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Research Paper

COUPLING OF TWO PURIFICATION METHODS: AMMONIUM SULPHATE PRECIPITATION AND AQUEOUS TWO PHASE SYSTEMS

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Aqueous two phase systems are specific and economic to be used for enzyme purification before high cost techniques such as chromatography. Using a commercially viable enzyme, horse radish peroxidase, as a model enzyme, a scheme was chalked out for its purification from crude extracts of horse radish roots employing precipitation and aqueous two phase systems, comprising polyethylene glycol (PEG) and ammonium sulphate. Based on results of the precipitation, two polyethylene glycol/ammonium sulphate systems were selected and using binodal curve diagrams, three compositions each of the two systems, were investigated. The enzyme showed preference for the top phase when PEG 400 was used whereas with PEG - 1500, no definite trend was followed. In one of the compositions of PEG 1500/ammonium sulphate, a greater partition of the enzyme into the top phase, with restoration and enhancement of enzyme activity was observed. A purification fold of 34 was obtained with this coupled purification method.

Keywords: Horse radish peroxidase, Polyethylene glycol, Ammonium sulphate, Purification

INTRODUCTION

In bioprocessing, the major challenge is to reduce the number of steps required to achieve a purified biomolecule. Chromatographic techniques though selective and efficient are not cost effective when introduced very early in the purification scheme. Chromatography is best suited where there are smaller volumes to deal with and when there are fewer contaminants to get rid of. Prior to chromatography several other methods may be employed such as precipitation, crystallization, flocculation, membrane separations, liquid-liquid extractions (Rosa *et al*., 2010). These methods mentioned are not target selective unless affinity ligands are involved which will contribute to the expense in the process.

Aqueous Two Phase Systems (ATPS) involving polymer/salt or polymer/polymer systems are unique as they can be target specific with or without the use of affinity tools. Separation of the biomolecule in these systems depends not only on its inherent physico-chemical properties (charge, molecular weight, hydrophobicity, conformation) but also on properties of system

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such as molecular weight of polymer, concentration and type of polymer, presence and concentration of ions, pH and ionic strength (Rito-Palomares, 2004; Aguilar and Rito-Palomares, 2010). Besides these factors, the partitioning of the biomolecule will also depend on its interaction with polymers comprising the ATPS.

ATPS involving polymer/salt systems are beneficial over polymer/polymer systems since they are low on cost and are easier to manage on the processing front. Polyethylene glycol (PEG), one of the commonly used polymers has the additional advantage of enhancing the refolding of complex molecules like proteins and enhancing their activity (Raja *et al.*, 2011). PEG is also low on toxicity and volatility and is biodegradable making it an attractive, environment friendly option to exploit for bioseparations (Chen *et al.*, 2005).

In this paper we will discuss the separation of the plant enzyme, Horse Radish Peroxidase (HRP) from the roots of horse radish (*Armoracia rusticana*). The paper investigates two nonchromatographic methods for purification used in tandem, ammonium sulphate precipitation and ATPS.

MATERIALS AND METHODS

Roots of horse radish were purchased from the local market. Ammonium sulphate, copper sulphate, Folin Ciocalteau reagent, o-dianisidine, PEG 1500, PEG 400 were purchased from SD. Fine Chemicals, India. Sodium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Merck India. Potassium tartarate was purchased from Fischer Scientific India.

Preparation of Crude Extract

Horse radish roots were washed with distilled water, and sliced into small pieces and homogenized in 0.1 M phosphate buffer pH 7 under ice cold conditions. The homogenate was centrifuged at 5000 g for 15 min at 4^{o}C and the supernatant was collected and stored at -20^{o}C.

Analysis of Protein Content and Enzyme Assays

Protein estimation was by Folin Lowry Method (Waterborg, 2002). HRP was assayed in 2 mL of test solution/extract by monitoring the change in absorbance at 436 nm after 10 min (25^{o}C) on addition of 1 mL substrate mixture comprising 0.5% o-anisidine (50 µL), 0.1 M hydrogen peroxide (100 µL), 1M phosphate buffer pH 7.0 (850 µL) (Lascu *et al*., 1986).

