Journal of Chromatography, 525 (1990) 307–318
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5070

PURIFICATION OF PHOSPHOLIPASE-C FROM *BACILLUS CEREUS* BY AFFINITY CHROMATOGRAPHY ON 2-(4-AMINOPHENYLSULPHONYL)ETHYL-CELLULOSE

EMANUEL KAMBEROV and ALEXANDER IVANOV*

Department of Biochemistry and Microbiology, Plovdiv University, Tsar Assen Street 24, 4000 Plovdiv (Bulgaria)

(First received July 19th, 1989; revised manuscript received October 4th, 1989)

SUMMARY

A new method for the purification of phospholipase-C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* has been developed, based on its affinity to 2-(4-aminophenylsulphonyl)ethyl derivative of beaded cellulose. The enzyme was adsorbed on the affinity sorbent through a site(s) that was clearly distinct from its catalytically active site, because it was still active in the immobilized state. A possible role of enzyme–inhibitor interaction in enzyme binding to the ligand used is discussed.

INTRODUCTION

Phospholipase-C (PLC) from *Bacillus cereus* (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) is a bacterial enzyme widely used for fundamental studies in the field of membranology, lipid metabolism, chemistry of lipids and blood coagulation. As pointed out by Van den Bosch [1], it is the best characterized bacterial PLC, both from a structural and a mechanistic point of view [2–8].

Various procedures have been published for the purification of this enzyme, using either conventional methods [9–12] or affinity chromatographic techniques [13–16]. Some of the reported methods are time-consuming or unsuitable for use on a large scale.

We report here a new kind of highly effective and very reproducable affinity chromatographic procedure for purification of PLC from *B. cereus*, and our

0378-4347/90/$03.50

attempts to establish the nature of the binding of the enzyme and its interaction with the ligand used.

EXPERIMENTAL

Materials

L-α-Phosphatidylcholine (from egg yolk, 99% pure) and *p*-nitrophenylphosphorylcholine (NPPC) were obtained from Sigma (St. Louis, MO, U.S.A.); Triton X-100, sodium deoxycholate and Coomassie Brilliant Blue R-250 were from Merck (Darmstadt, F.R.G.); tris(hydroxymethyl)aminomethane, D-mannitol and 2-propanol were products of Fluka (Buchs, Switzerland). Bovine serum albumin was from Serva (Heidelberg, F.R.G.). DEAE-cellulose (0.8 mequiv./g) was purchased from Reanal (Budapest, Hungary). D-Sorbitol was from Reachim (Moscow, U.S.S.R.) and Pharmachim (Sofia, Bulgaria), respectively. Sulphatiazole (ST), sulphamethoxazole and sulphadimethoxine were obtained from Pharmachim and were twice recrystallized in water before use. Unmodified beaded cellulose (MT 750) was from Spolchemie (Usti nad Labem, Czechoslovakia). Sulphanilic acid and *p*-aminosalicylic acid were products of Chemapol and Spofa (Prague, Czechoslovakia), respectively.

All other reagents were also of analytical grade or the highest purity available.

A method for synthesis of the 2-(4-aminophenylsulphonyl)ethyl (APSE) derivative of cellulose was first developed by Rogovin et al. [17] to make textile cellulose materials with new properties. In our experiments we used a sample of the APSE derivative of highly porous beaded cellulose with a low concentration of $-NH_2$ groups (ca. 0.2 mmol/g), which was synthesized and kindly provided by Dr. Milan Beneš (Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). The synthesis of this material was performed for biochemical applications, essentially as described by Burba and Lieser [18] and Burba et al. [19]. It is commonly used as a carrier after diazotization for coupling of some ligands for affinity chromatography [20].

This APSE-cellulose gel was used without any preliminary activation throughout our experiments.

