Three phase partitioning as a large-scale separation method for purification of a wheat germ bifunctional protease/amylase inhibitor

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Abstract

The technique of three phase partitioning (TPP) was used to purify a wheat germ bifunctional protease/amylase inhibitor. TPP is a relatively recent technique, which uses a combination of ammonium sulphate and *t*-butanol to precipitate proteins from crude extracts. The precipitated protein forms interface between the lower aqueous layer and the upper organic layer. The optimization of conditions for achieving efficient purification involved studying the effects of varying temperature, ammonium sulphate concentration and the amount of t-butanol. The ratio of t-butanol to crude extract of 1:1, temperature of 20 °C and 30% ammonium sulphate (w/v) gave best results. The single step of TPP led to 25-fold purification with an activity recovery of 85%. The sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis of enzyme showed considerable purification and its molecular weight was found to be 21 kDa. The results are compared with those obtained with expanded bed affinity chromatography of the same system.

Keywords: Three phase partitioning; Bifunctional protease/amylase inhibitor; *t*-Butanol

1. Introduction

Wheat germ is reported to contain three protease inhibitors namely PKI-1, PKI-2 and PKI-3. These inhibitors are single chain proteins with molecular weights of 11, 8 and 21 kDa, respectively. All these inhibitors are specific against proteases of the subtilisin family [1]. At higher concentration, PKI-1 and -2 also show limited inhibition of tryptic and chymotryptic activities. PKI-3, is infact, a bifunctional inhibitor and also inhibits alpha amylases from wheat and several insects [1].

Apart from the well known involvement of protease inhibitors in plant defence mechanism [2,3] and therapeutic application [4], these inhibitors are valuable affinity ligands for bioseparation of industrially important proteases and amylases [5,6]. Lately, affinity-based processes such as affinity precipitation [5,7] and expanded bed affinity chromatography [6,8] have been successfully used for large-scale purification of enzymes. Considering these applications, it is worthwhile to develop scalable and efficient purification procedures for the inhibitors. These inhibitors are generally purified by multi-steps protocols [3,9].

In this work, we have employed three phase partitioning (TPP) [10] for direct one step purification of protein inhibitor from wheat germ. TPP is an emerging bioseparation technique [10–14], which employs collective operation of principles involved in numerous techniques such as conventional salting out, isoionic precipitation, cosolvent precipitation and osmolytic and kosmotropic precipitation of proteins. It is easily scalable and can be used directly with crude suspensions. While the cost is only slightly higher than simple 'salting out' approach, fold purification is generally found to be much higher [10]. In this approach, a protein is precipitated as an interface between organic and aqueous phases, when an organic solvent (generally *t*-butanol) is added to the crude extract in the presence

of moderate concentrations of salt (generally ammonium sulphate) [14].

2. Materials and methods

α-Amylases (from porcine pancreas) and wheat germ were purchased from Sigma Chemical Co., St. Louis, MO. Starch was a product of E. Merck, India. All other chemicals used were of analytical grade.

2.1. Estimation of enzyme and enzyme inhibitory activities and amount of protein

Activity of α-amylases was estimated using starch as the substrate [15]. One enzyme unit liberates 1 μmole of reducing sugar (calculated as maltose) per min at 37 °C (for the enzyme from porcine pancreas) [15] and at 25 °C (for the enzyme from wheat germ) [16]. One unit of amylase inhibitor is defined as that amount, which inhibits the activity of porcine pancreatic α-amylase by 1 U under the assay conditions [17].

Protein content was estimated by the dye-binding method using bovine serum albumin as the standard protein [18].

2.2. Preparation of crude extract of wheat germ and removal of endogenous α-amylase activity

The crude extract of wheat germ was prepared according to O'Donnell and McGeeney [19]. Fifty grams of wheat germ was suspended in 150 ml of distilled water, stirred for 1 h at room temperature and centrifuged at $12{,}000 \times g$ for 20 min. The residue was resuspended in distilled water, stirred for 30 min at room temperature and centrifuged as before. The supernatants obtained in the two cases were pooled and lyophilized. Three grams of the lyophilized powder was dissolved in 30 ml of 0.02 M Tris–HCl + 0.25 M NaCl, pH 7.0 and heated for 30 min at 70 °C. After centrifugation ($12{,}000 \times g$ 20 min), the supernatant was diluted 100 times with the above buffer and heated for 60 min at 70 °C. The precipitate (after centrifugation) obtained was discarded. The supernatant obtained by this procedure is reported to be free of endogenous α-amylase activity [16] and was used further for TPP.

