pH 7.25. The reaction mixture consisted of enzyme, substrate, calcium chloride, dithiothreitol, and other additions when appropriate. The reaction was commonly initiated by addition of enzyme and was found to be linear for at least 10 min. Duplicate assays were reproducible within 10%. The identity of the phosphatidic acid products was verified on thin-layer chromatography using synthetic dihexanoylphosphatidic acid as a standard.

Results

Enzyme Purification. The results of two representative experiments are presented in Table I. The purification steps through application of solution II to the Sephadex G-200 column must be accomplished within 4 or 5 h, as the activity was labile during the initial steps. An elution profile of Sephadex chromatography is presented in Figure 1 (the first 80 mL of column eluate was collected in bulk). The gel filtration product was relatively stable and could be stored at −18 °C for 1 week without loss of activity. The combined active fractions of two Sephadex columns were pooled before hydrophobic affinity chromatography. Concentration of these pooled solutions III to 3–5 mL was required to achieve sufficient resolution on the affinity column. An elution profile of the affinity column is presented in Figure 2. Fractions 65–80 were combined and concentrated. Storage at −18 °C for several months resulted in no loss of activity. Enzyme activity

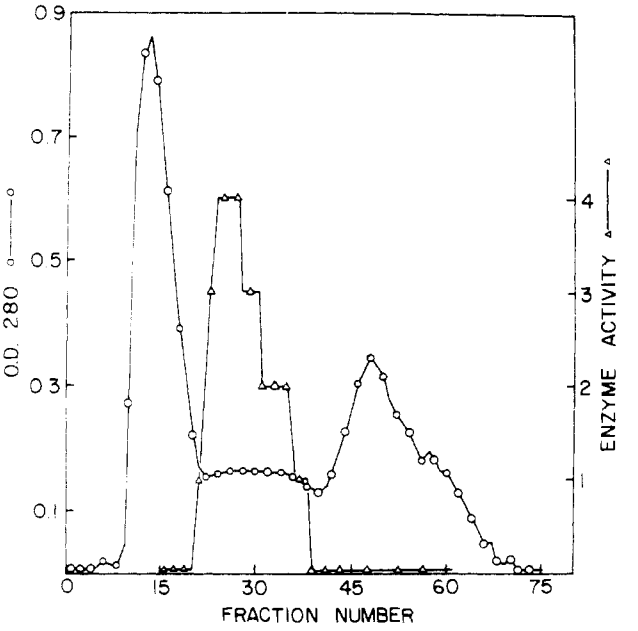

FIGURE 1: Results of gel filtration of phospholipase D on Sephadex G-200. A 2.5 × 90 cm column in Tes–EG buffer was eluted at 17 mL/h. Enzymatic activity was determined by the semiquantitative plate assay.

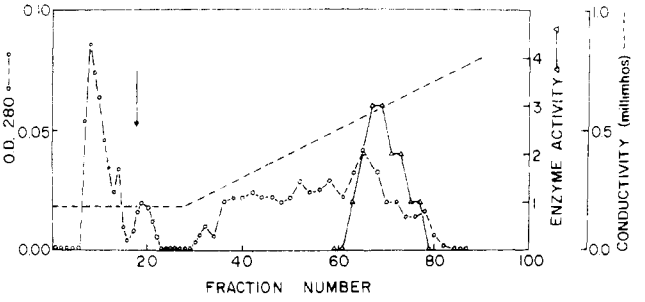

FIGURE 2: Hydrophobic affinity chromatography of phospholipase D on γ-aminopropane–agarose. A 1.5 × 60 cm column was eluted with Tes–EG buffer. At the arrow a linear salt gradient was started and monitored by measurement of conductivity. Enzymatic activity was determined by the semiquantitative plate assay.

could not be eluted under any conditions from affinity gels prepared from the longer chain homologues of 1,3-diaminopropane (diaminobutane or diaminopentane), and the enzyme was not retained by a gel prepared from the shorter homologue ethylenediamine.

The inclusion of ethylene glycol in the buffers at all stages after the primary solution steps was fundamental to the success of this purification. Gel filtration without ethylene glycol gave poorer recovery and resolution. The phospholipase D activity could not be recovered from the hydrophobic affinity column in the absence of ethylene glycol. The use of 50% ethylene glycol in the buffer solutions presented two technical problems: high buffer viscosity and susceptibility to bacterial contamination. The viscosity problem on gel filtration was managed as described under Materials and Methods; additionally, all columns were run at room temperature. Bacterial growth, which was particularly a problem in the Sephadex gel in spite of the use of sodium azide, was managed by filtering all buffer solutions prior to deaeration with a Millipore filtration apparatus and by maintaining buffer flow through the column at all times.