Assay of phospholipase-C activity

Phosphatidylcholine was subjected to five 30-s sonications as a 10 m*M* suspension in deionized water, containing 10% (v/v) ethanol, on a Fritsch Model sonifier, whilst being kept on ice. If not otherwise stated, the reaction mixture contained 0.2 ml of the above liposome preparation and 0.1 ml each of 0.2 *M* $Na_2B_4O_7$–HCl (pH 7.3), 0.5% sodium deoxycholate and enzyme in the same borax–HCl buffer. After incubation for 5–15 min at 37°C, the reaction was terminated by adding 0.1 ml of 50% (w/v) trichloroacetic acid, and the mixture was shaken vigorously on a vortex mixer with 2.5 ml of chloroform–methanol

(2:1, v/v). After centrifugation at 2000 *g* for 10 min, an aliquot of 0.2 ml was withdrawn from the upper layer and analysed for orthophosphate (P_i), as described by Takahashi et al. [21]. Reagent and zero-time blanks were run in all assays and the blank values were subtracted. One unit of the enzyme was defined as the amount that would catalyse the hydrolysis of 1 μmol of substrate per minute at pH 7.3 and 37°C. Reaction products were identified by thin-layer chromatography, essentially as described by Sonoki and Ikezawa [22], using a commercially obtained *B. cereus* PLC (Sigma) as a control.

For some routine assays, and when kinetic or inhibition analyses were carried out, a modification of the procedure described by Kurioka and Matsuda [23] was used. If not otherwise specified, the basal reaction mixture contained 20 μmol of NPPC in 0.1 *M* borax–HCl (pH 7.3) and the enzyme to be assayed, in a total volume of 1 ml (sorbitol was omitted). The assay was carried out at 33°C in a cuvette with a 10-mm optical path length, and the rate of *p*-nitrophenol formation was measured at various time intervals at 410 nm, by the use of a calibration curve.

Purification of enzyme

Production of PLC and collection of foam from culture. B. cereus FX-55 from our laboratory collection was used as a strain producing PLC. The strain was grown in the culture medium used by Gerasimene et al. [24] with some modifications. The medium (1 l) contained 10 g of yeast extract, 10 g of casein (enzymic hydrolysate), 4 g of glucose, 5 g of $NaHCO_3$, 5 g of NaCl, 10 mg of $ZnCl_2$ and 0.14 mg of biotin; the pH was adjusted to 7.0. Volumes of 150 ml of this medium in 500-ml conical flasks were inoculated directly from a plate colony and incubated for 20 h at 30°C with continuous shaking on a rotary shaker.

Culture solution (1 l) was poured into a conical flask with a side arm, and foam was produced and collected according to the procedure described by Myrnes and Little [16]. The liquified foam (115 ml) was then centrifuged for 15 min at 6000 *g* and 2°C.

2-Propanol precipitation. The supernatant from the previous step was saturated to 75% (by volume) with 2-propanol at a flow-rate of ca. 3 ml/min (kept at 0 to −10°C) under continuous stirring. The mixture was allowed to stand at −20°C for 1 h, and the precipitate was centrifuged for 15 min at 13 000 *g* and 0°C. The precipitate was then resuspended in 10 ml of 0.1 *M* borax–HCl (pH 7.3) containing 1 m*M* $ZnSO_4$ and dialysed twice against 2-l portions of the same buffer for 2 h.

DEAE-cellulose batch treatment. Precipitated material after dialysis was removed by centrifugation (6000 *g* for 10 min) and rejected. Equal volumes of dialysis residue and DEAE-cellulose, previously washed and equilibrated with the above borax–zinc buffer, were mixed by stirring for 20 min at 4°C and then filtered through a glass filter.

Affinity chromatography. The enzyme solution was saturated to 0.5 *M* with NaCl and loaded onto a column (100 mm×8 mm I.D.) of APSE-cellulose, previously washed and equilibrated with borax–zinc buffer containing 0.5 *M* NaCl. The flow-rate was 0.15 ml/min. The column was washed with three column volumes of the same buffer. Enzyme was then eluted using 5 *M* solution of urea in the borax–zinc buffer at a flow-rate of 0.5 ml/min. Fractions of 3 ml were collected throughout. The active fractions were pooled and dialysed twice against 1-l portions of borax–zinc buffer (3 h and overnight). This final preparation was stored at −20°C.

Other methods

Kinetic assays were carried out using six rate measurements over an NPPC concentration range 5–100 m*M*. For each assay, 2 μg of purified PLC were used. When the effect of Tris or ST was studied, they were applied at two different concentrations, 4 and 20 m*M* for Tris and 0.15 and 0.5 m*M* for ST.

In all experiments where inhibitory effects of different substances or enzyme adsorption to the affinity gel were studied, control samples with commercial *B. cereus* PLC were run.

Disc gel electrophoresis was run in 7.5% polyacrylamide in the system of Davis [25] at pH 8.9 and 2 mA per tube. Approximately 100 μg of protein were loaded into each gel. The gels were stained using Coomassie Brilliant Blue R-250.

