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Separation of enzymes by sequential macroaffinity ligand-facilitated three-phase partitioning

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Abstract

Pectinase and cellulase were separated from a commercial enzyme preparation called Pectinex Ultra SP-L. This was carried out using a process called macroaffinity ligand-facilitated three-phase partitioning (MLFTPP). In this method, a water-soluble polymer is floated as an interfacial precipitate by adding ammonium sulfate and *tert*.-butanol. The polymer (appropriately chosen) in the presence of an enzyme for which it shows affinity, selectively binds to the enzyme and floats as a polymer–enzyme complex. In the first step, pectinase was purified (with alginate as the polymer) 13-fold with 96% activity recovery. In the second MLFTPP step, using chitosan, cellulase was purified 16-fold with 92% activity recovery. Both preparations showed a single band on sodium dodecylsulfate–polyacrylamide gel electrophoresis. This illustrative example shows that the strategy of sequential MLFTPP can be used to separate important biological activities from a crude broth.

Keywords: Macroaffinity ligand-facilitated three-phase partitioning; Affinity partitioning; Enzymes; Pectinase; Cellulase

1. Introduction

Three-phase partitioning (TPP) is a process in which an aqueous solution of a protein [1,2] or a water-soluble polymer [3,4] when mixed with appropriate amounts of ammonium sulfate and *tert*.-butanol leads to the formation of the interfacial precipitate of the protein/polymer between the aqueous phase and *tert*.-butanol-rich phase. TPP of proteins is a rather nonselective process. It has been shown that the process can be made more selective by interfacing an affinity-based step into it [5].

Recently, we have developed a bioseparation process called macroaffinity ligand-facilitated three-phase partitioning (MLFTPP) for separation and purification of xylanase [6]. In this process, a water-soluble methyl methacrylate polymer, eudragit S-100, when subjected to TPP in the presence of xylanase molecules, floats in the form of its complex with the enzyme molecules. The affinity of eudragit S-100 for xylanases is known from earlier work on affinity precipitation [7]. From the same work, the method of recovery of enzyme activity from the interfacial precipitate of eudragit S-100-bound xylanase could also be adapted.

It was found that MLFTPP exhibited much higher selectivity than simple TPP of the same enzyme under the same or similar conditions. Thus, the fold purification of the xylanase was much higher than obtained with simple TPP of the same xylanase

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preparation. Also, unlike TPP, it is possible to design MLFTPP in a more predictable fashion by choosing an appropriate polymer. For example, it was shown that MLFTPP could also be used for purification of α-amylase by exploiting the reported affinity of alginate for α-amylases [8]. It has also been shown that MLFTPP could be used directly without any pre-clarification of suspended material in the crude broth [6,8].

The present work is aimed at further development of MLFTPP as a general separation technique for resolution of protein mixtures. Here, a commercially available preparation, Pectinex Ultra SP-L containing both pectinase and cellulase activities, was subjected sequentially to MLFTPP using alginate and chitosan. Chitosan is known to bind to cellulase [9] and alginate has been used for affinity-based purification of pectinase [10,11]. Using the two MLFTPP steps, with these two polymers, the two enzymes could be purified sequentially. The purity and % activity recovery obtained in this approach with both enzymes have been compared with the corresponding values obtained with purification using affinity precipitation and TPP (in a way MLFTPP can be looked upon as an integration of affinity precipitation and TPP).

It has been shown earlier that three-phase partitioning sometime leads to enhanced catalytic powers of the enzymes [1]. An X-ray diffraction study with proteinase K indicated that this could be due to an increase in overall flexibility in the protein structure [12]. The present work also investigates whether TPP and MLFTPP affect the catalytic efficiency of these two enzymes in a different way.

2. Materials and methods

Protanal LF10/60 (from brown seaweed, having a high guluronic acid content, i.e. 65–75%) was a product of Protan (Drammen, Norway). Polygalacturonic acid and chitosan (co-polymer of *N*-acetylglucosamine and glucosamine units) were purchased from Sigma (St. Louis, MO, USA). Pectinex Ultra SP-L (containing pectinase and cellulase from *Aspergillus niger*) was from Novo Nordisk (Switzerland). Carboxymethyl cellulose was a product of Loba Chemie (Bombay, India). All other chemicals used were of analytical grade.

