USE OF TRITON X-114 AQUEOUS TWO PHASE SYSTEM FOR RECOVERY OF MUSHROOM (*AGARICUS BISPORUS)* POLYPHENOLOXIDASE

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ABSTRACT

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Mushroom (*Agaricus bisporus*) polyphenoloxidase (PPO) (EC 1.14.18.1) was isolated and purified using an aqueous two phase system composed of octyl phenol (ethyleneglycol) $_{7\text{-}8}$ ether (Triton X-114/TX-114). TX-114 is a non-ionic surfactant which thermoseparates in water and forms an aqueous two phase system with a surfactant-depleted top phase and a surfactant-enriched bottom phase. Critical

micelle concentration (CMC) and cloud point of the surfactant are 0.17 mM and 22ºC respectively. The partitioning behavior of mushroom PPO in water/TX-114 aqueous two-phase systems was studied and the effects of TX-114 concentration, ionic strength, pH, temperature and crude extract preparation on PPO partitioning were studied. PPO generally partitioned to the surfactant-depleted top phase; whereas many other hydrophobic proteins and molecules partitioned to the surfactant-enriched bottom phase. When two-step ultrafiltration was used as a pretreatment, complete enzyme recovery was achieved with all studied TX-114 concentrations. Moreover, about 5 fold purification was achieved by using 8% TX-114. The purification increased to 10 fold by using polyvinylpolypyrolidone (PVPP) at pH 7.0 with a recovery of 72%. However changing pH from 7.0 to 6.0 increased the purification factor and enzyme recovery to 15 fold and 100%, respectively. Addition of potassium or sodium salts caused PPO molecules to partition in the surfactant-enriched bottom phase. Finally, crude enzyme can be concentrated besides being purified and recovered by doing aqueous two-phase separation at room temperature.

Keywords: Aqueous Two Phase Separation, Polyphenoloxidase, Triton X-114, Thermoseparation, Purification.

ÖZ

TRITON X-114 SULU İKİ-FAZ YÖNTEMİNİN MANTARDAN ELDE EDİLEN POLİFENOLOKSİDAZ'IN ELDESİ İÇİN KULLANIMI

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Mantardan (*Agaricus bisporus*) elde edilen polifenoloksidaz (PPO) (EC 1.14.18.1), oktil fenol (etilenglikol) $_{7\text{-}8}$ eter (Triton X-114/TX-114) içeren sulu iki faz sistemi kullanılarak ayrıştırılmıştır. TX-114, suda sıcaklığa bağlı deterjanca zengin alt faz ile deterjanca fakir üst faz olarak sulu iki faz sistemi oluşturan anyonik bir deterjandır. Kritik misel derişimi (CMC) ve bulut noktası sırasıyla 0.17 mM ve 22ºC'dir. Mantar

PPO'ının TX-114 sulu iki faz sistemlerindeki ayrılma davranışı çalışılmıştır ve TX-114 konsantrasyonu, ortamın iyonik gerilimi, pH, sıcaklık, ekstrakt hazırlanışı gibi parametrelerin PPO'nun ayrılmasındaki etkileri çalışılmıştır. PPO genellikle deterjanca fakir üst faza ayrılırken, istenmeyen çoğu hidrofobik protein ve fenolikler gibi diğer moleküller deterjanca zengin alt faza ayrılmıştır. Enzim hazırlanışı sırasında iki kademeli ultrafiltrasyon uygulandığı zaman, TX-114 ile çalışılan tüm konsantrasyonlarda, enzimlerin tümünün üst fazda korunumu sağlanmıştır. Ayrıca, 8% TX-114 kullanıldığı zaman yaklaşık 5 kat saflaştırma elde edilmiştir. pH 7.0' de polivinilpolipirolidon (PVPP) kullanılarak saflaştırma 10 kat artmış ve %72 enzim geri kazanımı elde edilmiştir. Bununla beraber pH 6.0'da çalışıldığı zaman saflaştırma 15.5 kata ve geri kazanım %100'e yükselmiştir. Sodyum veya potasyum tuzlarını eklemek, PPO moleküllerinin deterjanca zengin alt faza ayrılmasına yol açmıştır. Son olarak, sulu iki faz sistemi oda sıcaklığında yapıldığında, enzim saflaştırma ve geri kazanımının yanı sıra konsantre edilmiştir.

Anahtar sözcükler: İki Fazlı Sulu Sistem, Polifenoloksidaz, TX-114, Termal ayrılma, Saflaştırma.

To my father Mustafa...

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ABBREVIATIONS

CR	Concentration ratio
ER	Enzyme recovery
PF	Purification factor
PPO	Polyphenoloxidase
PR	Phenolics removal
PVPP	Polyvinylpolypyrrolidone
SA	Specific activity
TA	Total activity

CHAPTER 1

INTRODUCTION

Partitioning in an aqueous two phase system is a well established method for separation and purification of biomaterials, including proteins, cells, organelles, and biological membranes (Walter et al., 1985; Albertsson, 1986). In general these systems are formed in mixtures between two incompatible polymers, e.g., dextran (Dx) and polyethylene glycol (PEG) or one polymer (e.g., PEG) in a high concentration of salt (e.g., phosphate). Two aqueous phases will be formed above certain concentrations of the two components. In a two-polymer system each phase is enriched in one of the polymers, while in a polymer-salt system one polymer-rich phase is in equilibrium with a salt-rich phase. The PEG/Dx systems are used for small scale separations of macromolecules, membranes, cell particles and cells (Albertsson, 1986; Walter and Johansson, 1994). The PEG/salt systems are mainly used in large scale extractions (Hustedt *et al*., 1985; Kula and Selber, 1999). Both kinds of systems are suitable for biological samples because each phase contains 70-90% water, which offers a mild environment for biomolecules.

Aqueous two-phase systems (ATPS) have advantages when compared with other separation and purification techniques like chromatography, filtration, centrifugation and electrophoresis. The aqueous two phase separation technique is especially suitable for primary recovery. In one unit operation it is possible to combine cell and debris separation, concentration and purification of the target protein. Another advantage of aqueous two phase systems is the simplicity of scaling up from laboratory scale to large scale extractions (Collen *et al*., 2001). Other advantages are volume reduction, high capacity, cost, and being a fast separation.

1.1 Factors Influencing Protein Partitioning

Proteins, long polymers of amino acids, constitute the largest fraction (besides water) of cells. Some proteins have catalytic activity and function as enzymes, some other serve as structural elements, but the others carry specific signals or specific substances into or out of cells. They are perhaps the most versatile of all biomolecules. Proteins have the most well-defined physicochemical properties and they are generally easier to characterize and isolate than the other biomolecules such as nucleic acids, polysaccharides or lipids.

Extensive information has been gained about factors governing the partitioning of a protein, such as type of system components, molecular weight of polymer, pH, type and concentration of ions included in the system. The properties of a protein determining its partitioning behavior are molecular weight and the surface exposure of different amino acid residues which, in turn, characterize the protein with respect to hydrophilicity/hydrophobicity and charge. However, relatively little is known about how these surface properties contribute quantitatively to the partitioning behavior in different systems (Berggren *et al*. 1999). Partitioning may also be influenced by temperature, pH, salt type and concentration, polymer type and molecular weight.

The partitioning of proteins in two-phase systems is described by the partition coefficient, K. It is defined as the concentration of the protein in the top phase, C_T, divided by the concentration in the bottom phase, C_B.

Formally, logarithm of the partition coefficient can be split into several terms:

$$\ln K = \ln K^{o} + \ln K_{el} + \ln K_{hfob} + \ln K_{biosp} + \ln K_{size} + \ln K_{conf}$$

where el, hfob, biosp, size, and conf stand for electrochemical, hydrophobic, biospecific, size, and conformational contributions to the partition coefficient and $\ln K^{o}$ includes other factors. The different factors are more or less independent of each other though none is probably completely independent of the others. For

example, when a hydrophobic group is introduced on one of the phase-forming polymers, both the distributions of ions and the electrical potential may slightly be introduced (Albertsson, 1986).

1.1.1 Temperature

There is a general tendency for the K values of proteins in Dx-PEG systems, when smaller than 1.0, to increase with temperature. This may be due to the changes both in the composition of the phases (since the difference in composition between the two phases will be diminished) and in the relative solvation of proteins. As compared with aqueous buffer solutions, proteins are usually much more resistant to denaturation in the presence of phase polymers. This makes protein partitioning at high temperatures possible (Bamberger *et al*., 1985).

1.1.2 pH

Protein partitioning depends, quite generally, on the pH of the system. The variation of the protein K value in a specific system and over a given pH range is influenced by the ionic composition. Altering the pH changes the net charge of the protein from positive at low pH to negative at high pH. At a certain pH the net

charge of the protein is zero. This is the isoelectric point (pI). A number of proteins partition independently of pH under such conditions (Johansson, 1985).