Ammonium Sulphate Precipitation

Precipitation of proteins was carried out by addition of solid ammonium sulphate to the homogenized root extract to achieve cumulative saturations of 20%, 40%, 60%, 80%, 100% and 120%, as given in Scopes (Scope, 1993) and Burgess (Burgess, 2009). A precipitate and supernatant was obtained with each concentration of ammonium sulphate after centrifugation at 4000 g for 10 min at 4^{o}C. The supernatants and their precipitates that were individually reconstituted with 1 mL of 0.1 M phosphate buffer were analyzed for protein and enzyme content.

Aqueous Two Phase Systems

Two systems of ATPS were investigated for each enzyme. For purification of HRP, PEG/ ammonium sulphate systems were investigated since they were to be employed after the ammonium sulphate precipitation step. Binodal curves were prepared for PEG 400/ammonium

sulphate, PEG1500/ammonium sulphate, at 25°C by cloud point method according to Kaul (Kaul, 2000). For PEG/ammonium sulphate systems, 50% w/w PEG 400/50% w/w PEG 1500 and 40% w/w ammonium sulphate in 0.1 M phosphate buffer pH 7 was used. From the binodal curves obtained three system compositions were used for separation of the enzyme. The volume of the top phase and bottom phase were noted and the two phases were analyzed for total protein content and enzyme content.

The partition coefficient was calculated as $K = A_t / A_b$

where A_t and A_b denote the enzyme activity in the top phase and bottom phase respectively (Hamid and Eskander, 2008).

RESULTS AND DISCUSSION

Ammonium Sulphate Precipitation

Ammonium sulphate precipitation of proteins depends on their inherent hydrophobicity and molecular size. An estimate of the hydrophobic character of a protein may be done by assessing their ability to precipitate in presence of different concentrations of ammonium sulphate (Hagarova and Brier, 1995). The crude extract of horse radish peroxidase was subjected to ammonium sulphate precipitation, the results are shown in Table 1.

Ammonium sulphate concentration in 20 mL of crude enzyme extract was steadily increased from 0% saturation to 120% saturation. At 20% ammonium sulphate saturation 18.6 mg of protein was found in the pellet, with the enzyme present predominantly in the supernatant fraction. As the salt content in the extract was steadily increased, an increase in precipitation of non-enzymic proteins was observed as shown in Table 1. The HRP enzyme could be precipitated only at 120% saturation of ammonium sulphate. Although horse radish peroxidase could be precipitated only at 120% saturation, at 80% ammonium sulphate saturation a cumulative of 43.6 mg of protein accounting for 88% of the initial total protein was precipitated. The loss of protein from the supernatant at 80% ammonium sulphate saturation lead to negative purification of the enzyme resulting in a 14 fold increase in specific activity from 0.72 U/mg to 10.28 U/mg.

Table 1: Ammonium Sulphate Precipitation of Horse Radish Peroxidase from Crude Extract of Horse Radish Roots

Percent Saturation	Protein in Precipitate	Enzyme in Precipitate	Specific Activity	Protein in Supernatant	Enzyme in Supernatant	Specific Activity
0%				49.4 mg (20)	37.62 U	0.72 U/mg
20%	18.6 mg (1)	ND	NA	30.6 mg (23)	37.84U	1.23 U/mg
40%	7.2 mg (1)	ND	NA	22.4 mg (23)	37.52U	1.67 U/mg
60%	5.9 mg (1)	ND	NA	15.4 mg (23)	36.8 U	2.38 U/mg
80%	**12.2 mg (1)**	**ND**	**NA**	**3.42mg (23)**	**35.19 U**	**10.28 U/mg**
100%	1.5mg (1)	ND	NA	2.04 mg (23)	34.27 U	16.79 U/mg
120%	0.7 mg (1)	23.94U	34.2 U/mg	1.58mg (23)	7.4 U	4.68 U/mg

Note: Given in parenthesis are the volumes in milliliter of the precipitate and supernatant fractions. ND: Not detected NA: Not Applicable

The results of the ammonium sulphate purification indicated that horse radish peroxidase is a hydrophilic enzyme since the enzyme could be precipitated at only at high concentrations of ammonium sulphate. This was confirmed with the information received from the protein database wherein the sequence of horseradish peroxidase (GenBank: CAA00083.1) was investigated for its hydrophobicity. The enzyme of molecular weight 34KDa was found to have a negative GRAVY (Kyte and Doolittle, 1982) (Grand average of hydropathicity) value of –0.177 indicating that the enzyme is indeed hydrophilic. The amino acid composition also showed that 105 amino acids out of 309 amino acids were charged or polar amino acids (R (21), K(6), D(21), E (7), S(25), T(25)). The analysis was carried out using the ProtParam tool in the ExPASY Bioinformatics Resource Portal (http://web.expasy.org/protparam/).