2.1. Preparation of alginate solutions

Alginate (4%, w/v) was dissolved in distilled water. The solution was stored at 4 °C and diluted with appropriate buffer for further use [3].

2.2. Preparation of chitosan

Chitosan solution (50 ml, 1%, w/v) was prepared by dissolving 0.5 g of chitosan in 1% (v/v) acetic acid and then precipitating it by raising the pH to 8.0 by addition of 3 *M* NaOH. The precipitated chitosan was washed three times with 0.01 *M* Tris–HCl buffer, pH 8.0, and again dissolved in 50 ml of 0.01 *M* acetate buffer, pH 5.0, to get the final concentration of 1% (w/v) [4].

2.3. Enzyme assays

2.3.1. Pectinase assay

Pectinase activity was estimated by taking polygalacturonic acid as the substrate [11]. One enzyme unit is defined as the amount of enzyme which liberates 1 μmol of reducing group (calculated as galacturonic acid) per min at 30 °C. The amount of reducing group produced was estimated by the 3,5-dinitrosalicylic acid method [13].

2.3.2. Cellulase assay

Cellulase activity was measured by taking carboxymethylcellulose as the substrate [14]. One enzyme unit is defined as the amount of enzyme which liberates 1 μmol of reducing sugar (calculated as glucose) per min at 50 °C. The amount of reducing sugar produced was estimated by the 3,5-dinitrosalicylic acid method [13].

2.4. Protein estimation

Protein was estimated by the dye binding method [15] using bovine serum albumin as standard.

2.5. Sequential MLFTPP of pectinase and cellulase

Pectinex Ultra SP-L (0.5 ml containing 167 U of pectinase activity) was added to 1 ml of alginate (final concentration 1%, w/v). The total volume was made up to 4 ml with 0.05 *M* acetate buffer, pH 3.8. The solution was then subjected to MLFTPP for an hour by incubating at 37 °C with 30% (w/v) ammonium sulfate, and 8 ml of *tert*.-butanol. These conditions are known to precipitate alginate quantitatively as an interfacial layer between the aqueous and the organic phase [3]. The three phases formed, i.e. the upper layer of *tert*.-butanol, the interfacial precipitate (interfacial precipitate-1) containing alginate-bound pectinase and the lower aqueous layer (aqueous phase-1), were collected. The difference between the total enzyme activity in the starting solution and the activity in the aqueous phase-1 represented the amount of enzyme bound. The polymer-bound enzyme (interfacial precipitate-1) was recovered by following a slightly modified procedure as described earlier [11]. The precipitate was dissolved in 5 ml of 1 *M* NaCl (in 0.05 *M* acetate buffer, pH 5.9) and kept at 4 °C for 18 h. Enzyme was then recovered by precipitating alginate with 0.35 ml of 1 *M* $CaCl_2$ (final concentration of $CaCl_2$ in the solution was 0.07 *M*). This procedure was repeated twice in order to recover all the bound enzyme activity. Enzyme activity and protein in the supernatant were determined after extensive dialysis against 0.05 *M* acetate buffer, pH 5.0.

The lower aqueous layer (aqueous phase-1) was dialyzed to remove ammonium sulfate and *tert*.-butanol and subjected to a second MLFTPP by using chitosan as the polymer. Chitosan solution (final concentration 0.2%, w/v) was added to this aqueous phase and the total volume made to 4 ml with 0.05 *M* acetate buffer, pH 4.8. This was followed by the addition of 45% ammonium sulfate, and *tert*.-butanol in a ratio of 1:1 (aqueous phase to *tert*.-butanol, v/v). The mixture was gently vortexed and kept at 37 °C for an hour. These conditions are known to precipitate chitosan quantitatively as an interfacial layer between the aqueous and the organic phase [4]. Three phases were again formed, consisting of the upper *tert*.-butanol phase, an interfacial precipitate (interfacial precipitate-2) consisting of chitosan-bound cellulase and a lower aqueous phase (aqueous phase-2). The difference between the total enzyme activity in the crude extract and the activity in the aqueous phase-2 represented the amount of enzyme bound. The polymer-bound enzyme (interfacial precipitate-2) was recovered by incubating the precipitate in 4 ml of 1 *M* phosphate buffer, pH 7.0, for 18 h at 4 °C. The pH of the solution was then adjusted to 8.0 to precipitate chitosan. The suspension was centrifuged at 12,000 *g* for 15 min at 10 °C. Eluted cellulase activity and protein in the supernatant were determined after extensive dialysis against 0.05 *M* acetate buffer, pH 4.8.

