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Purification of phospholipase D by two-phase affinity extraction

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Abstract

An aqueous two-phase system of polyethylene glycol (PEG)–salt was used for purification of phospholipase D (PLD) from peanuts and carrots. Alginate, a known macroaffinity ligand for PLD, was incorporated in the PEG phase and resulted in 91 and 93% of the enzyme activity (from peanuts and carrots, respectively) getting partitioned in the PEG phase. The elution of the enzyme from alginate was facilitated by exploiting the fact that the latter can be reversibly precipitated in the presence of Ca^{2+}. The enzyme was eluted from the polymer by using 0.5 M NaCl. Peanuts and carrots PLD could be purified 78- and 17-fold with 82 and 85% activity recovery, respectively. The purified enzyme from both sources gave a single band on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis.

Keywords: Affinity extraction; Aqueous two-phase systems; Phospholipases; Enzymes; Alginate

1. Introduction

Two-phase affinity extractions have been widely used for separation of enzymes/proteins [1–3]. Such an approach combines the high selectivity of an affinity step with the well known advantage of two-phase aqueous system that one can directly deal with crude broths containing suspended matter (e.g. cell debris). Kamihira et al. [4] had outlined a useful strategy in which the affinity ligand incorporated in the polyethylene glycol (PEG) phase of PEG-Reppal PES 200 (modified starch) was a smart macroaffinity ligand. A smart macroaffinity ligand [5–7] in this context is an affinity ligand linked to a reversibly soluble–insoluble polymer, the solubility of which can be altered by an external stimulus such as change in pH, temperature or presence of chemicals. The approach of Kamihira et al. [4] allowed easy separation of complex of affinity ligand and the target protein from PEG phase. Thus, both PEG as well as affinity ligand could be reused. More recently, we have shown that this strategy could be utilized to purify wheat germ α-amylase [8], sweet potato β-amylase [8], xylanase [9] and pullulanase [9]. For all starch degrading enzymes, alginate was used as such as the smart macroaffinity ligand [10–12]. Alginate has also shown to be a smart macroaffinity ligand for peanut phospholipase D (PLD) [13]. PLD hydrolyses lecithin into phosphatidic acid and choline. The enzyme has been implicated in a number of cellular processes [14,15]. Recently, Servi [16] has described a number of important synthetic applications of PLD. Thus, a facile purification method for PLD should further the study of its enzymology and applications. The present work describes such a method for purification of phospholipase D from peanuts and carrots.

2. Materials and methods

2.1. Materials

Sodium alginate (catalog no. A-2158, composed primarily of mannuronic acid residues) and standard molecular weight markers were purchased from Sigma, St. Louis, MO, USA. Soybean lecithin (phosphatidyl choline) was obtained from BDH, E. Merck, Mumbai, India. Polyethylene glycol (PEG 6000) was from E. Merck, Mumbai, India. Peanuts and carrots were purchased from the local market. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of phospholipase D crude extracts

The crude extracts of the enzyme from peanuts and carrots were prepared as described earlier [13]. Dry *peanut seeds*

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(200 g) were washed with a commercial detergent containing approximately 4% (w/v) SDS, rinsed thoroughly with water and soaked overnight in water at 26 °C in an orbital shaker. The covers (testae) were removed and the dehusked seeds were homogenized for 1 min at 4 °C in a chilled Waring blender with 600 ml of a solution containing 0.25 M sucrose, 50 mM Tris, 2 mM EDTA and 3 mM 2-mercaptoethanol (pH 7.0).

Carrot roots (200 g) were washed, chopped and then homogenized in distilled water in a pre-chilled Waring blender at 4 °C for 3 min.

The actual purification work was carried out with a non-clarified crude extract. For optimization work, the clarified extract after centrifugation was used.

2.2.2. Preparation of alginate solution

Sodium alginate solution (2%) was prepared by dissolving 200 mg of sodium alginate in 10 ml of distilled water, pH adjusted to 7.0 [13].

2.2.3. Preparation of PEG–salt two-phase system

To 10 ml of graduated centrifuge tubes, the desired phase components in the order of PEG solution (2.5 ml from the stock solution of 22% (w/v) polyethylene glycol 6000 in 10 mM phosphate buffer, pH 7.0) and salt solution (2.5 ml stock solution of 10% K_2HPO_4 (w/v), and 12% (w/v), NaCl in 10 mM phosphate buffer, pH 7.0) were added. After vortexing for a minute, two distinct phases were formed within 5 min at 25 °C.