Polyethylene glycol is a polymer that has partial hydrophobic character and in ATPS systems, it is enriched in the top phase. Lowering the polymerization of PEG results in reduced molecular weight of PEG thereby reducing hydrophobicity of the PEG rich top phase (Prodanovic and Antov, 2008; Forciniti *et al*., 1991). The partitioning of a given protein into the top phase depends on polymer-protein hydrophobic interactions attributed to both molecular weight of PEG and surface hydrophobicity of the protein (Ramyadevi *et al.*, 2012). The behavior of a target protein in ammonium sulphate precipitation protocols gives an idea of the hydrophobic nature of protein (Hachem *et al.,* 1996). Since one of the factors that determine separation of proteins in ATPS systems is dependent on the molecular weight of target protein and its hydrophobic nature (Asenjo *et al*., 1994), the precipitation experiment gives an idea about the suitability of PEG molecular weight to be used in the ATPS, for separation and purification of the protein. Horse radish peroxidase, an enzyme of low molecular weight has a high degree of hydration as reflected by its precipitation behavior in ammonium sulphate solutions; therefore a lower molecular weight PEG if used for ATPS may be suitable for extraction of the enzyme into the PEG rich top phase in presence of high concentrations of salt. PEG 400 and PEG 1500 were therefore used in experiments to purify this enzyme. To increase the purification of the enzyme, ATPS was coupled to ammonium sulphate purification, i.e., the 80% ammonium sulphate saturation supernatant of the crude root extract was used as source of the enzyme in the ATPS experiments.

Aqueous Two Phase Systems

Binodal curves were prepared for the following systems, PEG 400/ammonium sulphate and PEG 1500/ammonium sulphate. To simulate conditions of the 80% saturation supernatant, binodal curves were prepared using 40% w/w ammonium sulphate and 50% w/w PEG. The binodal curves are shown in Figure 1A and B.

Based on the binodal curves three different compositions of PEG400/ammonium sulphate and PEG 1500/ammonium sulphate were used to further purify horse radish peroxidase from the 80% ammonium sulphate saturated supernatant. The results are tabulated in Table 2. The results of the ATPS purification (Table 2) show favorable partition of enzyme in the top phase of PEG-400 systems. The composition found optimum was of 30% w/w PEG-400/21% w/w ammonium

Figure 1: Binodal Curves of PEG/Ammonium Sulphate Systems Prepared by Cloud-point Method A) PEG 400-Ammonium Sulphate B) PEG 1500-Ammonium Sulphate

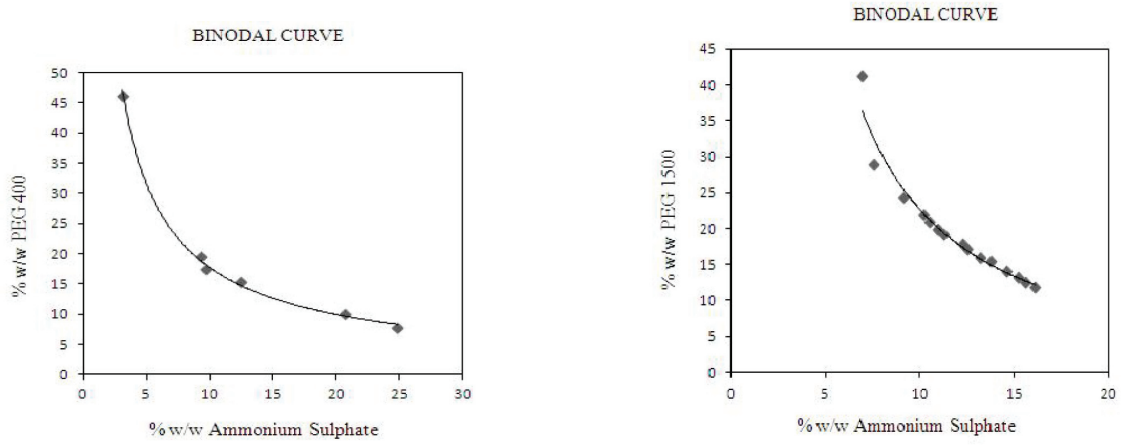

Table 2: Compositions of Aqueous Two Phase Systems for Purification of Horse Radish Peroxidase