1.1.3 Salt type and concentration

The *K* values of proteins, away from their isoelectric points, can be adjusted by incorporation of selected salts in the system. For a negatively charged protein the partition coefficient is reduced in the following series: phosphate > sulfate > acetate > chloride > thiocyanate > perchlorate and lithium > ammonium > sodium > potassium. The salt effect can be increased by raising the pH, thereby making the protein's charge more negative. Positively charged proteins behave in the opposite way. The above rules apply with moderate salt concentrations, i.e., less than 250 mM. Higher salt concentrations can be used to cause proteins to partition more into the upper phase (Johansson, 1985).

1.1.4 Concentration and molecular weight of polymer

Generally if the polymer concentration is increased, that is, the composition of the phase system deviates more from the critical point, the partition of the proteins will be more one-sided, that is, partition coefficient (*K*) will either increase above 1 or decrease below 1 (Albertsson, 1986).

If, for a given phase composition, one polymer is replaced by the same type of polymer having a smaller molecular weight, partitioned molecules such as proteins will favor more the phase containing the polymer with decreased molecular weight (Albertsson, 1986).

1.1.5 Hydrophobic Interactions

The hydrophobic effect can be increased by covalent binding hydrophobic groups to one of the polymers. If a protein, for example, contains surfaces or pockets which bind hydrophobic groups, its partition coefficient will be changed. This so-called hydrophobic affinity partition can be used for characterizing the hydrophobic properties of proteins or cell particles and also for separating molecules or particles differing in hydrophobicity (Albertsson, 1986).

1.2 Mechanism of Partitioning

The mechanism governing partition is largely unknown. When a particle is suspended in a phase, it interacts with the surrounding molecules in a complicated manner. Various bonds, such as hydrogen, ionic and hydrophobic, are probably involved, together with other weak forces. Their relative contributions are difficult

to estimate; however, their net effect is likely to be different in the two phases. If the energy needed to move a particle from one phase to the other is ΔE, at equilibrium, a relation between the partition coefficient and ΔE can be expressed as

$$\frac{C_1}{C_2} = e^{\Delta E/ kT} \quad (1)$$

where C_1 and C_2 are the concentrations of particles in phases 1 and 2, k is the Boltzman constant and T is the absolute temperature. Obviously ΔE must depend on the size of the partitioned particle since the larger it is, the greater the number of atoms that are exposed and can interact with the surrounding phase. Brønsted suggested the following formula for partition:

$$\frac{C_1}{C_2} = e^{\lambda M/ kT} \quad (2)$$

where M is the molecular weight and λ is a factor which depends on properties other than molecular weight. For a spherical particle, M should be replaced by A, the surface area of the particle. Thus,

$$\frac{C_1}{C_2} = e^{\lambda A/ kT} \quad (3)$$

and λ in this case is a factor which depends on properties other than surface area. The surface properties should be expressed by the surface free energy per unit area (surface tension). Both size and surface properties are, therefore of great importance in determining partition.

It is also expected that the net charge Z of a particle to play a role. If there is an electrical potential difference $U_1 - U_2$ between the phases, an energy term $Z (U_1 - U_2)$ has to be included into the relation.

$$\frac{C_1}{C_2} = \exp \frac{\lambda A + Z (U_1 - U_2)}{kT} \qquad (4)$$

where λ depends on factors other than size and net charge.

In this manner, the overall effect determining partition divides into a number of different factors, such as size, hydrophobicity, surface charge, and probably the conformation of the particle or macromolecule, which in turn determines the size and number of groups exposed to the surroundings (Albertsson 1986).

1.3 Thermoseparating Aqueous Two-Phase Systems

1.3.1 Thermoseparating polymer systems

A new development in aqueous two-phase separations has been the introduction of thermoseparating polymers. Two phases can be formed by heating an aqueous solution of a thermoseparating polymer above a critical temperature, the cloud point. One of the phases (often the bottom phase) is enriched with polymer, the other is depleted. The random copolymers of ethylene oxide (EO) and propylene oxide (PO) segments (EOPO copolymers) form a liquid polymer-rich phase in equilibrium with a water phase upon temperature increase. Most studies related with biomolecule partitioning in thermoseparated systems have been performed in EOPO copolymer-containing systems. In these systems, the concentration of EOPO polymer in the bottom phase is usually 40-60%, while the top phase contains almost 100% water. Various compositions of EOPO copolymers like 20% EO, 80% PO to 50% each of EO and PO have been used

In other studies, EOPO/dextran and EOPO/hydroxypropyl starch systems, where the usual top phase polymer PEG was replaced with an EOPO copolymer were used. Target proteins were partitioned to the EOPO top phase. Then, a water/EOPO two-phase system was formed by shifting the temperature above the cloud point of the EOPO random copolymer. Proteins used in these systems have been found to be excluded from the polymer-rich phase and partitioned 100% to

the water phase. Thus it became possible to separate the target protein from the EOPO copolymer (Persson *et al*., 1999a).

1.3.2 Thermoseparating surfactant systems

Aqueous two-phase systems can also be formed in solutions of surfactants which have a lower critical solution temperature (cloud point) to separate biomolecules. Phase separation is induced by a shift in temperature. Non-ionic surfactants, such as Triton X-114 and different alkyl polyoxyethylene surfactants (C_mEO_n), display such temperature-sensitive phase separation, and can form cloud point extraction (CPE) systems. These systems separate into a surfactant-rich phase in equilibrium with a surfactant-depleted phase (water rich phase). The separation is based on different distribution of biomolecules between the phases. The general properties of CPE systems have been studied by several researches (Albertsson, 1986; Hatti-Kaul, 2000).

Nonionic surfactants are used to lyse a biomembrane and to solubilize proteins that are membrane-associated without loss of their biological activity. Polyoxyethylene-type nonionic surfactants solubilized membrane proteins in a gentle fashion. During the solubilization, nonionic surfactant replaces most lipid molecules in contact with the hydrophobic domain of the amphiphilic protein and leads to a formation of a soluble protein-surfactant mixed micelle. Upon alteration

of the conditions of the micellar solution (temperature, pressure, addition of salt or other additives), a phase separation and partitioning are induced.

An important advantage of this extraction method is that using only one auxiliary chemical the desired biomolecules can be solubilized and after alteration of the temperature of the micellar solution an ATPS is formed, enriching amphiphilic/hydrophobic proteins in the small volume element of the surfactant-enriched phase, whereas hydrophilic proteins are recovered in the surfactant-depleted phase (Minuth, 2000).

1.3.2.1 Surfactants

Surfactants are amphipathic molecules that contain both polar and hydrophobic groups. These molecules contain a polar group (head) at the end of a long hydrophobic carbon chain (tail). In contrast to purely polar or non-polar molecules, amphipathic molecules exhibit unique properties in water. Their polar group forms hydrogen bonds with water molecules, while the hydrocarbon chains aggregate due to hydrophobic interactions. These properties allow surfactants to be soluble in water. In aqueous solutions, they form organized spherical structures called **micelles** (Figure 1.1), each of which contains several surfactant molecules. The micelle may be represented as a globular cylindrical or ellipsoidal cluster of individual surfactant molecules in equilibrium with its monomers. Because of

their amphipathic nature, surfactants are able to solubilize hydrophobic compounds in water.

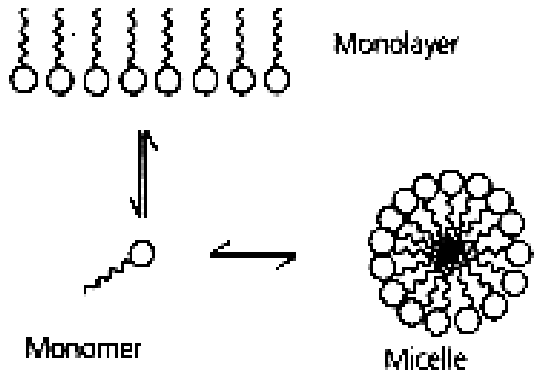


Figure 1.1 Surfactant monomer, surface monolayers and micelle in an aqueous medium. Micelles are in the interior and monolayers at the surface.

1.3.2.1.1 Classification of Surfactants

The primary classification of surfactants is made on the basis of the charge of the polar head group. They are classified as ionic, non-ionic, and zwitterionic surfactants.

