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A Simple Purification Scheme Yielding Crystalline Phospholipase C from *Bacillus cereus*

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A very simple and rather unusual purification scheme for phospholipase C from *Bacillus cereus* has been worked out. Air is bubbled vigorously through the bacterial culture and the foam collected. Liquified foam is centrifuged, dialyzed and heated at 74 °C for 5 min. After centrifugation, affinity chromatography is carried out on lipoprotein-Sepharose. The enzyme is then thermally denatured by exposure to 85 °C for 5 min and the precipitated material well washed and then renatured from solution in 4 M guanidinium chloride. The final enzyme preparations are electrophoretically homogeneous and easy to crystallize. The recovery of activity exceeds 80 %.

Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* is a very important and widely used tool in the study of membranes and phospholipids. Such studies require phospholipase C of the highest possible degree of purity, preferably crystallized enzyme, in order to exclude the possibility that the observed effects arise from low levels of contaminating enzyme activities. Several methods for the purification of this enzyme to apparent homogeneity have been published.[1-5] Of these procedures, by far the most convenient is that of Little *et al.*[3] This method, although very reliable, requires large amounts of an affinity gel (Sepharose-linked egg yolk lipoprotein) and is thus expensive. The observation that phospholipase C concentrates in foam produced on top of *B. cereus* cultures,[6] together with the finding that thermally coagulated totally inactive enzyme could be unfolded and refolded back to an active form[7] have now been used in a much modified and rather unusual purification scheme. The new scheme is probably the simplest so far published and has by far the greatest recovery of enzyme activity.

EXPERIMENTAL

Sepharose 4 B (Pharmacia) was activated by the method of Marsh *et al.*[8] Egg yolk lipoprotein was then coupled to the activated Sepharose by the method of Takahashi *et al.*[9] as described by Little *et al.*[3]

Enzyme assay. Enzyme activity was assayed titrimetrically[3] by measuring the rate of acid releas in the egg yolk substrate described by Zwaal *et al.*[1] Assays were carried out at 22–23 °C with the titration end point being pH 7.6. Under these conditions, one unit of enzyme activity is defined as liberating 1 μmol of titratable H^+/min.

Haemolytic activity was tested by incubating enzyme with a 2.5 % (v/v) suspension of fresh human erythrocytes in 0.15 M NaCl containing 5 mM tris-Cl buffer (pH 7.4) for 1 h at 37 °C.

Polyacrylamide gel electrophoresis. Gel electrophoretic studies were carried out in the discontinuous Tris-Cl/Tris-glycine system of Davis[10] using 7.5 % gels and in the sodium dodecylsulfate system described by Laemmli[11] using 10 % gels. Developed gels were stained for protein using Coomassie Brilliant Blue R-250. Protein was assayed by the method of Lowry *et al.*[12] with bovine serum albumin as standard. Unless otherwise mentioned, all operations were carried out at 0–4 °C.

Purification of enzyme

1. *Growth of bacteria. Bacillus cereus* strain ATCC 10987[13] was grown in the medium used by Zwaal *et al.*[1] After direct inoculation from a plate colony, the medium (4 l) was incubated in conical flasks with cotton wool plugs for 16–18 h at 27 °C in a

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shaker-incubator (150 rev./min). This incubation period corresponded to the maximum phospholipase C activity in the culture.

2. *Production and collection of foam from culture.* Culture (4 l) was poured into a 5 l conical flask with a side arm and compressed air was bubbled through sufficiently vigorously at room temperature to produce extensive foaming without direct splashing of liquid through the side arm. Foam was collected *via* the side arm into a flask (1 l) containing 2 drops of Antifoam C emulsion (Sigma). Bubbling was continued until a volume of liquified foam equal to about 10 % of the original volume of culture had been collected. The liquified foam was centrifuged (6000 *g* for 10 min) and the supernatant was then dialyzed for 2 h against 10 l of 0.15 M NaCl buffered at pH 7.4 by 2.9 mM sodium 5,5'-diethylbaribiturate and containing 0.1 mM Zn(II).

3. *Heat treatment.* After dialysis the enzyme solution was maintained at 73 – 74 °C for 5 min and then cooled on ice. Precipitated material was removed by centrifugation (6000 *g* for 10 min) and was rejected.

4. *Affinity chromatography.* The supernatant from the previous step was loaded onto a column (2.8 × 3 cm) of Sepharose-linked egg yolk lipoprotein at a flow rate of 150 ml/h. The column was then washed with 6 column volumes of the above saline-barbiturate-Zn^{2+} buffer. Enzyme was eluted from the column using a freshly prepared solution of 8 M urea in the saline-barbiturate-Zn^{2+} buffer[3] and 5 ml fractions were collected at a flow rate of 2 – 3 ml/min. Fractions containing activity were pooled and dialyzed twice against 2 l portions of 0.1 M sodium acetate buffer (pH 6.0) for 1 h at room temperature.