Protein was determined by the method of Hartree [26] with a bovine serum albumin as a standard, or by measuring the UV absorption at 280 nm.

All data are presented as the mean value of at least a triplicate of two separate experiments.

RESULTS

A typical purification, starting with 1 l of bacterial culture, yielded 5–7 mg of purified PLC with an overall recovery of 63% (Table I). The enzyme was purified 724-fold over the supernatant of the culture. The final enzyme preparation appeared as a single protein band in polyacrylamide disc electrophoresis (Fig. 1).

Affinity adsorption was sensitive to pH changes and to the kind of buffer system used. Thus at pH 8.5 (in 0.1 *M* borax or in 0.05 *M* sodium 5,5′-diethylbarbiturate) a significant amount (ca. 76%) of enzyme activity passed straight through the column. On the contrary, at pH 6.0 (0.05 *M* sodium acetate) PLC bound tightly to the affinity ligand, but the specific activity of the preparation eluted was only 38% of the maximum. Best results were obtained when the adsorption was carried out in the pH range 7.0–7.5 in 0.1 *M* borax–HCl or in 0.05 *M* barbiturate–HCl buffers, but in the latter case some preparations with lower specific activity were obtained. When 0.1 *M* Tris–HCl was

TABLE I

PURIFICATION OF PHOSPHOLIPASE-C

Summary of purification starting with 1 l of *B. cereus* supernatant. Details are described in the text.

Step	Total volume (ml)	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (*n*-fold)
B. cereus supernatant	1000	19 000	7200	2.6	100	–
Liquified foam	115	16 600	828	20	87.3	7 6
2-Propanol precipitate	10.5	15 750	77.7	202.7	82.9	78
DEAE-cellulose (batch)	11.8	15 380	58	265	81	102
Affinity column	12.3	12 054	6.4	1883	63	724

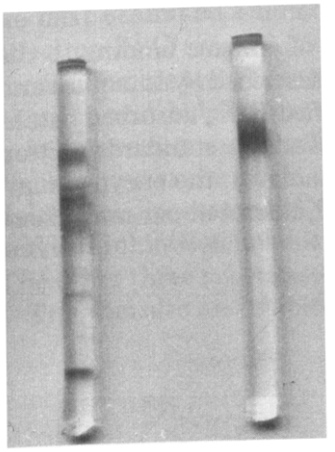

Fig. 1. Gel electrophoretic patterns. Electrophoresis in 7.5% polyacrylamide at pH 8.9 was carried out with 100 μg of protein at 2 mA per tube (stained with Coomassie Brilliant Blue), according to the method of Davis [25]. (Left) Enzyme after DEAE-cellulose batch treatment; (right) enzyme after affinity column.

used in the same pH interval, the affinity sorbent bound less than 25% of the enzyme activity.

Solutions of NaCl up to 1.5 *M* did not appreciably influence the enzyme binding, so it could be included in the sample in order to limit non-specific binding. Surprisingly the presence of 30% (w/v) sorbitol resulted in a complete loss of binding activity of the gel. Thus the buffer used for routine affinity chromatography of PLC was 0.1 *M* borax–HCl (pH 7.3) containing 0.5 *M* NaCl and 1 m*M* $ZnSO_4$. At these conditions unmodified beaded cellulose adsorbed less than 1.5% of enzyme activity, presenting in an equivalent sample (data not shown).

An amount of enzyme equal to 16 000 U was close to the maximum binding capacity of a column with dimensions of 100 mm×8 mm I.D., since a significant leakage of activity was observed when larger amounts were applied.

We next examined different substances for elution of PLC from the affinity column. Solutions of NaCl up to 3.5 *M* were not effective and resulted in elution of a maximum of 8±2% of the bound enzyme. Only partial elution was achieved by applying detergents, such as Triton X-100 or sodium deoxycholate, at concentrations up to 0.1% (w/v) and 0.3% (w/v), respectively. Very broad elution profiles were obtained, exceeding by more than ten-fold the volume of the sample applied. The most suitable elution medium was found to be 5 *M* urea in the borax–zinc buffer. Urea was first proposed by Little et al. [15] for elution of the same enzyme from lipoprotein–Sepharose affinity columns. A sharp elution profile and a high recovery of activity were obtained (Fig. 2 and Table I). Apart from being the best eluting agent, urea effectively regenerated our affinity gel.