Analytical polyacrylamide gel electrophoresis of the purified protein using 7.5% gels gave a single band following staining with Coomassie Brilliant Blue. Spectrophotometric scans of

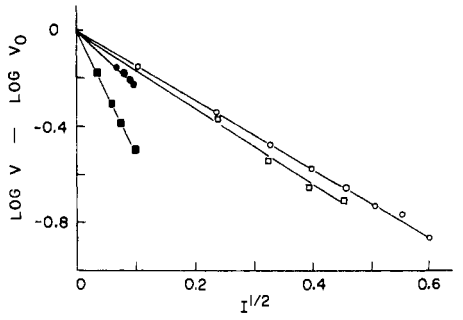

FIGURE 3: Effect of ionic strength ($I^{1/2}$) on the velocity of phospholipase D hydrolysis of dihexanoyllecithin (DiC_6). Velocity (V) of DiC_6 hydrolysis at varying ionic strength was corrected for the velocity at zero ionic strength (V_0). All assays were at pH 7.25 and 30 °C. Variation in ionic strength was accomplished by addition of KCl. (■) 1 mM DiC_6 and 0.25 M $CaCl_2$ (spectrophotometric assay); (●) 1 mM DiC_6 and 2 M $CaCl_2$ (pH stat assay); (□) 4 mM DiC_6 and 2 mM $CaCl_2$ (pH stat assay); (○) 20 mM DiC_6 and 2 M $CaCl_2$ (pH stat assay).

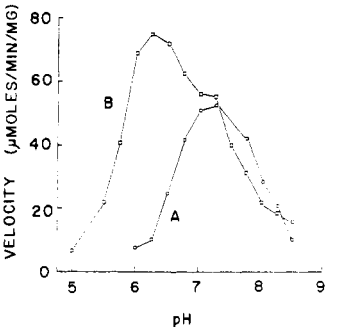

FIGURE 4: pH dependence of the phospholipase D catalyzed hydrolysis of DiC_6 at different Ca^{2+} concentrations. (A) 1 mM DiC_6 and 0.5 mM $CaCl_2$; (B) 2 mM DiC_6 and 50 mM $CaCl_2$. All assays used the pH stat.

such gels indicated (assuming equivalent protein staining) that contamination in the preparation was less than 1%. Analytical gels run using 5, 12, and 15% acrylamide also showed a single band. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ also gave a single band and an estimated molecular weight of 112 500 ± 7500.

The purified protein was subjected to sedimentation velocity ultracentrifugation in Tes–EG buffer. Analysis of a plot of log r vs. elapsed run time as an unweighted linear regression gave a correlation coefficient of 0.9998 and an observed sedimentation coefficient, $s_{20,w}$, of 5.59 S. High-speed sedimentation equilibrium ultracentrifugation of the purified protein was conducted at 16 000 rpm in Tes–EG buffer. A plot of log A_{280} vs. r^2 gave a correlation coefficient of 0.9970. Calculation of a molecular weight estimate from sedimentation equilibrium data gave a value of 116 600 ± 6900 (average of six scans).

Kinetic Assays. The purified protein was routinely stored in the Tes–EG buffer; however, since primary alcohols act as acceptors in the transphosphatidylation reaction, the protein was removed from ethylene glycol solution prior to use in kinetic experiments by dialysis into 10% (w/v) inositol. Inositol, a fully secondary and polyhydric alcohol, maintained the stability of the protein but did not have a detectable kinetic effect. The inositol–protein solution could be stored at 4 °C for a month with no appreciable loss of activity. Dithiothreitol (DDT) resulted in activation of the purified protein, presumably through reduction of a labile sulfhydryl group, and was routinely included in kinetic assays at 10^{-5} M, which was twice the concentration required for maximal activation.