2.3. Purification of wheat germ inhibitor by three phase partitioning

The crude extract of wheat germ after removal of endogenous α-amylase activity (1 ml containing appropriate amount of α-amylase inhibitor activity, pH 7.0) was saturated with ammonium sulphate to the desired level at the specified temperature, vortexed gently to dissolve the salt followed by the addition of *t*-butanol. After 1 h, the mixture was centrifuged ($2000 \times g$ for 10 min) to facilitate separation of phases. The lower aqueous layer and the interfacial precipitate were collected, the latter was dissolved in 1 ml of distilled water pH 7.0. Both of these were assayed for amylase inhibitor activity. The activity of the crude extract initially added (25 U) was taken as 100%.

All the experiments were run in duplicate and the difference in the readings in duplicates was less than ± 5%.

2.4. Polyacrylamide gel electrophoresis

SDS-PAGE of the protein samples using 15% gel was performed according to Hames [20] on a Genei gel electrophoresis unit (Bangalore Genei Pvt. Ltd., Bangalore, India) with standard molecular weight markers (Sigma Chemical Co.)

3. Results and discussion

There is a reciprocal relationship between the amounts of *t*-butanol and ammonium sulphate required to precipitate the protein in TPP [21]. Fig. 1A shows that the ratio to crude extract of wheat germ amylase inhibitor to t-butanol is important and 1:1 ratio gives the maximum purification of 24 fold. This optimum ratio presumably arises as a result of two factors. If the amount of t-butanol is less, it does not adequately synergize with ammonium sulphate. If it is higher, it is likely to cause protein denaturation. As TPP operates through multiple effects, which includes conformational tightening and changes in protein hydration, it was considered worthwhile to explore the effect of varying process temperature (Fig. 1B). The temperature of 20 °C was found to be the best for achieving greatest fold purification and for obtaining maximum yield. It is difficult to speculate about the reasons for 20–30 °C to be better range for carrying out TPP in view of complexity of the factors involved.

Fig. 1C illustrates the critical role played by the amount of ammonium sulphate present in aqueous phase during TPP for recovery of inhibitor in the process. Sulphate anion is an excellent kosmotrope [14]. It binds to cationic sites in the protein [10]. As TPP exploits the synergistic effect of both sulphate and *t*-butanol, the sulphate ion concentration used is much lower than required for 'salting out' of the protein. The optimum concentration of 30% (w/v) determined here seems to be, in general, the best concentration to operate TPP with various system. Pike and Dennison [21] and Lovrein et al., [10] have also reported that 30% (w/v) is the best salt concentration for carrying out TPP efficiently.