We attempted to establish the type and the site of enzyme binding to the affinity ligand used. An experiment similar to that described by Imamura and Horiuti [14] was carried out. When 0.05 μg of purified PLC, adsorbed batchwise on the affinity gel (35 mg wet weight), was applied to a standard reaction medium with phosphatidylcholine (without deoxycholate), the enzyme manifested its total catalytic activity of 0.075 μmol of P_i liberated per min. How-

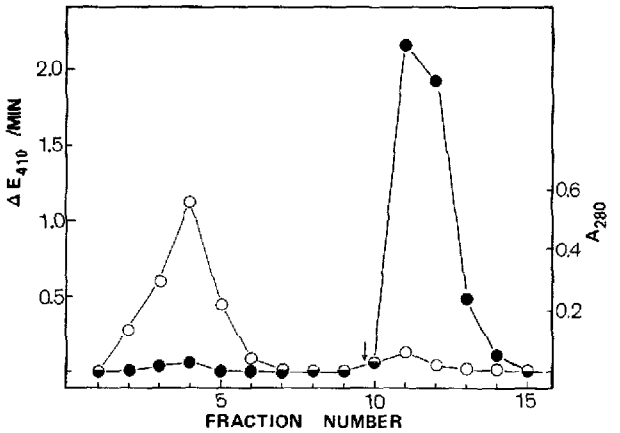

Fig. 2. Elution profile for phospholipase-C from affinity column. The affinity column (100 mm×8 mm I.D.), packed with APSE-cellulose gel, was washed and equilibrated with 0.1 *M* borax-HCl buffer (pH 7.3) containing 0.5 *M* NaCl and 1 m*M* $ZnSO_4$. A solution (11.8 ml) of DEAE-cellulose filtrate was saturated to 0.5 *M* with NaCl and applied to the column at a flow-rate of 0 15 ml/min. After washing with three column volumes of the same buffer, enzyme was eluted with 5 *M* solution of urea in 0.1 *M* borax-HCl buffer (pH 7.3). Fractions of 3 ml were collected throughout. Enzyme activity was determined using 50 μl of each fraction in a standard reaction mixture with NPPC (see Experimental). (●) Extinction at 410 nm (E_{410}); (○) A_{280} protein content. The arrow indicates the beginning of elution.

ever, the complex could not be reused after extensive washing on a glass filter with the borate buffer, i.e. complete desorption of the enzyme took place during its incubation with substrate liposomes.

In a standard system with NPPC as a substrate, the enzyme (20 μg adsorbed on 100 mg wet weight of the gel) remained in an immobilized state and its activity was almost 97% of the original (29 ± 0.2 nmol of *p*-nitrophenol liberated per minute, against 30 ± 0.3 nmol/min for the free PLC) in the first repeated reaction. This complex was reused several times without loss of activity (29 ± 0.3 nmol/min, mean value of three runs of the second, third and fourth reactions).

Gerasimene et al. [13] and Aakre and Little [27] have demonstrated that amines, such as Tris, are able to interact with and, in some cases, to inhibit PLC from *B. cereus*. We presumed that the affinity adsorption in our case could be based on a kind of enzyme–inhibitor interaction. To check this hypothesis we next examined a number of APSE structural analogues for an inhibitory effect on PLC (Fig. 3). Sulphathiazole was the most potent inhibitor among the substances tested. Its half-inhibiting concentration (IC_{50}) was 83 μM. Sulphanilic acid, sulphadimethoxine and sulphamethoxazole were less active.

In contrast, *p*-aminosalicylic acid had no effect at the concentrations used. Table II shows the effect of ST on PLC activity in a reaction with phosphatidylcholine liposomes. ST was an inhibitor with an IC_{50} value of ca. 0.8 mM, and this inhibition did not increase at ST concentrations greater than 1.5 mM. The other three substances were not active at the same concentrations.