2.2.4. Enzyme assay

Phospholipase D activity was assayed by a titrimetric method using soybean lecithin as substrate. One unit is defined as the amount of enzyme which liberates one micromole of acid from soybean lecithin per minute at 25 °C at assay pH [17]. The activity measurements for various systems were carried out by withdrawing aliquots in the range of 100–500 μl.

2.2.5. Glycoprotein assay (phenol sulphuric 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 sulphuric acid was added and kept at 25 °C for 10 min. Absorbance was read at 489 nm [18].

2.2.6. Protein estimation

Protein was estimated according to the dye binding method using bovine serum albumin as the standard protein [19]. These estimations were done by withdrawing aliquots from various systems in the range of 100–500 μl.

2.2.7. Binding of phospholipase D from peanut and carrot with alginate as macroaffinity ligand in aqueous two-phase system

Aqueous two-phase separation using the PEG–salt system was tried for phospholipase D after incorporating 0.2% (w/v) alginate solution. The crude preparations of peanut (2.0 ml containing 10 U) and carrot (2.0 ml containing 35 U) were added to the respective systems. These two systems were prepared as described earlier. During the purification work, unclarified crude extracts were used. In such cases, PEG- and salt phases were separated by an interface consisting of cell debris and other insoluble matter. After vortexing, the phases were separated after 30 min. The volume of the total system before adding the crude extracts were 5.0 ml (without alginate) and 6.0 ml (with alginate), respectively. Alginate distribution was restricted to the PEG phase with less than 5% (of the initially added amount) partitioning to the bottom phase. Alginate concentration in the two phases was estimated by phenol sulphuric acid method [18]. The top phase containing the enzyme bound alginate was removed with a pipette. The alginate was precipitated in the presence of 70 mM Ca^{2+} [20,21] by incubating for 20 min at 25 °C. The precipitate was centrifuged at 8000 × *g* for 10 min at 25 °C. The supernatant and subsequent washings with buffer (till no enzyme activity was detected in the washings) were collected. Bound phospholipase D was calculated by the difference of initial activities loaded on alginate and recovered in supernatant and washings. The elution of the bound enzyme was tried using 2.0 ml of 0.5 M NaCl and were kept at 4 °C for overnight incubation. Polymer was recovered by precipitation with $CaCl_2$, followed by centrifugation and enzyme activity was estimated in the supernatant [20,21]. After removal of the top phase, the lower aqueous phase was separated by using a pipette which pierced through the interface. The interface left was also analyzed for protein and activity.

2.2.8. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the protein samples was performed using 12% gel according to Hames [22] on a Genei gel electrophoresis unit (Bangalore Genei, Bangalore, India).

3. Results

Earlier work has shown that alginate could be incorporated in the PEG–salt two-phase system [8,9]. Fig 1a shows the variation in extent of activity bound and eluted (to alginate in PEG–salt two-phase systems) with the different starting amounts of peanut phospholipase D activity in the crude extract. As number of enzyme molecules increase, they start occupying available affinity sites on the incorporated alginate molecules. As the extent of occupancy increases, the "crowding effect" prevents easy access to incoming enzyme molecules. At the point, the available affinity sites are mostly occupied. This gets reflected in the "approach to saturation" phase with decreased extent of binding of initial activity load. About 10 U activity as starting load was found to be optimum. The elution throughout was in the range of 89–94% of the bound activity. Table 1 shows that peanut PLD activity (10 U) got distributed more or less evenly in PEG–salt

Table 1
Purification of peanut phospholipase D using PEG-alginate–salt two-phase system

Steps	Activity (U)	Protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Fold purification
Unclarified crude extract	10	15	0.7	100	1
Lower phase (salt)					
No alginate	4.5	7.3	0.6	45	1
+Alginate	0.6	7.5	0.1	6	–
Interface					
No alginate	1.5	3.2	0.5	15	1
+Alginate	0.3	2.7	0.1	3	–
Upper phase (PEG)					
No alginate	3.5	2.5	1.4	35	2
+Alginate (supernatant + washing)	0.4	1.0	–	4	–
Elution	8.2	0.15	56	82	78

Purification was carried out using 0.2% sodium alginate solution. The elution was carried out using 2.0 ml of 0.5 M NaCl at 4 °C for overnight. The reaction velocity was measured titrimetrically using 0.02 M NaOH by measuring the rate of proton liberation during hydrolysis of soybean lecithin. All the experiments were performed in duplicate and the difference in the readings in the duplicates was less than ±5%.

two-phase system. However, incorporation of alginate in the PEG phase dramatically improved the partition of the enzyme activity in the PEG phase. The alginate–enzyme complex could be separated from PEG phase by precipitation with Ca^{2+} [20,21] and the enzyme activity was recovered by dissolving the precipitate in 0.5 M NaCl and precipitating alginate alone by adding Ca^{2+} [20]. 82% enzyme activity could be recovered with 78-fold purification. Fig. 2a shows the SDS-PAGE of the purified enzyme which appeared as a single band at molecular mass position of 22 000 Da. This molecular mass is in agreement with what has been reported earlier for this enzyme [13,23].