1.3.2.1.1.1 Ionic Surfactants

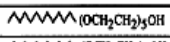
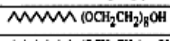
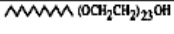
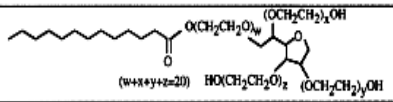
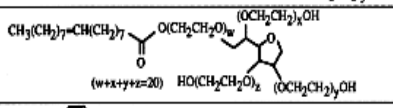
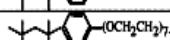
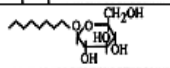
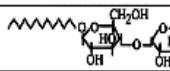
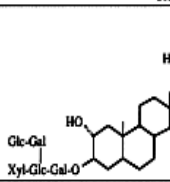
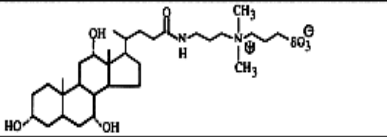
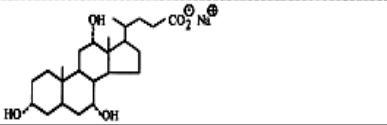
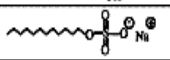
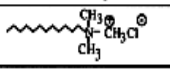
Ionic surfactants contain a head group with a net charge. They can be either negatively (anionic) or positively charged (cationic). For example sodium dodecyl sulfate (SDS), which contains the negatively charged sulfate group, is an anionic surfactant while cetyl trimethyl-ammonium bromide (CTAB), which carries the positively charged trimethylammonium group, is a cationic surfactant. Furthermore, the ionic surfactants either contain a hydrocarbon (alkyl) straight chain as in SDS and CTAB, or a more complicated rigid steroidal structure as in sodium deoxycholate. Cationic and anionic surfactants neutralize each other when present in the same solution together. The oppositely charged surfactant ions join each other to form a water-insoluble salt, and if stoichiometrically equivalent amounts are present, no surfactancy properties remain evident. Anionic surfactants are commercially very important and represent the major fraction of surfactants in use. Cationic surfactants are of interest in the surfactant industry principally because of their bacteriostatic or germicidal property. Their performance as surfactants is rather poor and represent only a minor fraction of the surfactants being used (Swisher, 1970).

1.3.2.1.1.2 Non-ionic Surfactants

Non-ionic surfactants contain uncharged, hydrophilic head groups that consist of either polyoxyethylene moieties as in Triton and BRIJ or glycosidic groups as in octyl glucoside and dodecyl maltoside. This type comprises about 25% of all surfactants. In general, non-ionic surfactants are better suited for breaking lipid-lipid and lipid-protein interactions than protein-protein interactions. Hence, they are considered non-denaturant and are widely used in the isolation of membrane proteins in their biologically active form. Unlike ionic surfactants, salts have minimal effect on the micellar size of the non-ionic surfactants. They have the additional property that they may be combined for use with either anionic or cationic surfactants. Examples are the Igepals, Tritons, Tergitols, Suronics, Plurafacs (Levitt, 1967).

Surfactant molecules form micelles in aqueous solutions when the concentration exceeds a certain value, and this concentration is generally called the critical micelle concentration (CMC). Little or no uptake of solute occurs until the CMC is reached, which suggests that the solute molecules are taken up in some way by the micelles. Among non-ionic surfactants, the polyoxyethylene type represents a large group whose formula are shown in Table 1.1.

Table 1.1 Structures and properties of different surfactants (Sivars & Tjerneld, 2000).

Detergent	*Structure*	*T*	*M_r (monomer/ micelle)*	*CMC (mM)*	*HLB*	*n*	*CP (°C)*
$C_{12}EO_5$		N	406.6	0.065[39]		160[13], i, ii	31.5[40]
$C_{12}EO_8$		N	540/28 600	0.071[39]	12.8[41]	62-120[13]	76.8[39]
$C_{12}EO_{23}$ (Brij 35)		N	1200/48 000	0.09[40]	16.9	40	
Tween 20		N	1228	0.059[40]	16.7	<58[i, 42]	
Tween 80		N	1 310/76 000	0.01	15.0	58	
Triton X-100		N	628/90 000	0.31[40]	13.5	140	65
Triton X-114		N	536	0.17[40]	12.4	>140[i, ii]	22
Octyl glucoside		N	292/24 500	15		84[40]	
Dodecyl maltoside		N	511/50 000	0.15[43]		98	
Digitonine		N	1 229/74 000	0.67-0.73[40]	0.4[40]	60[43]	
CHAPS		Z	615/6 000	4.2-6.3[40]		9-10[40]	
Cholate		A	431/1 300	14[40]	18[40]	3[40]	
SDS		A	288/18 000	8.1[40]	40[43]	60-100	
DTAC		C	308/19 000	20.3[42]		61[43]	

Data are from [13, 39–42]. Where not stated, data are from Von Jagow and Schägger [3]. T, type; N, non-ionic; Z, zwitterionic; C, positively charged; A, negatively charged; M_r, relative molecular mass of monomer/micelle; CMC, critical micelle concentration; HLB, hydrophilic–lipophilic balance; n, aggregation number; CP, cloud-point temperature. [i]Micelle size (aggregation number) increase with shorter polyoxyethylene chain length and longer alkyl chains [43]. [ii]Highly dependent on condition such as temperature and concentration.

The structure is normally abbreviated to C_nE_m, where C_n represents the length of the alkyl group and E_m the number of oxyethylene units. These surfactants are solubilized in water by the hydration of the ether oxygens of the polyoxyethylene groups. An increase in temperature leads to a decrease in the number of hydrogen bonds, which raises the micellar mass and decreases the CMC. If the temperature continues to increase, the micelle becomes so large and the number of intermicellar interactions increase to such an extent that a sudden onset of turbidity is perceptible even to the naked eye. This temperature is called the cloud point. A further rise in temperature causes the solution to begin to separate into two phases, one surfactant-rich and the other surfactant-depleted with no or few micelles present.

The cloud point depends on the length of the hydrophilic (oxyethylene units) and hydrophobic (alkyl) chains. In the Triton series, the cloud point ranges from 22ºC for TX-114 (7 units) to 67ºC for Triton X-100 (TX-100) (9 units).

Surfactant concentration is also important because, for example, a 3% (w/v) concentration is necessary for C_6E_3 to partition at 37ºC. However, surfactant concentration has less influence than the concentration of the additives, which are usually inorganic salts and hydrocarbons (Sánchez-Ferrer *et al*., 1994).

The addition of most neutral electrolytes (e.g., chlorides, sulfates, carbonates) typically depresses the cloud point due to their salting-out effect in proportion to

their concentration, with the effect of a given salt depending on the hydrated radii of both ions. The lower the lyotropic number of the electrolyte, the greater the effect. On the other hand, salting-in-type electrolytes, such as nitrates, iodides, and thiocyanates, typically increase the cloud point. The addition of shorter hydrocarbons generally does not lower the cloud point very much, whereas more nonpolar organic compounds that can be solubilized in the interior of the micelle normally raise the cloud point. The presence of many denaturants (such as urea or substituted ureas) also increases the cloud point. Polar organic compounds, such as aliphatic alcohols, fatty acids, phenols typically depress the cloud point remarkably. Last, studies indicate that an increase in the pressure typically causes a slight increase in the cloud point temperature of nonionic surfactant solutions. The cloud point of dilute non-ionic surfactant solutions increases upon addition of charged ionic surfactants (Hinze and Pramauro, 1993).

1.3.2.1.1.3 Zwitterionic Surfactants

Zwitterionic surfactants contain both two charged group of different sign. Whereas the positive charge is almost invariably ammonium, the source of negative charge may vary, although carboxylate is by far the most common. Common types of zwitterionic surfactants are *N*-alkyl derivatives of simple amino acids such as glycine, betaine and amino propionic acid. Zwitterionic surfactants are unique in that they offer the combined properties of ionic and non-ionic

surfactants. Zwitterionics as a group are characterized by having excellent dermatological properties. They also exhibit low eye irritation and are frequently used in shampoos and other cosmetic products. Since they possess no net charge, including CHAPS, they lack conductivity and electrophoretic mobility, and do not bind to ion-exchange resins. However, like ionic surfactants, they are efficient at breaking protein-protein interactions. They are compatible with all other classes of surfactants (Holmberg *et al.* 2003).

1.4 Studies on Triton X-114-water systems

There are many useful applications of the biological materials that have been extracted and isolated or partially purified using the cloud-point extraction technique based upon the phase separation behavior of nonionic surfactants mostly TX-114. Alcaraz *et al*. (1984) examined the phase-separation extraction and distribution of the receptor for immunoglobulin E (IgE) and its subunits in the TX-114 system. They found that the beta and gamma chains, once dissociated from the alpha chain, were readily separated from the latter, provided that the alpha chains remain attached to IgE. In another application, Holm *et al*. (1986) separated hormone-sensitive lipase from ATP-citrate lyase using the TX-114 cloud point extraction. They extracted over 80% of the hormone lipase into the surfactant phase with 78% enzymatic activity, while the hydrophilic ATP-citrate was almost exclusively in bulk aqueous phase. Sivars *et al*. (1996) showed that

the partitioning of the hydrophilic proteins was strongly affected by the addition of ionic surfactants to surfactant/polymer aqueous two-phase system. In addition, Tani *et al*. (1998) found an increased purification of membrane bound cytochrome b_5 by using a mixture of TX-114 and charged dextran, dextran sulphate, compared when only the surfactant was used.