Tris was also an inhibitor of PLC, which is in agreement with the observa-

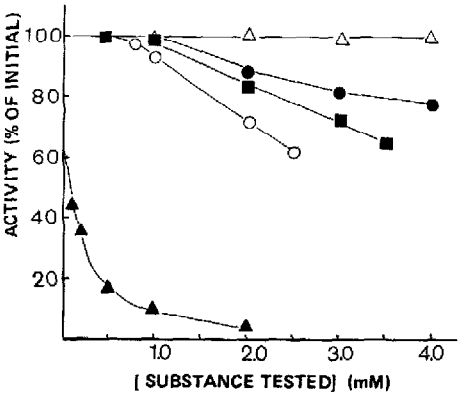

Fig. 3. Inhibition of phospholipase-C by different structural analogues of APSE Enzyme activity was measured using 20 mM NPPC as substrate in 0.1 M borax-HCl buffer (pH 7.3) in the presence of specified concentrations of APSE analogues. The final concentration of PLC was 5 μg/ml. Values represent the mean in triplicate samples. Substances used were: (▲) ST; (○) sulphadimethoxine; (■) sulphamethoxazole; (●) sulphanilic acid; (△) *p*-aminosalicylic acid. The basal phospholipase-C activity corresponds to 7 6 nmol/min.

TABLE II

INHIBITION OF PHOSPHOLIPASE-C BY SULPHATHIAZOLE

Enzyme activity was measured using 4 m*M* phosphatidylcholine as substrate in 0.1 *M* borax-HCl buffer (pH 7.3) in the presence of the indicated concentrations of ST. Deoxycholate was omitted. The final concentration of phospholipase was 0.1 μg/ml. Values represent means ± S.D. for three experiments. For more details see Experimental.

ST concentration (m*M*)	Enzyme activity (nmol P, liberated per min)	Activity (% of control)
0	75.0±0.2	100
0.1	72.7±0.2	97
0.2	63.5±0.4	85
0 5	48.3±0.3	64
1 0	31.7±0.3	42
1.5	30.9±0.2	41
2.0	30.9±0.2	41
2.5	30.9±0.2	41

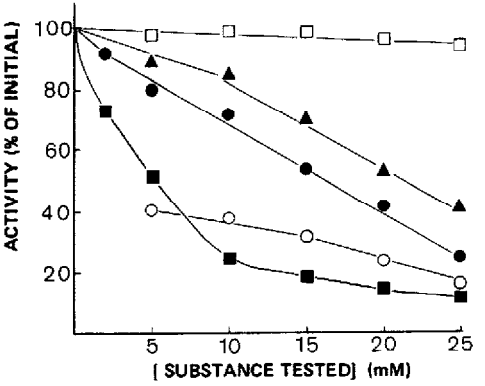

Fig. 4. Inhibition of phospholipase-C by Tris and polyvalent alcohols. Experimental conditions were the same as described for Fig. 3, except that the activity of phospholipase-C was assayed in the presence of various amounts of (■) Tris, (▲) mannitol, (□) glycerol and (●) sorbitol alone or (○) with IC_{50} of Tris. Values represent the mean in triplicate samples. For more details see text.

tion of Aakre and Little [27]. The IC_{50} value for Tris was 5 m*M*, with respect to the rate of NPPC hydrolysis (Fig. 4). Contrary to the findings of Kurioka and Matsuda [23] for *Clostridium perfringens* PLC, we found that different polyvalent alcohols inhibit the rate of hydrolysis of NPPC. Thus, IC_{50} values for sorbitol and mannitol were calculated to be 16.4 and 21.4 m*M*, respectively. Glycerol had little effect at the concentrations used, but significant inhibition was observed above 40 m*M*. In the presence of Tris (IC_{50}), an increase in the sorbitol concentration resulted in an increased rate of inhibition (Fig. 4).

TABLE III

EFFECT OF DIFFERENT REACTION CONDITIONS ON THE RATE OF HYDROLYSIS OF NPPC BY PHOSPHOLIPASE-C

The reaction mixture contained 25 μg of PLC and 20 μmol of NPPC in a total volume of 1 ml. Incubation was carried out at 33 °C in sodium borate or Tris buffer as indicated, with or without 60% (w/w) of sorbitol. Values represent means ± S.D. for three experiments.

Reaction conditions	Rate of NPPC hydrolysis (nmol/min)
0 1 *M* Borax-HCl (pH 7.3)	38.0 ±0.5
0.1 *M* Borax-HCl (pH 7.3) with 60% of sorbitol	–[a]
0.25 *M* Tris-HCl (pH 7.3)	0.58±0.007
0.25 *M* Tris-HCl (pH 7.3) containing 0.5 m*M* ST	0.58±0.007
0.25 *M* Tris-HCl (pH 7.3) with 60% of sorbitol	0.39±0.005

[a]No detectable hydrolysis.