Ionic Strength Effect. Increasing the ionic strength of the reaction mixture produced a decrease in the velocity of phospholipase D hydrolysis of DiC_6 so that the log of the velocity depended linearly upon the square root of the ionic strength (I); this is illustrated in Figure 3.

pH and Calcium Dependence. The pH dependence of the velocity of phospholipase D hydrolysis of DiC_6 in 0.5 mM Ca^{2+} is illustrated in curve A of Figure 4. The reaction displayed an apparent optimum around pH 7.25 and ascending and decending limbs with approximate pK values of 6.5 and 8.1, respectively. This behavior is consistent with a diprotic model in which only the singly protonated enzyme form displays activity. The pH dependence of the reaction velocity at high Ca^{2+} concentration (50 mM) is illustrated in curve B of Figure 4. This latter curve may be resolved into two approximately bell-shaped curves, one with an apparent optimum at pH 6.25 and a second with an apparent optimum at pH 7.25, as was demonstrated at low Ca^{2+}. Calcium appears to bind to the protein with low affinity and convert the pH 7.25 optimum form into the pH 6.25 optimum form. The conversion from the pH 7.25 to 6.25 optima was not specific for Ca^{2+} since a curve similar to B was observed by using 0.5 mM Ca^{2+} and 50 mM Mg^{2+}. This conversion was not achieved with high concentrations of monovalent cations.

As a result of the compound effects of Ca^{2+}, velocity vs. [Ca^{2+}] plots below pH 7.0 were not hyperbolic. However, above pH 7.0 the plots did show a hyperbolic dependence upon calcium concentration. At pH 7.50, a constant ionic strength of 0.002 (KCl), and 1 mM DiC_6, the value of $K_{Ca^{2+}}$ (K_m for Ca^{2+}) was 0.21 mM. Kinetic determinations were routinely made at pH 7.25 and at low Ca^{2+} concentrations.

Substrate Concentration Dependence. A major aim of this study was to carry out kinetic analyses using monomeric substrates, as has been done for phospholipase A_2 (Wells, 1972). A number of unexpected problems were encountered with phospholipase D which were not observed with phospholipase A_2. We will report a few of these problems by using data obtained with dihexanoyllecithin (DiC_6), and note that similar problems were encountered with DiC_4, DiC_5, and DiC_7.

The dependence of initial velocity of DiC_6 hydrolysis at various concentrations on the amount of phospholipase D added is illustrated in Figure 5A. The velocity at each substrate concentration was found to be a linear function of enzyme addition. Since the substrate at these concentrations was expected to be monomeric (Tausk et al., 1974), the dependence of the velocity upon substrate concentration was expected to be hyperbolic. When the slopes of the plots in Figure 5A were replotted as a function of substrate concentration (Figure 5B), the dependence of velocity upon substrate concentration was found to be parabolic, whereas the dependence of the square root of the velocity upon the substrate concentration was linear. This behavior represented a significant departure from the predicted kinetics. For establishment that this anomalous behavior was not the result of product activation of the enzyme, the dependence of initial velocity upon concentration was determined in the presence of 0.2 mM dihexanoylphosphatidic acid. The resulting data demonstrated the same V and $V^{1/2}$ dependencies on substrate concentration, indicating that product activation was not a factor.

At higher concentrations of DiC_6 the velocity dependence upon [S] went through an inflection point and became hyperbolic in character (Figure 6). By use of a phase-separation model (Wells, 1974), i.e., the substrate concentrations were corrected by subtracting a "critical concentration", the data above 5 mM were fit to an Eadie–Scatchard replot (V/[S] vs. V) by assuming various values for the critical concentration. The best fit (correlation coefficient of 0.9937) was achieved