2.6. Purification of pectinase by affinity precipitation

Affinity precipitation involves selective precipitation of the target protein by a smart macroaffinity ligand [16].

Pectinase from Pectinex Ultra SP-L was purified by affinity precipitation method as described earlier [11]. To 35 U of pectinase activity, 0.2% (w/v) of alginate was added. After 30 min of incubation at 25 °C, the polymer–enzyme complex was precipitated by adding 0.1 *M* $CaCl_2$.The polymer-bound enzyme was washed with 0.05 *M* acetate buffer, pH 3.8, containing 0.1 *M* $CaCl_2$ till no enzyme activity could be detected in the washings. Elution was done by incubating the alginate–pectinase precipitate with 0.05 *M* acetate buffer, pH 5.9 (containing 0.5 *M* NaCl and 0.2 *M* $CaCl_2$) at 25 °C for 1 h. Enzyme activity was determined after dialysis against the assay buffer.

2.7. Purification of pectinase by TPP

TPP of pectinase was carried out as described earlier [17].

To a solution of Pectinex Ultra SP-L (containing 38 U of pectinase), 30% (w/v) of ammonium sulfate and *tert*.-butanol in a ratio of 1:1 (v/v; aqueous to *tert*.-butanol phase) were added and the solution incubated at 25 °C for 1 h. Three phases formed (i.e. the upper layer of *tert*.-butanol, the interfacial precipitate and the lower aqueous layer) were separated and the enzyme activity in the interfacial precipitate (76% of the starting activity) was determined after dissolving it in 0.05 *M* acetate buffer, pH 5.0.

2.8. Purification of cellulase by affinity precipitation

Cellulase from Pectinex Ultra SP-L was purified by affinity precipitation as follows. No optimization was carried out. Instead, the polymer concentration, temperature, starting activity of cellulase were the same as mentioned above in MLFTPP of cellulase with chitosan. Hence, 54 U of cellulase activity were added to 0.2% (w/v) of chitosan and the solution incubated at 37 °C for 1 h. The polymer–enzyme complex was precipitated by increasing the pH of the solution to 8.0. Enzyme was recovered by incubating the chitosan–cellulase complex in 1 *M* phosphate buffer, pH 7.0, for 18 h at 4 °C and estimated after dialysis against 0.05 *M* acetate buffer, pH 4.8.

2.9. Purification of cellulase by TPP

TPP of cellulase was carried out as follows. No optimization of the conditions was carried out. Instead, ammonium sulfate, the ratio of aqueous solution to *tert*.-butanol (v/v), temperature and starting activity were chosen identical to MLFTPP of cellulase by chitosan. Thus, to a solution of Pectinex Ultra SP-L (containing 54 U of cellulase activity), 30% (w/v) ammonium sulfate and *tert*.-butanol in a ratio of 1:2 (v/v; aqueous solution to *tert*.-butanol phase) was added and the reaction mixture incubated at 37 °C for 1 h. Three phases formed (i.e. the upper layer of *tert*.-butanol, the interfacial precipitate and the lower aqueous phase) and were collected. Cellulase activity was estimated in the interfacial precipitate after dissolving it in 0.05 *M* acetate buffer, pH 4.8.

2.10. Determination of kinetic parameters

K_m and V_{max} values of enzymes purified by affinity precipitation, TPP and MLFTPP were determined by measurement of enzyme activity with varying concentration of substrate (which in the case of pectinase was polygalacturonic acid and in the case of cellulase was carboxymethylcellulose). Kinetic constants were calculated using the Leonora software program [18]. This software uses the Lineweaver–Burke equation to calculate K_m and V_{max}.