Similar results were obtained in the case of carrot PLD. Fig. 1b shows that 39 U of enzyme activity was optimum load in this case for binding maximum amount of activity. Again, extent of bound activity which got eluted with different starting activity loads did not show much variation. It was in the range of 92–96% of bound activity. Table 2 shows that incorporation of alginate again led to partition of 90% of applied activity (35 U) to the PEG-alginate phase. Following similar procedure as in the case of peanut PLD, 85% of initial activity could be recovered with 17-fold purification. SDS-PAGE (Fig. 2b) shows a single band for the purified preparation in this case as well. Again the molecular mass obtained (60 000 Da) was in agreement with the value reported earlier [24].

4. Discussion

The various purification protocols described for PLD generally consists of multi-step procedures [25,26]. In the case of PLD from peanut and carrot, simpler and shorter purification procedures have been developed in this laboratory [13,24]. Thus, it may be worthwhile to compare the results obtained here with those described earlier [13,24]. PLD

Table 2
Purification of carrot phospholipase D using PEG-alginate–salt two-phase system

Steps	Activity (U)	Protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Fold Purification
Unclarified crude extract	35	500	70	100	1
Lower phase (salt)					
No alginate	14	200	70	40	1
+Alginate	1.8	220	8	5	–
Interface					
No alginate	3.8	100	39	11	1
+Alginate	0.7	140	5	2	–
Upper phase (PEG)					
No alginate	15.8	110	143	45	2
+Alginate (supernatant + washing)	1.4	70	20	4	3
Elution	29.8	25	1190	85	17

Purification was carried out using 0.2% sodium alginate solution. The elution was carried out using 2.0 ml of 0.5 M NaCl at 4 °C for overnight. The reaction velocity was measured titrimetrically using 0.02 M NaOH by measuring the rate of proton liberation during hydrolysis of soybean lecithin. All the experiments were performed in duplicate and the difference in the readings in the duplicates was less than ±5%.