1.5. Polyphenol oxidases

Polyphenol oxidase (PPO; EC 1.14.18.1) is an oxidoreductase that is widely distributed in plants and, due to its wide specificity for several phenolic substrates, generally recognized as being responsible for the enzymatic browning reaction which occurs during the handling, storage, and processing of fruits and vegetables. The enzyme is known by various names such as tyrosinase, phenolase, polyphenolase, catechol oxidase, cresolase or catecholase. PPO is particularly high concentration in mushrooms, potatoes, apples, bananas, pears, peaches, avocados, tea leaves, tobacco leaves and cocoa beans. The enzyme has been most extensively studied in mushrooms and potatoes and obtained in homogeneous state (Motoda, 1979).

The enzyme catalyzes two different reactions in the presence of molecular oxygen. The first, and the only specific reaction catalyzed by this enzyme, is the hydroxylation of monophenols to *o*-diphenols, a reaction that is usually termed

monophenolase or cresolase activity. The second, diphenolase activity, consists of the oxidation of the self-generated *o*-diphenols to the corresponding *o*-quinones, which are highly reactive molecules and which polymerize to brown, red, or black pigments depending on the natural components present in a given plant material.

PPO (tyrosinase) has received considerable attention in the last 20 years as an indispensable tool to perform studies over a wide range of topics. Tyrosinase found in mushrooms (*Agaricus bisporus*) has been utilized in the majority of these studies since it is readily obtainable in relatively large quantities. Commercial preparations of this enzyme are available. However, commercial *A. Bisphorus* tyrosinase is usually tanned, i.e., modified by its self-generated quinones, which results in great enzyme heterogeneity. That makes its purification difficult (Resigno *et al*., 1997).

Mushroom tyrosinase has a molecular weight of 128 kDa (Yada *et al*., 1994) and its isoelectric point (pI) is identified by isoelectric focusing as 4.28 (Flurkey, 1991),and as 4.1-4.3 (Dicko *et al*., 2002).

1.5.1 Studies on PPO purification

Partington and Bolwell (1996) described the purification of PPO from potato tuber (*Solanum tuberosum cv Cara*) to homogeneity including critical step of

hydrophobic chromatography on Octyl-Sepharose which was sufficient to completely remove patatin. Paul and Gowda (2000) purified and characterized PPO from the seeds of field bean (*Dolichos lablab*) to apparent homogeneity by a combination of ammonium sulfate precipitation, DEAE-Sephacel chromatography, phenyl agarose chromatography, and Sephadex G-200 gel filtration. Shi *et al*. (2001) purified PPO from fresh leaves of tobacco (*Nicotiana tobaccum*) using acetone powder, ammonium sulfate precipitation and column chromatography on DEAE-Sephadex A-50, CM-Sephadex G-75. Nagai and Suziki (2001) purified and characterized PPO from Chinese cabbage by ammonium sulfate precipitation and DEAE-Toyopearl 650M column chromatography. Roy *et al*. (2002) separated an isoenzyme of PPO from aqueous extracts of seeds of *Duranta plumieri* by expanded bed chromatography. Wititsuwannakul *et al.* (2002) isolated PPO from the B-serum obtained after repetitive freeze-thawing of the bottom fraction isolated from ultracentrifuge fresh latex.

1.5.2 Studies on the purification of PPO with Triton X-114 induced phase separation

There are a few studies on mushroom polyphenol oxidase partitioned by TX-114 based aqueous two-phase systems. Sánchez-Ferrer *et al.* (1993) partially purified soluble potato PPO using a two-phase partitioning approach with TX-114. They

achieved 5-fold purification with an 18% recovery of activity and reduced the phenolics 3% of the original content. Sojo *et al*. (1998) partially purified banana pulp PPO in a latent form using sequential aqueous two-phase system based on TX-114 and PEG 8000/phosphate. They achieved 5-fold purification with a 50% recovery. They reduced the (poly) phenols including tannins to 6% of the original avoiding post purification tanning of the enzyme. Finally, Núñez-Delicado *et al*. (1996) partially purified mushroom tyrosinase using an aqueous two-phase system with TX-114. They achieved 5.5-fold purification with a high recovery of 84%. They reduced the phenols to 8% of the original content, avoiding pre and post-purification tanning of the enzyme.

1.5.3. Purification of mushroom PPO with Triton X-114/water systems

Núñez-Delicado *et al*. (1996) used the following procedure for the purification of mushroom PPO.

A 20 g sample of mushroom pileus was cleaned by removing the earth and 40 ml of 100 mM sodium phosphate buffer pH 7.3 were added. The mixture was homogenized for 30 s in a high-speed blender and centrifuged at 10,000xg for 30 min at 4ºC.

This supernatant was subjected to temperature phase partitioning by adding TX-114 at 4ºC, so that the final surfactant concentration was 6%. The mixture was kept at 4ºC for 10 min and then warmed to 37ºC in a thermostatic bath. After 10 min, the solution became spontaneously turbid due to the formation, aggregation and precipitation of large mixed micelles of surfactant, which contained hydrophobic proteins and phenolic compounds. This solution was centrifuged at 10,000xg for 15 min at room temperature. The surfactant-rich phase was discarded. The clear surfactant-poor supernatant of 6% (w/v) TX-114 was subjected to additional phase partitioning to remove the remaining phenols. For this, fresh TX-114 was added to obtain a final concentration of 4% (w/v) and the mixture brought to 37ºC. After centrifugation at room temperature at 10,000xg, the surfactant-poor supernatant of 4% (w/v) TX-114 containing the soluble PPO, was brought to 25% saturation with solid $(NH_4)_2SO_4$ under continuous stirring at 4ºC.

After one hour, the solution was centrifuged at 60,000xg for 30 min at 4ºC and the pellet was discarded. $(NH_4)_2SO_4$ was added to the clear supernatant to give 75% saturation and stirred for 1 h at 4ºC. The precipitate obtained between 25% and 75% was collected by centrifugation at the same rotor speed and dissolved in a minimal volume of 100 mM phosphate buffer, pH 7.3, containing 20% glycerol. The salt content was removed by dialysis and the enzyme stored at -20ºC.

1.6 The aim of the study

The aim of the study was to isolate, purify and concentrate PPO from mushroom (*Agaricus bisporus*) extract by using Triton X-114/water aqueous two-phase system and to investigate the partitioning behavior of the enzyme. In this respect, PPO was required to partition to the surfactant depleted top phase to prevent an extra step necessary for removal of surfactant. The contaminating molecules like phenolics and other proteins, were required to partition to the surfactant enriched bottom phase. The effects of surfactant concentration, ionic strength, pH, temperature, PPO and crude extract concentration, as well as crude extract preparation on PPO partitioning were investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Non-ionic surfactant TX-114 [octyl phenol (ethyleneglycol) $_{7\text{-}8}$ ether], catechol, Bovine Serum Albumin (BSA) were purchased from Sigma (Germany) and used without further purification. Folin Ciocalteau phenol reagent was obtained from Sigma. All salts and other chemicals were obtained either from Sigma or Merck and were of analytical grade.

2.2 Methods

2.2.1 Enzyme Activity

PPO activity was determined spectrophotometrically at 410 nm with catechol. The reaction was performed at 20ºC in 2 ml reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 0.2 M catechol as substrate and a suitable amount of enzyme. One unit (U) of enzyme was defined as the amount of enzyme increased the absorbance by 0.01 per min due to the oxidation of catechol at 20ºC, under the reaction conditions.

2.2.2 Protein Content

Protein content was determined according to the dye binding method of Bradford (Bradford, 1976), using bovine serum albumin as a standard and measuring the absorbances at 595 nm. Bradford reagent was prepared according to Bradford and stored in a dark bottle at 4ºC.

2.2.3 Phenolics Content

Phenolics were determined by the method of Folin & Ciocalteau (Folin *et al*., 1927). In this method 1.5 ml sodium carbonate (200 gl^{-1}) and 0.5 ml Folin-Ciocalteau phenol reagent were added successively to 10 ml sample. After one hour incubation at room temperature, the absorbances were measured at 750 nm in 4 ml cuvettes against distilled water and corrected for the absorbance of a distilled water reagent blank (Box, 1983).

2.2.4 TX-114 Content

TX-114 concentration was estimated by using absorption data at 268 nm absorption up to the surfactant concentrations of 6% (w/v) in 1 cm quartz cuvettes (Werck-Reichhart *et al*., 1991).

2.2.5 Cloud Point Determination

The cloud point was taken as the temperature at which solution became turbid. The cloud point of the system was simply determined by heating up a stirred one-

phase system in a water bath until clouding occurred. The cloud point of TX-114 in pure system was determined as 22ºC. In systems containing Triton X-114, both total protein and phenolics concentrations were measured at 4ºC due to the low cloud point of TX-114.