Each set of the above experiments was carried out in duplicate and the difference in the values within a pair was found to vary within 4%.

2.11. Polyacrylamide gel electrophoresis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the protein samples was performed using 12% gel according to Hames [19] on a Genei gel electrophoresis unit (Bangalore Genei, Bangalore, India) and standard molecular mass markers (Bangalore Genei).

3. Results and discussion

Alginate has been reported to bind *A. niger* pectinase activity at pH 3.8 [11]. It is also reported that alginate precipitates as an interfacial precipitate (between the aqueous and *tert*.-butanol phase) to the extent of 95% when subjected to three-phase partitioning from its solution in distilled water [3]. It was found that even at pH 3.8, 92% (w/v) alginate precipitated (as an interfacial precipitate) while keeping other conditions (30%, w/v ammonium sulfate; 1:2 ratio of alginate to *tert*.-butanol solution, v/v; 37 °C) unchanged (data not shown). Fig. 1 shows that if pectinase is present along with alginate at pH

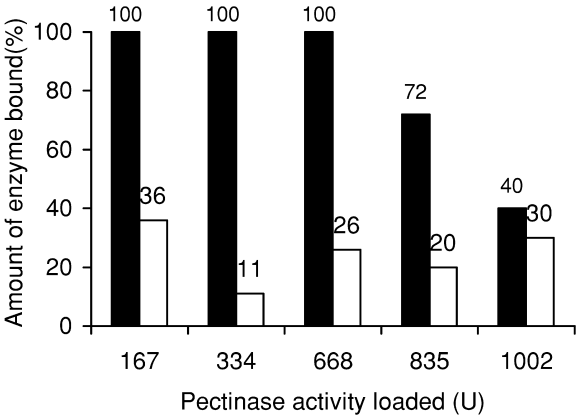

Fig. 1. MLFTPP of alginate with different pectinase units. Varying enzyme units of pectinase were subjected to MLFTPP with alginate under the conditions specified in Section 2. The difference between the total enzyme in the crude preparation and that in the aqueous phase represented the amount of enzyme bound to the polymer, shown as percentage enzyme activity bound (■) and percentage protein bound (□).

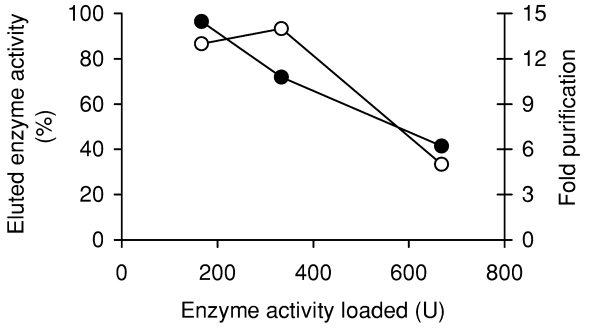

Fig. 2. Elution of pectinase from alginate with different starting enzyme unit loads. Varying enzyme activity units were subjected to MLFTPP with alginate (as shown in Fig. 1). The bound enzyme activity was eluted with 1 *M* NaCl as described in Section 2. Eluted enzyme activity (●) and fold purification (○) were measured taking the initial enzyme activity loaded as 100%.

3.8, alginate and pectinase together float as precipitate. This process, similar to that described earlier with xylanase [6] and α-amylase [8] is called MLFTPP. Fig. 1 also shows the changes in extent of pectinase binding and total protein binding to alginate with varying amounts of starting pectinase activity present along with 1% (w/v) alginate solution. Up to 668 U, 100% enzyme binds to the polymer. The selectivity at the binding stage, however, varies within the 167–668 U range and enzyme molecules seem to be most selectively picked up at the middle value of 334 U. However, Fig. 2 shows that it was better to work with a lower range of around 167 U since greater activity (96%) could be eluted (as compared to 72% when 334 U was the starting enzyme amount). The fold purification was also nearly identical whether one started with 167 or 334 U. Thus the selectivity at the elution stage operates in a different way as compared to selectivity at the binding stage and in fact dictates the final outcome.