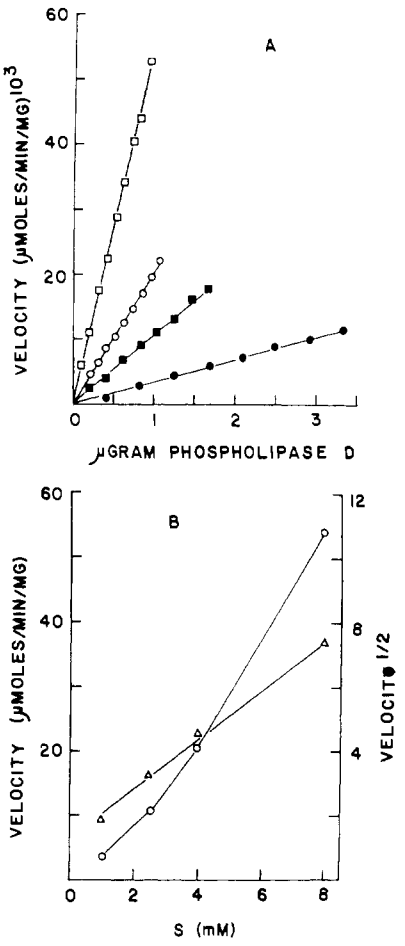

FIGURE 5: Dependence of the velocity of phospholipase D hydrolysis of DiC_6 on the enzyme concentration and substrate concentration. Panel A: dependence of velocity on enzyme concentration. These data were obtained by conducting assays as a function of added enzyme at various fixed concentrations of DiC_6: 1 (●); 2.5 (■); 4 (○); and 8 (□) mM. The lines represent unweighted least-squares plots with correlation coefficients. Panel B: dependence of the velocity (○) and the square root of the velocity (△) upon substrate concentration. These data were obtained from the slopes of the lines in panel A. The line of the $V^{1/2}$ plot is a least-squares line, $r^2 = 0.9955$. All assays were conducted with 4 mM Ca^{2+}, at pH 7.25, 30 °C, and ionic strength = 0.2.

with a critical concentration of 4.3 mM, V_{max} of 338 μmol/(min mg), and K_m of 2.64 mM. No kinetic contribution from "monomer" was considered. Estimates of V_{max} and K_m by the method of Eisenthal & Cornish-Bowden (1974) using the same critical concentration were 339 μmol/(min mg) and 2.66 mM, respectively. These values were used to generate the solid curve drawn in Figure 6.

In the application of the phase-separation model to phospholipase A_2, the critical concentration was equal to the critical micelle concentration, which has a value of ~10 mM (Wells, 1974). The fact that the critical concentration is 4.3 mM and that no large rate acceleration is observed above the cmc indicates that the response of phospholipase D to substrate aggregation is entirely different than that observed with phospholipase A_2.

Multiple-site enzymes which demonstrate positive cooperativity with respect to substrate binding may yield sigmoidal velocity curves which are in some respects similar to the velocity curves of phospholipase D. The degree of site–site interaction of such an enzyme may be characterized by use of the logarithmic Hill plot, i.e., a plot of log $V/(V_{max} - V)$ vs. log [S]. The data for phospholipase D hydrolysis of DiC_6

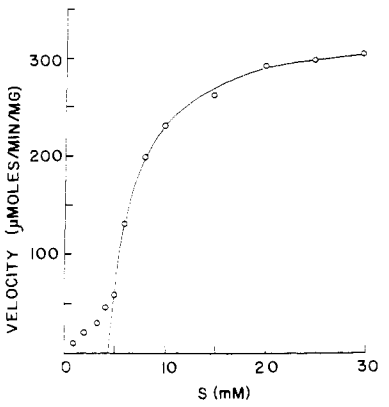

FIGURE 6: Dependence of the velocity of phospholipase D hydrolysis of DiC_6 on substrate concentration. The circles represent experimental data. Conditions: 2 mM Ca^{2+}, 0.1 M KCl, pH 7.25, and 30 °C on pH stat. The curve was calculated from the Henri–Michaelis–Menten equation by assuming a phase-separation model, neglecting monomer contribution, and using a critical concentration of 4.3 mM and V_{max} and K_m estimates of 339 μmol/(min mg) and 2.66 mM, respectively.

did not give linear Hill plots; therefore, it would appear that the sigmodial velocity curves do not arise from site–site interactions in the enzyme.

Discussion

A procedure has been established for the successful purification of phospholipase D from cabbage. The interesting aspect of the purification is that inclusion of ethylene glycol in the buffer solutions is mandatory to ensure the stability of the protein during the chromatographic separations. Equilibrium sedimentation ultracentrifugation of phospholipase D in the absence of ethylene glycol demonstrated large polydispersity.

The stabilizing influence of high concentrations of polyhydric alcohols from ethylene glycol to polyethylene glycol on the quaternary structure of oligomeric proteins appears to be a general phenomenon (Shifrin & Parrott 1975). Timasheff et al. (1976) have shown that a variety of proteins in glycerol and sucrose solutions are preferentially hydrated; i.e., the cosolvent is partially excluded from the immediate domain of the protein. They have also described the mechanism by which preferential hydration can exert a stabilizing influence on oligomeric proteins by increasing the protein self-association constant. Preliminary $NaDodSO_4$ gel electrophoresis experiments in the presence of high concentrations of urea indicate that phospholipase D is an oligomeric protein and that the molecular weight estimates by ultracentrifugation and $NaDodSO_4$ gel electrophoresis without urea are of an associated species.