2 .2.6 Aqueous Two-Phase Extraction

Mushrooms were cleaned by removing earth and stored at constant temperature (4ºC). A 10g sample was homogenized with 200 ml sodium phosphate buffer at pH 7.0 for 30 s in a high speed blender. In the case of polyvinylpolypyrrolidone (PVPP) use, crude enzyme was prepared by homogenization of 10 g mushroom containing 2.5 g PVPP with 200 ml sodium phosphate buffer. The homogenate was filtered and centrifuged at 11,000xg for 15 min at 25ºC. The supernatant was filtered through filter paper and used as a crude enzyme extract. This supernatant was subjected to temperature-induced phase partitioning by adding TX-114 to various final concentrations at 4ºC, and then kept at 37ºC for one night. This solution was centrifuged at 19,000xg for 15 min at 25ºC. Phase volumes were determined. Samples from the top and bottom phases were taken with an automatic pipette and were analyzed for the PPO activity, protein concentration and phenolics concentration. In each aqueous two-phase experiment 2 ml of crude enzyme extract was used and the total volume of the system was 5 ml. All the experiments were performed at least two times. In each experiment, duplicate

analysis of the parameters was done. Averages of these four data points were used in the partitioning parameter calculations.

Partitioning Parameters

Enzyme recovery (ER) was defined as the ratio of the total enzyme activity in the top phase to the total enzyme activity in the original extract.

$$ER = \frac{\text{total activity in the top phase}}{\text{total enzyme activity added to the system}} \text{, dimensionless} \qquad (1)$$

The partitioning of a substance in an aqueous two phase system was described by the **partition coefficient K**,

$$K = \frac{C_T}{C_B} \text{, dimensionless} \qquad (2)$$

where C_T and C_B are the equilibrium concentrations of the enzyme (U/ml) in the upper and lower phases, respectively.

Specific activity (SA) was defined as the activity of an enzyme in a solution divided by the total protein content in that solution.

$$SA = \frac{\text{enzyme activity in one phase}}{\text{total protein in that phase}} \text{, U/mg protein} \quad (3)$$

Purification factor (PF) was defined as the ratio of the specific activity in the top phase to that of the original solution.

$$PF = \frac{\text{specific activity in the top phase}}{\text{specific activity in the crude extract}} \text{, dimensionless} \quad (4)$$

Concentration ratio (CR) was defined as the ratio of the volume of the crude extract added to the system to the volume of the top phase.

$$CR = \frac{\text{volume of original extract}}{\text{top phase volume}} \text{, dimensionless} \quad (5)$$

Phenolic removal (PR) was defined as the ratio of the total phenolic compounds in the bottom phase to that of added to the system.

$$PR = \frac{\text{total phenolics in the bottom phase}}{\text{total phenolics in the original extract}} \text{, dimensionless} \quad (6)$$

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Partitioning Behavior of Mushroom PPO in TX-114/Water Systems

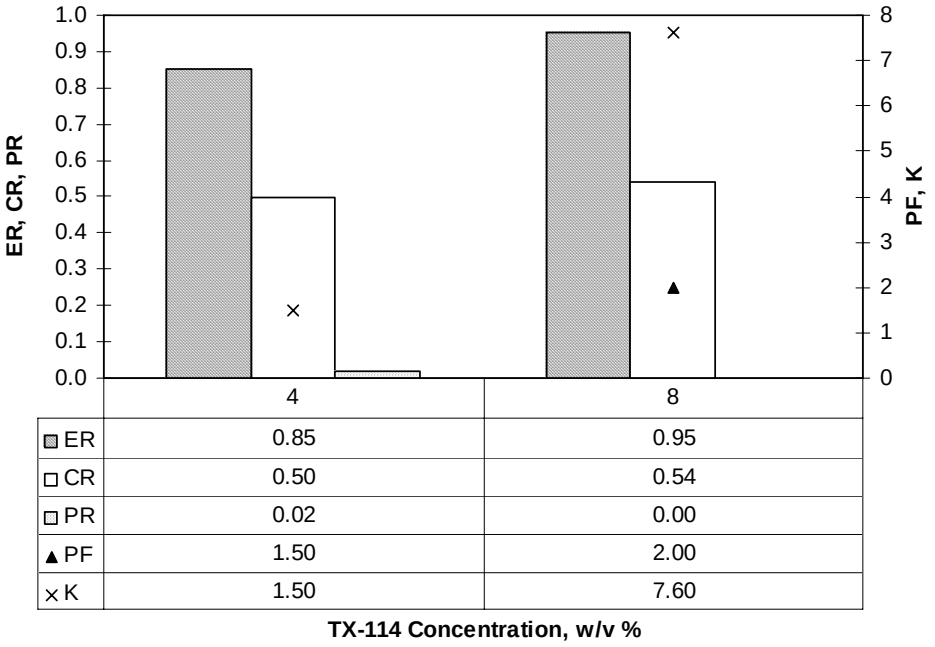
In Triton X-114/Water systems, like the other aqueous-two phase systems, proteins can be separated from each other depending mainly on their hydrophobicity/ hydrophilicity. More hydrophobic proteins partition to the surfactant-rich phase while, more hydrophilic ones prefer surfactant-depleted phase (Bordier, 1981). A literature survey reveals few reports on the use of TX-114/water systems in partial purification of PPO. Triton X-114 was used to remove phenols from potato tubers to an extent where no browning was produced during the partial purification of PPO (Sánchez-Ferrer *et al*., 1993). In one study, banana pulp PPO was partially purified in a latent form using sequential aqueous two-phase system based on Triton X-114 and PEG 8000/phosphate (Sojo *et al*., 1998). In another study, mushroom tyrosinase was partially purified using an

aqueous two-phase system with TX-114, with a recovery of 84% (Nuñez-Delicado *et al*., 1996). The aim of these studies was not to examine the partitioning behavior of PPO. However, in this study, partitioning behavior of mushroom PPO in TX-114/water system was evaluated. The effects of surfactant concentration, ionic strength, pH, temperature, PPO and crude extract concentration, as well as crude extract preparation on PPO partitioning were investigated. The aim was to partition, purify and concentrate PPO in the surfactant depleted phase to prevent an extra step for surfactant removal and to remove phenolics to the surfactant-enriched phase as much as possible.

3.2 Preliminary Trials for PPO partitioning

In ATPS composed of TX-114 and sodium phosphate buffer, PPO partitioned to the surfactant-depleted top phase with 85% and 98% recovery when 4% and 8% TX-114 were used respectively (Figure 3.1). Increasing the TX-114 concentration from 4% to 8% (w/v) increased the partition coefficient of the enzyme from 1.5 to 7.6 and purification factor from 1.5 to 2. However, almost none of the phenolics were removed from the top phase. Another set of experiment was performed by using 6 and 8% TX-114 and 10 times diluted mushroom extract. The results were given in Figure 3.2. PPO was successfully partitioned to the top phase with 100% recovery by using 6% TX-114. However, all of the contaminating proteins together with phenolics were also partitioned to the surfactant depleted top phase,

so that no purification was achieved. Use of diluted crude enzyme extract decreased purification factor and enzyme recovery when 8% TX-114 was used.



	4	8
ER	0.85	0.95
CR	0.50	0.54
PR	0.02	0.00
PF	1.50	2.00
K	1.50	7.60

Figure 3.1 Effect of TX-114 concentration on partitioning. ATPS performed at T: 37ºC; pH: 7.0.

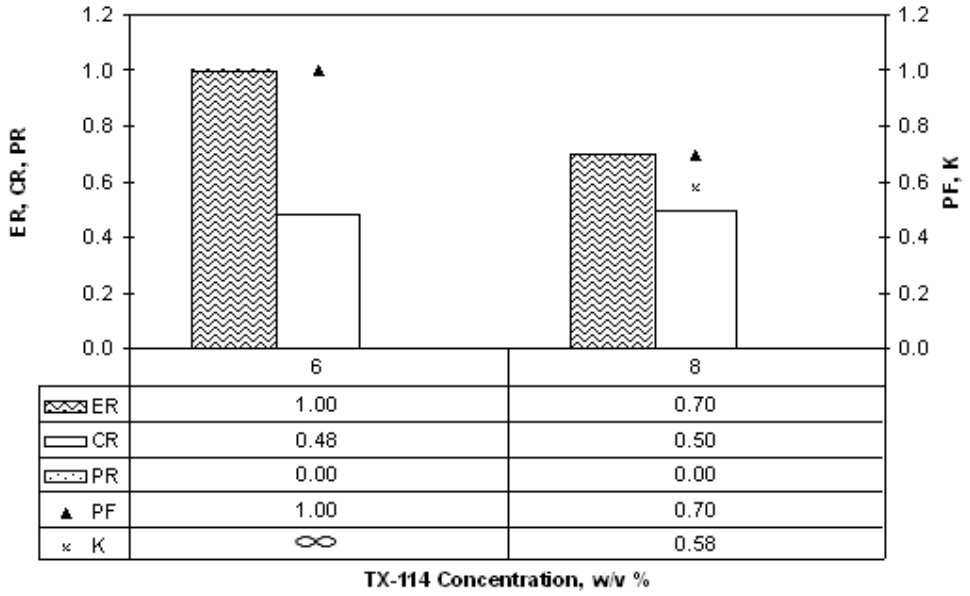


Figure 3.2 Dilution effect on PPO partitioning. ATPS performed at T: 37ºC; pH: 7.0.