Table 1 gives detailed purification data for pectinase corresponding to the above steps. Thus, 96% activity could be recovered with 13-fold purification. The preparation showed a single band on SDS–PAGE (Fig. 3). The molecular mass of 48 000 observed on the SDS–PAGE agrees with the earlier reported value [20].

After separation of pectinase, the protein solution was again subjected to the process of MLFTPP but with chitosan. It was found that starting with cellulase activity in the range of 9–54 U along with 4 ml of 0.2% (w/v) of chitosan, 97–98% of enzyme activity appeared in the interfacial precipitate during MLFTPP. The conditions used for floating chitosan

Table 1
Sequential MLFTPP of pectinase and cellulase by using alginate and chitosan

Steps	Activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude (pectinase activity)	167	0.55	304	100	1
Aqueous phase-1	0	0.30	–	–	–
Washing of interfacial precipitate-1 (0.05 *M* acetate buffer, pH 5.0)	0.20	0.05	4	–	–
Eluted pectinase activity (1 *M* NaCl in 0.05 *M* acetate buffer, pH 5.0)	161	0.04	4025	96	13
Aqueous phase-1 (cellulase activity)	54	0.30	180	100	–
Aqueous phase-2	1.6	0. 25	6.4	3	–
Washing interfacial precipitate-2 (0.05 *M* acetate buffer, pH 4.8)	0.8	0.03	27	1.5	
Eluted cellulase activity (1 *M* phosphate buffer, pH 7.0)	50	0.01	5000	92	16

Pectinex Ultra SP-L (containing 167 U pectinase activity and 54 U of cellulase activity) was added to 1 ml of alginate (final concentration 1%, w/v). MLFTPP was carried out under the conditions described in Section 2. Three phases formed were collected after incubating this solution at 37 °C for 1 h. The lower aqueous layer (aqueous layer-1) was then subjected to MLFTPP by adding chitosan (0.2%, w/v, final concentration) followed by addition of 45% (w/v) ammonium sulfate and *tert*.-butanol in a ratio of 1:1 (v/v). The bound and eluted pectinase and cellulase activity were estimated according to the procedure given in Section 2. The activity initially added (for both pectinase and cellulase) was taken as 100%.

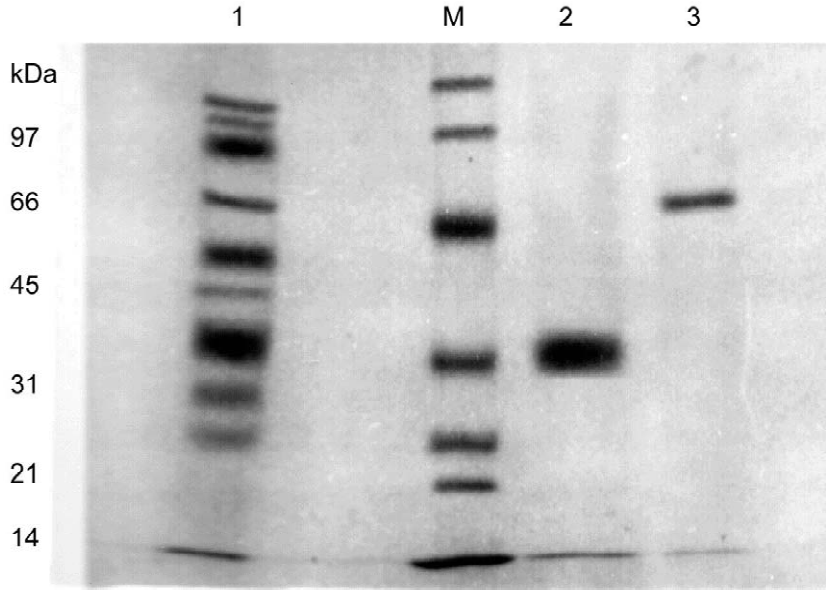

Fig. 3. SDS–PAGE of purified pectinase and cellulase. Lanes M, marker proteins; 1, crude Pectinex Ultra SP-L (20 μg); 2, purified cellulase (20 μg); 3, purified pectinase. The gel was stained with Coomassie Brilliant blue R-250 for 60 min and then destained in a solution consisting of 40% (v/v) methanol and 10% (v/v) acetic acid.

were the same as found to be optimal in earlier work [4]. Table 1 shows the data corresponding to 54 U of starting activity; 92% cellulase activity could be recovered with 16-fold purification. Purified cellulase showed a single band on SDS–PAGE (Fig. 3). The observed molecular mass of 31 000 agrees with the earlier reported value [21].