System	Concentrations %w/w PEG/%w/w Ammonium Sulphate	Protein (Top phase) mg	Enzyme (Top Phase) Units	Specific Activity (Top Phase) U/mg	Protein (Bottom Phase) mg	Enzyme (Bottom Phase) Units	Specific Activity (Bottom Phase) U/mg	Partition Coefficient K (Enzyme)
PEG	20/25	0.39	2.3(10)	5.89	0.176	0.88(8)	5	2.61
400/	30/21	**0.286**	**4 (16)**	**13.98**	**0.314**	**0.54 (2)**	**1.719**	**7.4**
ammonium	50/15	0.317	2.21(17)	6.917	0.17	1.25(2)	7.35	1.768
sulphate								
PEG	15/16	0.11	0.1(12)	0.9	0.431	3.30(7)	7.65	0.030
1500/	20/13	0.338	0.42 (14)	1.242	0.21	1.82 (6)	8.667	0.230
ammonium	30/10	**0.5**	**12.49(17)**	**24.98**	**0.12**	**0.73(3)**	**6.083**	**17.10**
Sulphate								

Note: Total weight of the aqueous two phase systems =20g given in parenthesis are the volume in milliliter of the top phase and bottom phase fractions.

Table 3: Final Results of Purification Scheme from Horse Radish Roots

Purification Step Specific	Activity U/mg	Purification fold
Crude horse radish root extract (homogenization)	0.72	–
80% ammonium sulphate saturation	10.48	14.55
30% w/w PEG 1500/10% w/w ammonium sulphate	24.98	34.69

sulphate which purified the enzyme by 4.5 fold (13.98 U/mg Vs. loaded 3.12 U/mg). For PEG 1500 systems, the enzyme did not always favor the PEG rich phase. Only when the volume of the PEG 1500 phase was 5.6 times higher than that of the bottom salt rich phase, did the enzyme partition into the top phase, implicating solubility of the enzyme was playing a role. This was observed at composition of 30% w/w PEG 1500/ 10% w/w ammonium sulphate indicated by a sharp increase in the partition coefficient of the enzyme and a purification fold of 8 (24.98 U/mg vs. loaded 3.12 U/mg). Since the objective of enzyme purification was satisfied, other compositions of PEG 1500/ammonium sulphate were not investigated. We also observe an increase in enzyme activity in the PEG phase observed for both the PEG 400/ammonium sulphate and PEG 1500/ammonium sulphate highlighted compositions (Table 2). PEG 1500 probably enhances the refolding and hydration of the enzyme reflected by the marked increase in activity, 12.49U vs. loaded 1.83 U. Enhancement of the activity of an enzyme in presence of PEG has been reported elsewhere (Kucera and Paulus, 1988) in another context. Refolding of denatured protein in PEG has also been previously reported (Cleland *et al.*, 1992).

CONCLUSION

Aqueous two phase systems have been used previously for purification of horse radish peroxidase from crude root extracts of *Armoracia rusticana*. The system used was 10% w/w PEG 1540, phosphate (pH 7) 14.8 % w/w, NaCl 8 % w/ w. The purification factor obtained was 4.8 with 90.8% recovery of the enzyme in the top phase (Miranda *et al*., 1995). In this paper an aqueous two phase protocol to be complemented with ammonium sulphate precipitation for HRP purification from roots of *Armoracia rusticana,* was investigated. On basis of the recovered enzyme activity and protein content we could obtain around 34 fold purification of the enzyme from the crude horse radish root extract (Table 3).

Ammonium sulphate precipitation studies may enable a researcher to choose a suitable PEG polymer to be employed for purification of the target protein by ATPS. The partitioning of protein in PEG is attractive since the polymer can enhance the folding of the protein which may be compromised due to high concentrations of salt. The ATPS experiments illustrate that the behavior of a protein in an aqueous two phase polymer-salt systems is unpredictable and seems to depend on the complex interplay of, volume of the two phases, solubility of proteins in the two phases, concentration and type of salt, concentration and molecular weight of the polymer, hydropathicity of the protein.

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