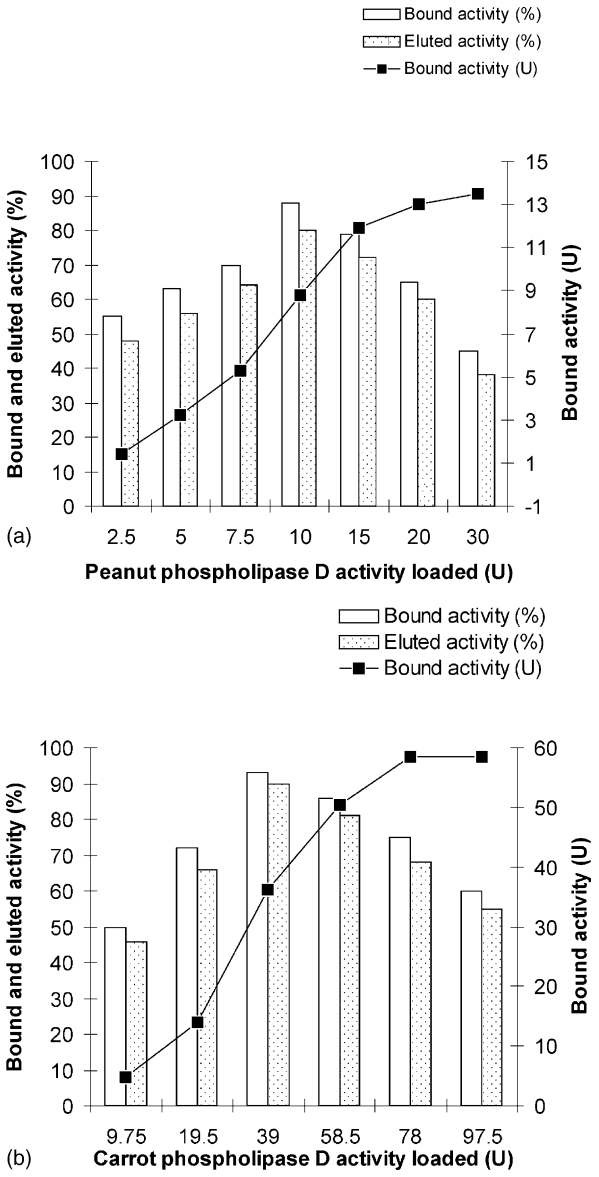

Fig. 1. (a) Effect of enzyme activity loaded (2.5–20 U) on the binding and elution of peanut phospholipase D to alginate (0.2%) solution. The binding of phospholipase D was carried out as described in Section 2. For the optimization experiment, the clear crude extract after centrifugation (15 000 × *g*, 30 min, 10 °C) was used. Bound phospholipase D activity was calculated by the difference of initial activity loaded and the activities of the supernatant and washing. Phospholipase D activity was determined titrimetrically using soybean lecithin as substrate. (b) Effect of enzyme activity loaded (9.75–78 U) on the binding and elution of carrot phospholipase D to alginate (0.2%) solution. For the optimization experiment, the clear crude extract after centrifugation (15 000 × *g*, 30 min, 10 °C) was used. The binding and elution of phospholipase D was carried out as described in Section 2. Bound phospholipase D activity was calculated by the difference of initial activity loaded and the activities of the supernatant and washing. Phospholipase D activity was determined titrimetrically using soybean lecithin as substrate.

from peanut has been purified earlier by affinity precipitation with alginate itself [13]. The yield as well as fold purification in that case were lower (55% and 34-fold, respectively) than those obtained in the present work (82% and 78-fold).

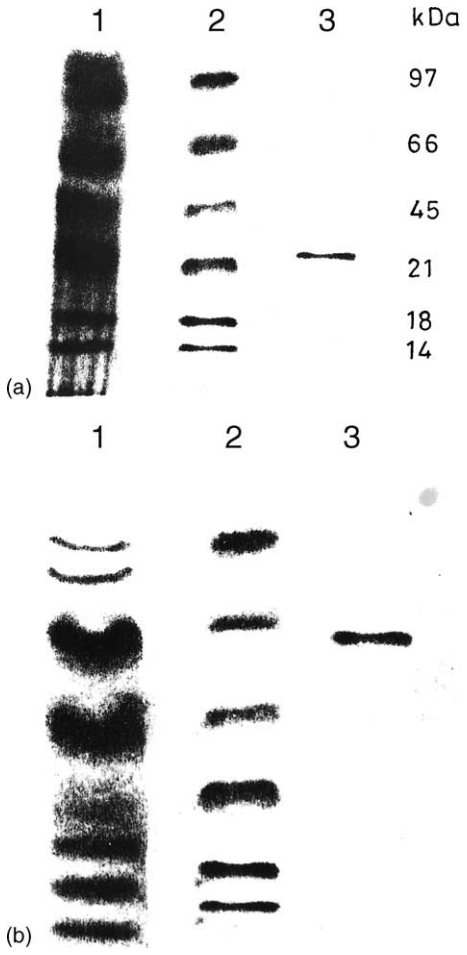

Fig. 2. (a) SDS-PAGE pattern of peanut phospholipase D. Lane 1: Crude peanut phospholipase D; lane 2: marker proteins; lane 3: purified preparation; (b) SDS-PAGE pattern of carrot phospholipase D. Lane 1: crude carrot phospholipase D; lane 2: marker proteins; lane 3: purified preparation.

Besides, in the case of affinity precipitation approach, the starting material had to be centrifuged before carrying out the process. In the present work, no such step was used/required.

PLD from carrot has been purified earlier by three-phase partitioning (TPP) with an activity recovery of 72% and 13-fold purification [24]. Again, better recovery of activity and fold purification were obtained in the present case. In the case of this enzyme as well, the TPP work was carried out with the crude extract after centrifugation.

The results described here show that PLD from peanut and carrot can be purified without any preprocessing/clarification step by two-phase affinity extraction. Apart from Kamihira et al. [4], polymer–dye conjugates have also been used as smart macroaffinity ligand in two-phase system [27]. In both cases, the two-phase system is of polymer–polymer systems. In the present work, more economical PEG–salt system has been used. The second important aspect of the present approach is that inherent affinity of a naturally occurring polysaccharide has been used. Thus, no conjugation

step of crafting a polymer-affinity ligand was involved. The usefulness of exploiting this unexpected affinity of both naturally occurring and synthetic polymer in bioseparation has been discussed earlier [28,29]. Thus, the present approach can be extended to use of various economical and easily available polymers for purification of large number of enzymes/proteins.

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