The above results illustrate that a sequence of MLFTPP with polymers of different selectivity can in principle separate all key biological activities present in a crude broth. Earlier work with affinity precipitation in quite a few cases has shown that it is possible to design a water-soluble polymer of a specific selectivity by incorporating a suitable affinity in the polymer [22,23]. Thus, the strategy outlined above is quite generic in application and is not limited by the fortuitous availability of a water-soluble polymer with inherent affinity towards a chosen target protein.

It should be added that one may have to be cautious while deciding about the order in the sequence of MLFTPP. For example, in this work, it is necessary to first remove pectinase before MLFTPP with chitosan is carried out. This is because chitosan shows some limited binding for pectinase as well. Cellulase on the other hand does not bind to alginate (data not shown).

It has been mentioned in the Introduction that TPP sometime enhances the catalytic efficiency of an enzyme. Table 2 summarizes the V_{max}/K_m value of pectinase purified by affinity precipitation, TPP and MLFTPP. The purification by affinity precipitation and TPP was carried out as reported earlier [11,17]. However, the kinetic data have been gathered as a part of this work only. It is interesting to observe that TPP enhances V_{max}/K_m in a significant way. It largely originates in the better K_m observed with the TPP-purified preparation. X-ray studies with proteinase K have shown that increased access to the active site was observed after subjecting that enzyme to TPP [12]. Thus, the decrease in K_m value of pectinase is not entirely unexpected. It is also interesting to observe that subjecting the same population of pectinase molecules to TPP when bound to alginate (i.e. during MLFTPP) did not significantly alter the V_{max}/K_m value. Thus, binding to the polymer prevents structural changes when the enzyme undergoes TPP.

Similar data with cellulase were also obtained (Table 3). In this case, TPP itself did not result in

Table 2
Determination of kinetic parameters for pectinase purified by different methods. K_m and V_{max} values for pectinase purified by TPP, MLFTPP and affinity precipitation were determined as given in Section 2

Kinetic parameters	Crude	TPP	MLFTPP	Affinity precipitation
K_m (mg/ml)	0.90	0.50	0.75	0.86
V_{max} (mmol/h nmol enzyme^{-1})	2.57	2.55	2.41	2.47
V_{max}/K_m	2.85	5.10	3.21	2.87

Table 3
Determination of kinetic parameters for cellulase purified by different methods. K_m and V_{max} values for cellulase purified by TPP, MLFTPP and affinity precipitation were determined as given in Section 2

Kinetic parameters	Crude	TPP	MLFTPP	Affinity precipitation
K_m (mg/ml)	41	36	38	40
V_{max} (mmol/h nmol enzyme^{-1})	37.20	37.64	50.23	50.31
V_{max}/K_m	0.91	1.06	1.32	1.26

any significant change in the V_{max}/K_m value. So cellulase purified by any one of the three methods showed similar V_{max}/K_m values. However, it may be pointed out that yields with MLFTPP (92%) are higher than observed with either affinity precipitation (81%) or TPP (76%). MLFTPP also gave better fold purification, i.e. 16-fold as compared to ninefold purification obtained by affinity precipitation or fivefold with TPP.

To conclude, the present work indicates that a series of suitable MLFTPP steps can directly recover enzymes/proteins present in a crude broth. Both, alginate and chitosan, used in the present work, are inexpensive food grade polymers. Thus, pectinase and cellulase purified here can be used even for food processing applications. Being non-chromatographic, the approach, potentially, is easy to scale up. As it is an affinity-based approach with reasonably high selectivity, the purity of protein preparations obtained with its use should be adequate for most applications.

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