3.3 Phenolics Partitioning By Using TX-114 ATPS

Mushroom crude extract contains various phenolic compounds and presence of phenolics at high concentrations may inactivate PPO. So, it is beneficial to partition the phenolics to the other phase. For this purpose, initially, experiments were performed by using pure catechol as phenolic compound to observe the effect of TX-114 concentration on phenolics removal. As TX-114 concentration was increased, phenolics content in the top phase decreased (Figure 3.3).

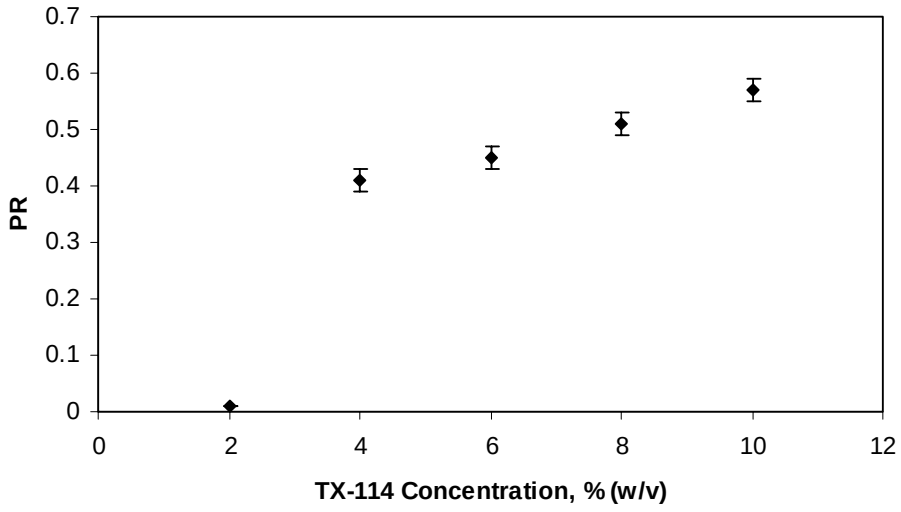


Figure 3.3 Effect of surfactant concentration on catechol separation. Total catechol content in the ATPS was 0.1 mg. ATPS performed at T: 37ºC; pH: 7.0.

About 60% of catechol was removed, when 10% (w/v) TX-114 was used. Then, phenolics removal was performed by using mushroom extract. Phenolics removal increased with increasing TX-114 concentration (Figure 3.4). However; 50% of the phenolics could only be removed by using 20% TX-114 which means the presence of other compounds affected phenolics removal.

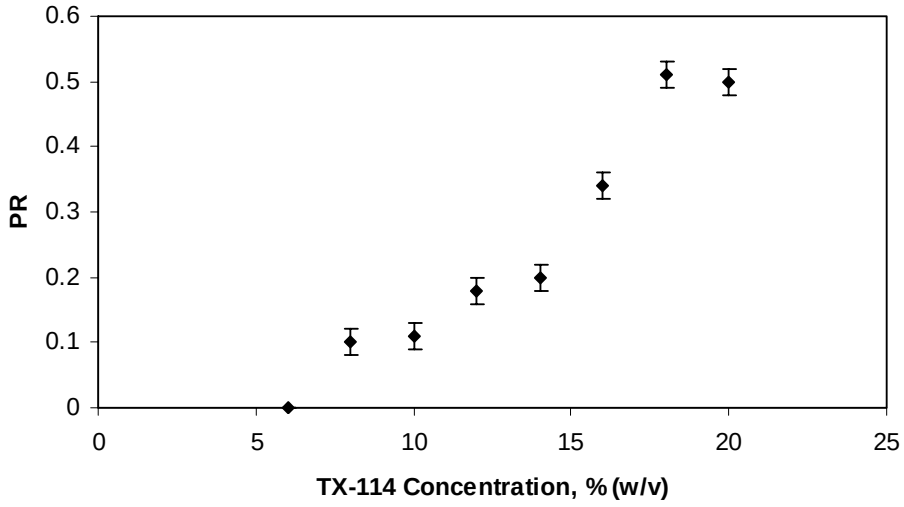


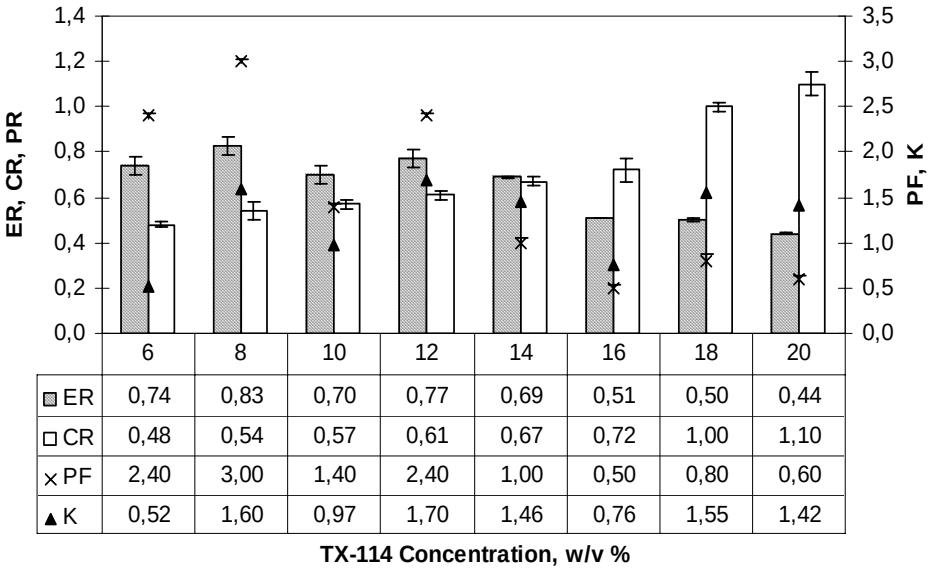
Figure 3.4 Effect of surfactant concentration on phenolics removal from mushroom crude extract. Total phenolics content in ATPS was 0.09 mg/ml. ATPS performed at T: 37ºC; pH: 7.0.

3.4 Effect of TX-114 Concentration on PPO Partitioning

Preliminary experiments showed that, surfactant concentration strongly affected protein and PPO partitioning. Therefore, further experiments were done in a TX - 114 concentration range of 6%-20%. Higher surfactant concentrations were not used to prevent the formation of an additional surfactant phase (Albertson, 1986). Figure 3.5 shows how PPO partitioning changed by changing TX-114 concentration. PPO mostly partitioned to the water-enriched phase in the concentration range of 6% to 14% TX-114. Both PF and ER values were higher in this range. The strongest partitioning to the water-enriched phase was obtained at

8% TX-114 with highest recovery and PF. Afterwards, ER and PF decreased by increasing TX-114 concentration.

In summary, enzyme recovery and purification factor were better at low surfactant concentrations, but phenolic removal and concentration ratio were better at high surfactant concentrations. Although one of the aims was to concentrate the product, it was diluted up to 18% TX-114. As observed in Figure 3.5, increasing surfactant concentration decreases the top phase volume. The highest ER and PF were obtained when 8% TX-114 was used in the expense of diluted product and poor phenolics removal.



	6	8	10	12	14	16	18	20
ER	0,74	0,83	0,70	0,77	0,69	0,51	0,50	0,44
CR	0,48	0,54	0,57	0,61	0,67	0,72	1,00	1,10
PF	2,40	3,00	1,40	2,40	1,00	0,50	0,80	0,60
K	0,52	1,60	0,97	1,70	1,46	0,76	1,55	1,42

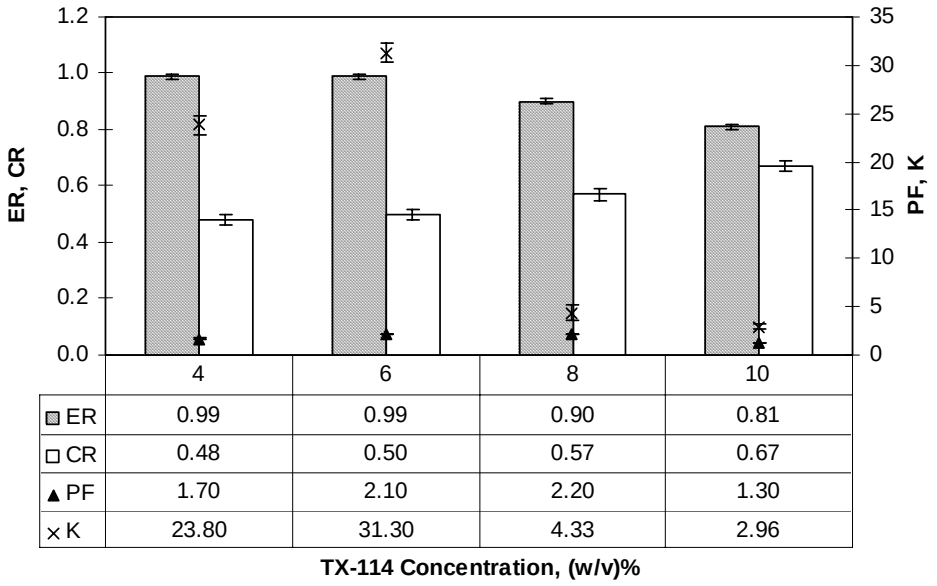
TX-114 Concentration, w/v %

Figure 3.5 Effect of Triton X-114 concentration on PPO partitioning. ATPS performed at T: 37ºC; pH: 7.0.