The homogeneity of the purified protein was evaluated by analytical gel electrophoresis, $NaDodSO_4$ gel electrophoresis, and high-speed sedimentation equilibrium ultracentrifugation. The observation of a single visible band upon $NaDodSO_4$ gel electrophoresis and upon analytical gel electrophoresis using gels of four different acrylamide contents testifies to the purity of the preparation. Likewise, the linearity of the plots of the sedimentation equilibrium data is indicative of a homogeneous preparation.

The use of short-chain lecithins as substrates has been useful in the analysis of the mechanism of action of phospholipase A_2 (Wells, 1972). It was anticipated that a similar study with phospholipase D might simplify many of the complex results obtained with long-chain substrates (see Heller (1978) for a recent review). It is clear from the data presented, and considerably more not presented, that the anticipated simplifica-

tion was not realized. It is not clear at present whether the complex kinetics arise from heretofore unrecognized substrate self-association at concentrations below the cmc or from problems arising from the apparent multisubunit structure of the enzyme and its apparent instability in aqueous solutions lacking polyhydric alcohols. These problems are currently under investigation.

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Isolation and Characterization of Argininosuccinate Synthetase from Human Liver†

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ABSTRACT: This communication describes the purification and characterization of argininosuccinate synthetase from human liver. By numerous criteria including electrophoresis in sodium dodecyl sulfate containing gels, electrophoresis in nondissociating gels, and analytical ultracentrifugation, the protein is homogeneous at a specific activity of 4.2 μmol/(min mg) assayed at 37 °C in the direction of argininosuccinate synthesis. The enzyme has a molecular weight of 183 000, as determined by gel filtration. Electrophoresis in the presence of sodium dodecyl sulfate yielded a single band migrating with an R_f corresponding to 43 000 daltons. Thus, the enzyme is considered to contain four subunits of identical molecular weight. The $s_{20,w}$ of the enzyme is 8.2 S. Antibodies were prepared in rabbits directed against the purified protein. These antibodies react specifically with argininosuccinate synthetase, as determined by electrophoretic analysis of the immunoadsorbed product from crude extracts of human liver. The human enzyme has very similar properties to those published for the beef and rat liver enzymes.

Argininosuccinate synthetase (EC 6.3.4.5) catalyzes reaction 1. The enzyme was described first by Ratner and her colleagues in beef liver (Ratner & Petrack, 1951, 1653) and since has been studied extensively (Rochovansky et al., 1977; Rochovansky & Ratner, 1961, 1967). The enzyme also has been isolated from hog kidney (Schuegraf et al., 1960) and rat liver (Saheki et al., 1975) and has been found in numerous human cultured cell lines (Lockridge et al., 1977; Schimke, 1964; Irr & Jacoby, 1978).

$$\text{citrulline} + \text{aspartate} + \text{ATP} \rightleftharpoons \text{AMP} + \text{argininosuccinate} + \text{PP}_i \quad (1)$$

In ureatelic organisms this enzyme plays a vital role in the disposal of ammonia via the Krebs–Henseleit urea cycle. This metabolic process occurs predominately in the liver, and this organ has many-fold higher levels of enzyme than other tissues. In tissues not capable of ureagenesis, the enzyme presumably serves to synthesize arginine from citrulline. Due to the bifunctional role of the enzyme, it might be expected that different tissues would have evolved different mechanisms to control the levels of the enzyme. In cultured lymphocytes and in Hela and KB cells argininosuccinate synthetase levels are influenced by the availability of arginine and citrulline in the culture fluid (Schimke, 1964; Irr & Jacoby, 1978). In rat and monkey liver the levels of the urea cycle enzymes including argininosuccinate synthetase are influenced by the amount of protein in the diet (Nuzum & Snodgrass, 1971; Schimke, 1962). Nothing is known about the mechanism by which these control processes occur. Our interest in inborn errors of metabolism involving urea cycle enzymes in humans and the control of these enzymes necessitated that more information be made available concerning these proteins. Carbamylphosphate synthetase (Pierson & Gilbert, 1978), ornithine transcarbamylase (Kalausek et al., 1978), and arginase (Beruter et al., 1978) have been isolated from human liver and their properties described. This report describes the isolation and characterization of argininosuccinate synthetase from human liver.

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