5. *Denaturation and renaturation.* The enzyme solution was then maintained at 85 °C for 5 min and cooled on ice. The precipitated material was isolated by centrifugation (20 000 *g* for 10 min) and washed twice by resuspension in 30 ml portions of distilled water followed by centrifugation (20 000 *g* for 10 min). The precipitate was then dissolved in 30 ml 4 M guanidinium chloride in 0.1 M sodium acetate (pH 6.0) buffer and incubated at 22 – 23 °C for 1 h. Afterwards the solution was dialyzed twice against 4 l portions of the saline-barbiturate-Zn^{2+} buffer (2 h and overnight). Precipitated material was removed by centrifugation (20 000 *g* for 10 min) and discarded.

6. *Crystallization.* Enzyme (3 – 4 mg/ml) dissolved in unbuffered 30 % saturated ammonium sulfate solution was centrifuged (20 000 *g* for 10 min) to remove dust particles, placed in a small plastic container and crystallized by vapour phase diffusion against a 45 % saturated ammonium sulfate solution essentially as described by Hough *et al.*[14] Crystals (see Fig. 1) appeared after two days and were harvested after 10 days. The crystals were washed by resuspension in 0.15 M NaCl in which they dissolve very slowly and finally dissolved in the saline-barbiturate-Zn^{2+} buffer and stored at – 20 °C. Crystallization has no significant effect on the specific activity of the enzyme.

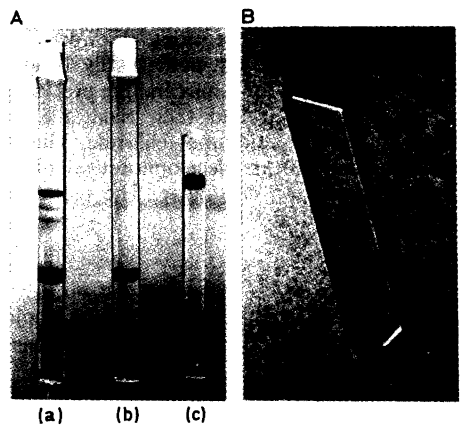

Fig. 1. A. Gel electrophoretic patterns. (a) enzyme after affinity chromatography (b) and (c) enzyme denaturation/renaturation. (a) and (b) were run in the system of Laemmli[11] and (c) in the system of Davis.[10] B. Photomicrograph of enzyme crystal.

Table 1. Purification of phospholipase C.

Step	Total volume/ml	Total activity/units	Protein/mg	Specific activity/ units mg^{-1}	Recovery/%
B. cereus culture	4000	34 000	–	–	100
Liquified foam	420	33 600	2789	12	99
Heat (74 °C)	465	32 550	1897	17	96
Affinity column	36.5	31 110	33	943	92
Denaturation/ Renaturation	37.8	27 450	19.5	1410	81

Enzyme purity. Prior to crystallization, enzyme samples appeared homogeneous in disc gel electrophoresis under both denaturing and non-denaturing conditions (see Fig. 1). The specific activity is very similar to that of enzyme samples prepared according to our earlier method[3] which have been demonstrated to be homogeneous according to electrophoretic, immunological and amino acid sequencing criteria.[3,15] The purified enzyme has almost no haemolytic activity towards fresh human erythrocytes and caused <2 % lysis when incubated at 37 °C for 1 h at a concentration of 0.01 mg/ml.

Enzyme samples obtained by the present method were compared with those from our previous method[3] to examine whether the denaturation-renaturation process had produced a modified enzyme conformation. Enzyme samples prepared according to the two different procedures had identical mobilities in the two gel electrophoretic systems, identical isoelectric points, very similar specific activities and produced crystals of identical forms.

DISCUSSION

The results of a typical purification are given in Table 1. The method was found to be very reliable and the overall recovery of activity is very high and normally exceeds 80 %. The removal of activity from the culture by foaming is extremely effective resulting in near total transfer of the enzyme activity. This foaming method was first used in the purification of phospholipase C from *B. cereus* by Zwaal *et al.*[6] who grew the bacteria under conditions of vigorous aeration and collected the foam throughout the growth. In the present method, foam is produced and collected after growth and has the advantage that the specific activity in the liquified foam is about 3-fold greater. The affinity chromatography is the single most effective step but does not yield a homogeneous preparation (see Fig. 1) and a further purification step is required. During step 5 in the scheme, thorough washing of the heat-precipitated enzyme is essential if a homogeneous product is to be obtained.

The crystallizion procedure was found to be very simple to reproduce and the very low rate of dissolution of the crystals in 0.15 M NaCl means that any amorphous material can easily be removed by washing. The present purification scheme is even easier to carry out than our previous one,[3] has a higher recovery of enzyme activity and is considerably cheaper to operate since the amount of affinity gel required/l bacterial culture used is decreased by about 80 %. This new scheme would appear very suitable for the large scale purification of phospholipase C from *B. cereus.*

Note added in proof. If porous airstones as used in the aeration of aquaria are employed in the foaming step, the enzyme activity can be recovered in a volume of liquified foam equal to $0.5-1$ % of the culture volume. This further simplifies the subsequent purification

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