3.5 Effect of Pretreatments Used During Crude Extract Preparation

3.5.1 Ultrafiltration

To remove the phenolics to the bottom phase, high TX-114 concentrations should be used. However, PPO recovery and purification decreased with increasing TX-114 content and highest values of enzyme recovery, purification and partition coefficient were achieved when 8% (w/v) TX-114 was used. To remove phenolics without affecting the enzyme recovery and purification, an ultrafiltration step using 30 kDa cut-off membrane was included to the crude extract preparation step. Phenolic compounds were reduced to 25% of original content with small loss of PPO activity. The partitioning results were given in Figure 3.6. Using ultrafiltration as a pretreatment, enzyme recovery and partition coefficients increased significantly. Especially at 4-6% TX-114, almost 100% recovery was achieved with high *K* values. The increase in recovery may be related to the removal of low molecular weight solutes like ions, monosaccharides, phenolics, and lipids, which can alter many properties of the phases important in PPO partitioning such as ionic strength, hydrophobicity and hydrophilicity. Although CR values increased with increasing TX-114 concentrations, PPO could not be concentrated in the top phase and a slight reduction in PF was observed.



	4	6	8	10
ER	0.99	0.99	0.90	0.81
CR	0.48	0.50	0.57	0.67
PF	1.70	2.10	2.20	1.30
K	23.80	31.30	4.33	2.96

Figure 3.6 Effect of using ultrafiltration on PPO partitioning. ATPS performed at T: 37ºC; pH: 7.0.

3.5.2 Two-step ultrafiltration

To prevent membrane fouling and remove phenolics and other small molecules better, two-step ultrafiltration by using 0.2µ and 30kDa cut-off cellulose acetate membranes was used. Phenolic compounds were reduced to 4.5% of the original content. Better removal of phenolics and small molecules before partitioning, resulted in perfect enzyme recovery and partition coefficients independent of TX-114 concentration and about two fold higher purification factors. PF and CR

values increased with increased TX-114 concentration; however PPO still could not be concentrated in the top phase (Figure 3.7).

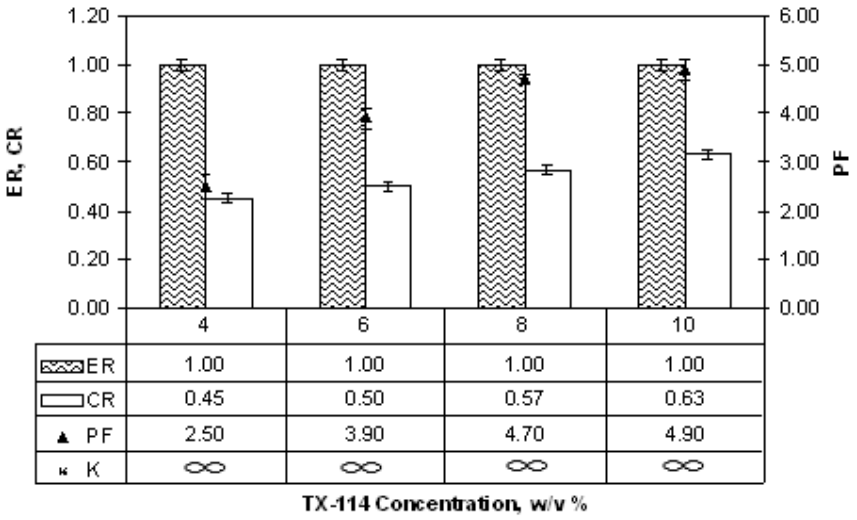


Figure 3.7 Effect of using two-step ultrafiltration on partitioning. ATPS performed at T: 37ºC; pH: 7.0.

3.5.3 PVPP precipitation of phenolics

The partitioning and purification results of previous experiments in which two-step ultrafiltration was used were successful except concentration effect. Therefore, another technique, phenolic precipitation with polyvinylpolypyrrolidone (PVPP) was tried as a pretreatment step and 15% of the phenolics were precipitated with the use of PVPP. The partitioning results were given in Figure 3.8. PF, CR, PR values increased by increasing surfactant

concentration. Highest values of *K* and ER were achieved at 8% TX-114. These results showed that the importance of the presence of other molecules (ions, phenolics, small proteins, lipids, amino acids, carbohydrates etc.) in enzyme partitioning. Presence or absence of other molecules changes enzyme recovery and purification abruptly.

In general, when compared with ultrafiltration experiments, purification increased two fold despite the decrease in recovery. Since the increase in purification is considered to be more important than the complete recovery, following experiments were done by using phenolics precipitation by the addition PVPP.

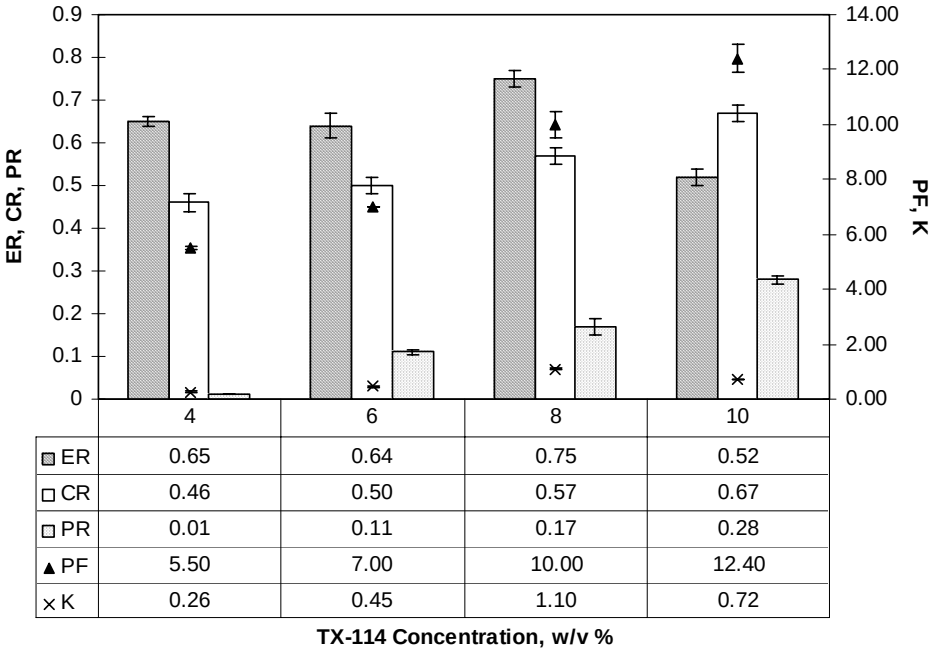
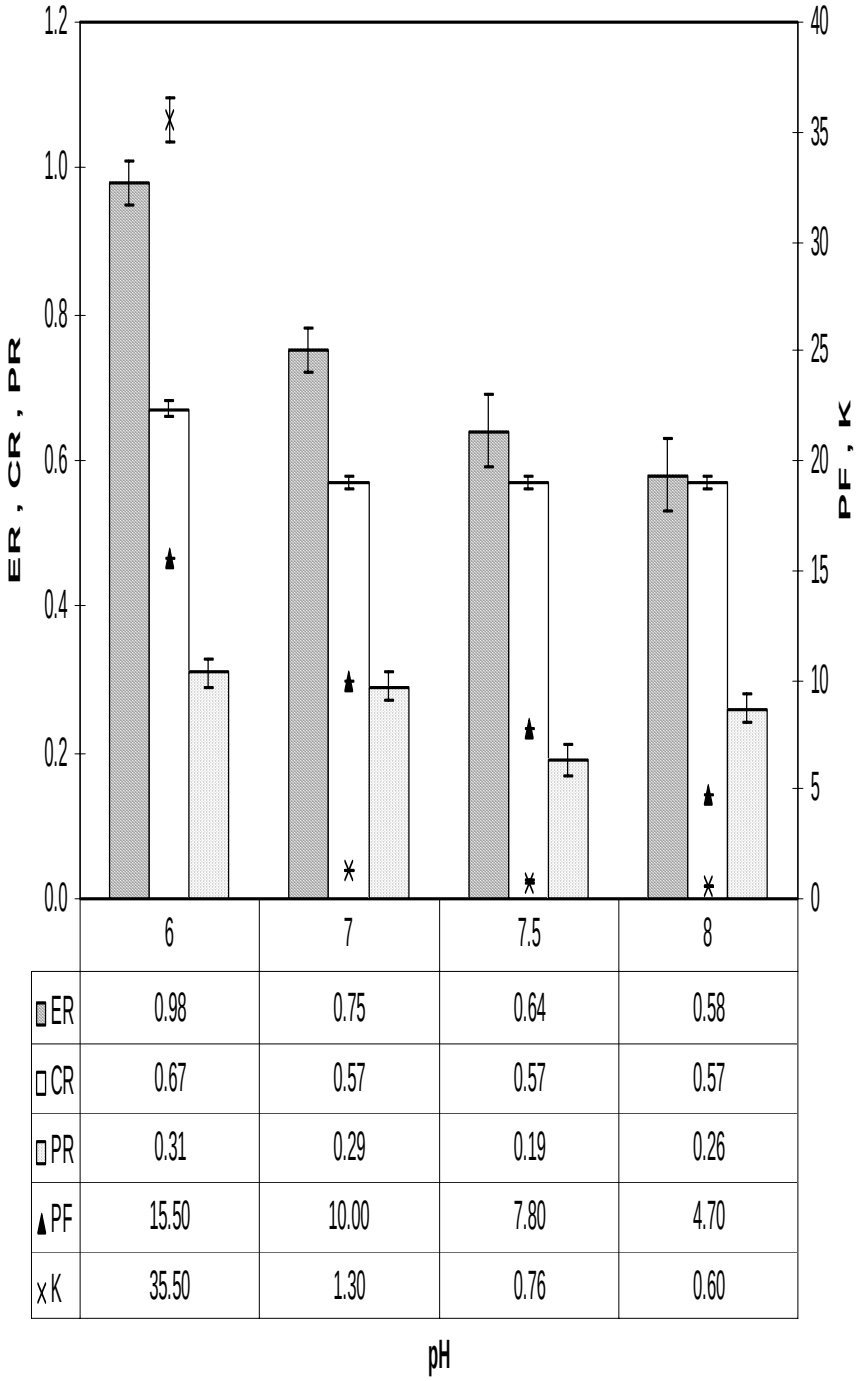