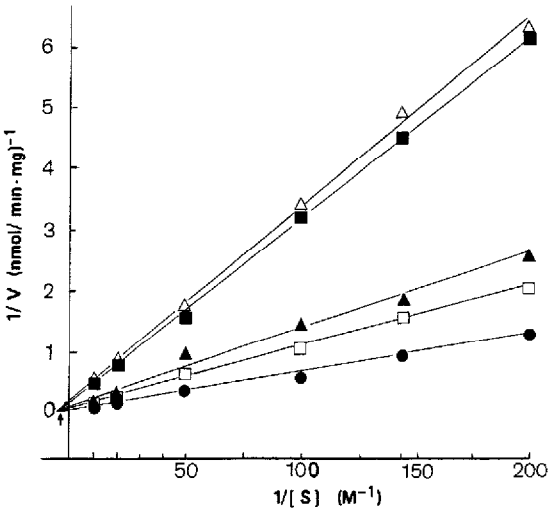

Fig. 5. Double-reciprocal plots of the inhibition of phospholipase-C by ST and Tris Phospholipase-C activity was assayed in the presence or absence of indicated concentrations of ST or Tris along with various amounts of NPPC. Incubation was carried out in 0.1 *M* borax-HCl buffer (pH 7.3) at 33 °C. The final concentration of PLC was 2 μg/ml. For more details see Experimental. The concentrations used were: (●) vehicle; (□) 4 m*M* Tris; (■) 20 m*M* Tris; (▲) 0 15 m*M* ST; (△) 0.5 m*M* ST

In 0.1 *M* borax–HCl buffer without sorbitol, the reaction rate was 96 times that in 0.25 *M* Tris–HCl with 60% (w/w) sorbitol, at the same pH (7.3). In the borate buffer inclusion of 60% sorbitol led to complete inhibition of enzymic hydrolysis of NPPC (Table III).

Kinetic analysis showed that ST and Tris inhibit PLC activity in the same non-competitive manner (Fig. 5). The apparent Michaelis constant (K_M) value for *B. cereus* PLC, read from the double reciprocal plots, was 0.2 *M* and coincides with the value calculated for the *Clostridium* enzyme [23].

K_i values for ST and Tris were estimated to be 0.12 and 4.8 m*M* respectively (mean values of two independent readings from Dixon plots, and secondary plots of the slope as a function of inhibitor concentration).

Another interesting observation was that in the presence of high concentrations of Tris, ST did not inhibit the rate of NPPC hydrolysis (Table III).

DISCUSSION

Since Zwaal et al. [12] first purified PLC from *B. cereus* to a homogeneous state, a number of purification schemes for this enzyme have been reported, using affinity chromatographic procedures. Little et al. [15] and Myrnes and Little [16] used Sepharose-linked egg-yolk lipoprotein to obtain preparations with the highest specific activity reported (2900 U/mg, at 37°C, with respect to the egg-yolk test). Imamura and Horiuti [14] used hydrophobic chromatography on palmitoyl cellulose, and their preparation had a similarly high activity (1320 U/mg, assayed by the rate of P_i liberated). Gerasimene et al. [13] succeeded in purifying PLC by negative adsorption on aminoalkyl derivatives of Sepharose and Sephadex.

Our procedure is comparable with these methods, in that it has a high efficiency and is very simple to perform. It does not include time-consuming procedures or expensive materials. In addition, our affinity column can be used at least 30 times, without any loss of binding capacity or other properties of practical interest. A very high affinity of APSE-cellulose was also confirmed for a new phosphatidylcholine-hydrolysing PLC, isolated in our laboratory from the culture broth of a Gram-negative bacterium that has yet to be identified (unpublished data).

The affinity adsorption of PLC was sensitive to pH changes, but was not influenced appreciably by the ionic strength. This effect is most probably due to the reversible dissociation of the amino group of APSE. Only partial elution could be achieved by detergents and NaCl, and the only suitable substance for elution was the chaotropic agent urea. It thus appears that combined weak interactions, i.e. hydrophobic (π–π aromatic) and hydrogen-bond formation, take place in PLC binding to our affinity sorbent, and the latter type proved to be predominant.

Our results show that in the presence of high Tris and/or sorbitol concen-

trations, both enzymic hydrolysis of NPPC and enzyme adsorption on APSE-cellulose, were almost completely inhibited. On the other hand, structural analogues of APSE were shown to inhibit PLC activity. It is thus very likely that the affinity binding is due to enzyme–inhibitor interaction, although more detailed experiments are needed to establish the mode of this interaction.