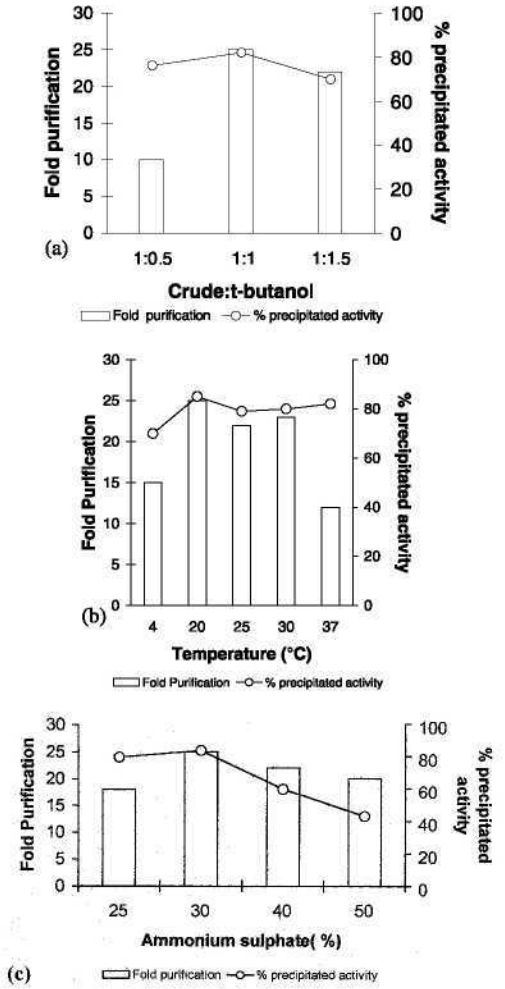

Fig. 1. (A) Effect of varying ratio of crude extract to tertiary butanol on the recovery of wheat germ inhibitor activity. The crude extract (1 ml containing 26 U) was brought to 30% (w/v) ammonium sulphate saturation (w/v). Varying amount of *t*-butanol was added to this in the following volumetric ratios of crude extract to t-butanol viz. 1:0.5, 1:1 and 1:1.5. The mixtures were then incubated at 20 °C. (B) Effect of temperature on the recovery of crude extract of wheat germ inhibitor. The crude extract (1 ml containing 26 U) was brought to 30% (w/v) ammonium sulphate saturation (w/v) followed by the addition of 1 ml of *t*-butanol. The mixture was kept at different temperatures 1 h for three phase formation. (C) Effect of ammonium sulphate concentration on the recovery of wheat germ after TPP. Different amount of ammonium sulphate (25, 30, 40 and 50% w/v) were added to 1 ml of crude wheat germ amylase inhibitor (containing 24.5 U activity) keeping the volume of t-butanol constant (1 ml). The interfacial precipitate formed was collected after keeping these systems at 20 °C.

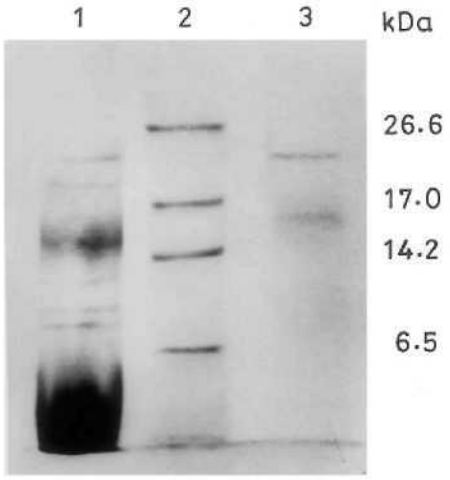

Fig. 2. SDS-PAGE pattern of purified wheat germ amylase inhibitor. Lane 1: crude wheat germ extract; (15 μg); Lane 2: marker proteins (15 μg); Lane 3: purified wheat germ amylase inhibitor (25 μg).

Thus, ratio of *t*-butanol to crude extract of 1:1, temperature of 20 °C and 30% ammonium sulphate (w/v) give the recovery of about 85% and 25-fold purification. These results are summarized in a purification table (Table 1).

SDS-PAGE analysis (Fig. 2) reflects the considerable purification achieved in the single step process. The main band at 21 kDa agrees well with the reported molecular weight for PKI-3 [1]. The purified inhibitor was also found to inhibited caseinolytic activity of proteinase K (data not shown), which is in agreement with the reported bifunctional nature of PKI-3.

It may be interesting to compare this approach with that of immobilized metal affinity chromatography, which was reported earlier for purification of PKI-3 [16]. The bifunctional inhibitor PKI-3 also bound to Cu (II)–Streamline™-chelating resin and could be eluted with imidazole. Both packed and expanded bed gave similar activity yield of around 83% and 23-fold purification. However, the product purified by immobilized metal affinity chromatography was found to be a single band on SDS-PAGE. This apparent discrepancy (viz. the purified preparation described in the present work with similar fold purification showing a minor impurity) may be explained as follows: It has been reported by Dennison and Lovrein that *B. subtilis* protease, *S. cerevisiae* invertase, *C. cylindracea* lipase gave a yield of

Table 1
Purification of amylase inhibitor from wheat germ by subjecting to TPP

Step	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	25.9	0.56	46	1	100
After TPP	22	0.02	1157	25	85

The crude extract of wheat germ inhibitor (1 ml containing 25.9 U) was saturated with 30% (w/v) ammonium sulphate followed by addition of *t*-butanol (1 ml).

300, 100 and 900%, respectively after TPP [14]. These unusual percentage yields were believed to be due to removal of inhibitors during purification. However, recent work from our laboratory with proteinase K using X-ray diffraction [13] shows that TPP results in a number of amino acid residues adopting more than one conformation. The increased flexibility of the molecule probably is mostly responsible for higher activity observed with TPP treated proteinase K. Thus, TPP, at least in some cases results in enhancing the activity of the enzymes. Hence any fold purification obtained using TPP may result from two factors (a) actual purification i.e. removal of contaminating proteins (b) activation of the enzyme molecules. This may be the reason that purified protein obtained here with similar fold purification as obtained by immobilized metal affinity chromatography is slightly more impure.

This, scalable single step protocol may be valuable for isolating other protease and bifunctional inhibitors as well in gram amounts for use as macroaffinity ligands for separation of proteases and amylases [5].

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