In addition to the main differences between PLCs from *B. cereus* and *C. perfringens* [8], our results show that the rate of NPPC hydrolysis by the former enzyme is inhibited even at low concentrations of polyvalent alcohols, whereas high alcohol concentrations accelerate hydrolysis of the same substrate by the latter enzyme [23].

In recent years sulphonamides have been shown to inhibit a number of calmodulin-dependent enzymes and cellular functions [28]. The observation that PLC from *B. cereus* is inhibited by sulphonamides (if true for phospholipases of other origin) may play an important role in the study of their physiological effects and interactions with some key enzymes of cell metabolism.

ACKNOWLEDGEMENT

This work was supported by grants from the Bulgarian Committee of Science (Ministry of Culture, Science and Education).

REFERENCES

1 H. van den Bosch, in J.N. Hawthorne and G.B. Ansell (Editors), Phospholipids, Elsevier, Amsterdam, 1982, Ch. 9, pp. 313–357.
2 K. Aalmo, L Hansen, E. Hough, K. Jynge, J. Krane, C Little and C Storm, Biochem. Int., 8 (1984) 27.
3 B. Aurebekk and C. Little, Int. J. Biochem., 8 (1977) 757.
4 R Bicknell, G. Hanson, B. Holmquist and C. Little, Biochemistry, 25 (1986) 4219.
5 Ø Hetland and H. Prydz, Scand. J. Clin. Lab. Invest., 42 (1982) 57
6 C. Little, Acta Chem. Scand., B31 (1977) 267.
7 C. Little, Biochem. J., 175 (1978) 977.
8 A.-B. Otnaess, C. Little, K. Sletten, R. Wallin, S. Johnson, F. Flengsrud and H. Prydz, Eur J. Biochem., 79 (1977) 459.
9 J. Kleiman and W. Lands, Biochim. Biophys. Acta, 187 (1969) 477.
10 A -B. Otnaess, H. Prydz, E. Bjorklid and Å. Berre, Eur. J. Biochem., 27 (1972) 238.
11 J. Shiloach, S. Bauer, I. Vlodavsky and Z. Selinger, Biotechnol Bioeng., 15 (1973) 551.
12 R. Zwaal, B Roelofsen, P. Comfurius and L. van Deenen, Biochim. Biophys. Acta, 233 (1971) 474
13 G. Gerasimene, A. Glemzha, V. Kulene, Yu. Kulis and Yu. Makaryunaite, Biokhimiya, 42 (1977) 919.
14 S. Imamura and Y. Horiuti, J. Lipid Res., 20 (1979) 519.
15 C. Little, B. Aurebekk and A.-B Otnaess, FEBS Lett., 52 (1975) 175.
16 B. Myrnes and C Little, Acta Chem. Scand., B34 (1980) 375.
17 Z. Rogovin, Sun-Tun, A. Virnik and N. Khvostenko, Visokomolekulyarnie Soedineniya, 4 (1962) 571.
18 P. Burba and K. Lieser, Angew Makromol. Chem., 50 (1976) 151.

19 P. Burba, M. Rober and K. Lieser, Angew. Makromol. Chem , 66 (1978) 131.
20 M. Turkova, M. Beneš, M. Kühn, V. Stepanov and L. Lyublinskaya, in J. Chaloupka and V. Krumphanzl (Editors), Extracellular Enzymes of Microorganisms, Plenum Press, New York, London, 1987, p. 99.
21 T Takahashi, T. Sugahara and A. Ohsaka, Methods Enzymol., 71 (1981) 710
22 S. Sonoki and H. Ikezawa, Biochim. Biophys. Acta, 403 (1975) 412.
23 S. Kurioka and M. Matsuda, Anal. Biochem., 75 (1976) 281.
24 G. Gerasimene, Yu. Makaryunaite, V. Kulene and A. Glemzha, Priklad. Biokhim. Mikrobiol., 16 (1980) 523.
25 B.J. Davis, Ann. N.Y. Acad. Sci., 121 (1964) 404.
26 E.F. Hartree, Anal. Biochem., 48 (1971) 422.
27 S.-E. Aakre and C. Little, Biochem. J., 203 (1982) 799.
28 B.D. Roufogalis, in D. Marme (Editor), Calcium and Cell Physiology, Springer-Verlag, Berlin, 1985, pp. 148–169