Figure 3.8 Effect of PVPP addition during crude enzyme preparation on PPO partitioning. ATPS performed at T: 37ºC; pH: 7.0.

3.6 Effect of pH on Partitioning

Changes in pH alter the partition coefficients of biomolecules (Albertsson, 1986). The effect of the pH value of the system on PPO partitioning was evaluated in a phase system of 8% TX-114 and water. For this purpose, sodium phosphate buffer in a pH range of 6-8 and acetate buffer of pH 4-6 were used for preparation of mushroom extract and aqueous two-phase system. Performing experiments in a pH range of 4-6, caused surfactant molecules to partition more to the top phase which affected the formation of phase separation.

Isoelectric point (pI) of mushroom was given in a range of 4.1-4.3 in the literature (Dicko *et al*., 2002). Therefore, at the pH range used in the experiments, the net charge of PPO is negative. As observed in Figure 3.9, ER and PF increased considerably by decreasing pH from 8.0 to 6.0 indicating a major effect of charge on partitioning. The net charge of PPO is less negative at pH 6.0 at which highest recovery and purification were achieved. At high pH, being more negatively charge, PPO may form salt bridges with positively charged other protein molecules causing a decrease in charged regions available to the hydrophobic solvent. As a result, the protein complexes may prefer to partition to the more hydrophobic surfactant enriched phase. PPO, being a less negatively charged molecule at pH 6.0, preferred to partition to the hydrophilic water phase. However, partitioning direction changed for the other proteins, they partitioned to the more hydrophobic surfactant enriched phase.



	6	7	7.5	8
ER	0.98	0.75	0.64	0.58
CR	0.67	0.57	0.57	0.57
PR	0.31	0.29	0.19	0.26
PF	15.50	10.00	7.80	4.70
K	35.50	1.30	0.76	0.60

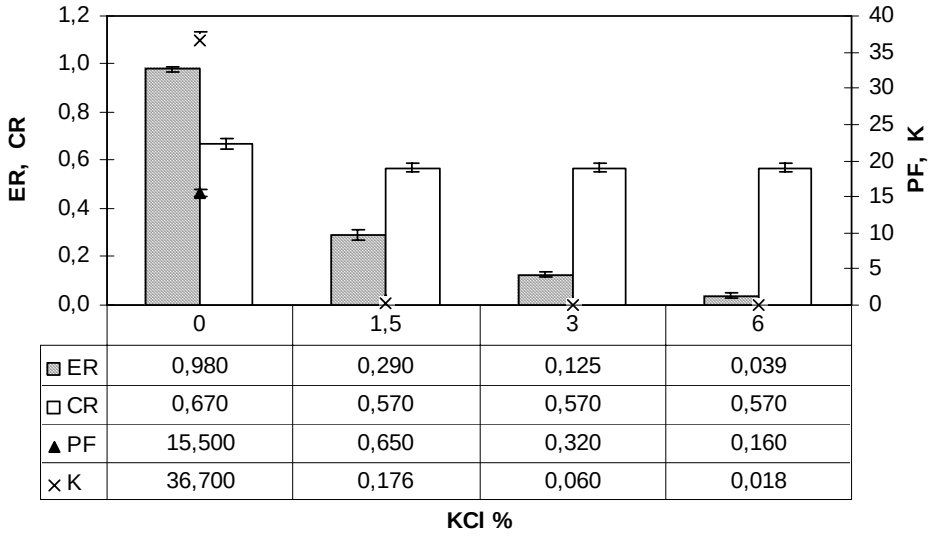
Figure 3.9. Effect of pH on partitioning. System composition 8% TX-114, T: 37ºC

3.7 Effect of Ionic Strength on Partitioning

Salts have been used to enhance partitioning of proteins in aqueous two-phase systems. As different ions have different affinity for the two phases there will be a driving force toward uneven partitioning of the ions between the phases, although electroneutrality must be retained in each phase. The electrochemical driving force in partitioning has been explained by the formation of an electrostatic potential difference over the interface. This potential difference is created by the different affinities of the ions for the two phases. The electrostatic potential difference will affect the partitioning of proteins or other charged molecules present in the phase system. It has been established that the partitioning of ions in aqueous two-phase systems follows the Hofmeister or lyohopic series with the more chaotropic (hydrophobic) ions partitioning stronger to the more hydrophobic surfactant enriched phase (Persson *et al*., 1999b).

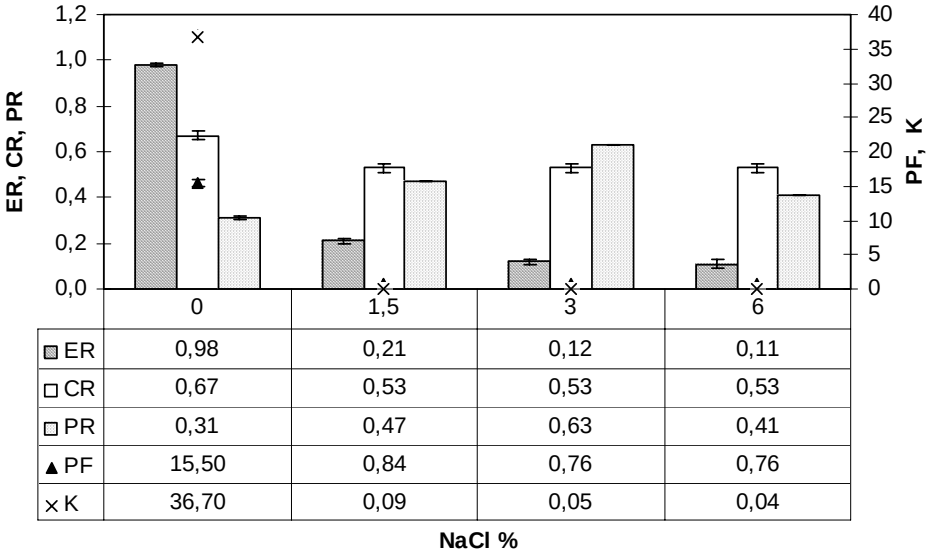
NaCI and KCl in a concentration range of 0-6% were added to investigate the ionic strength effect on partitioning. PPO partitioned strongly to the bottom phase by adding salt and this increased by increasing salt concentration in both cases. Therefore all the parameters, ER, PF and K decreased sharply (Figures 3.10 and 3.11).

The effect of sodium for negatively charged proteins was given higher than potassium, i.e., partition coefficient of negatively charged proteins decreased more in presence of sodium than potassium, when the salt concentration was less than 250 mM (Walter *et al.*, 1985). As shown in Figures 3.10 and 3.11, the effect of NaCl was higher than KCl at 1.5% salt concentration, which is in agreement with the rule written above. At higher concentrations than 1.5%, the rule could not be used.



	0	1,5	3	6
ER	0,980	0,290	0,125	0,039
CR	0,670	0,570	0,570	0,570
PF	15,500	0,650	0,320	0,160
K	36,700	0,176	0,060	0,018

Figure 3.10 Effect of KCl addition on partitioning. System composition: 8% TX-114, pH: 6.0; T: 37ºC



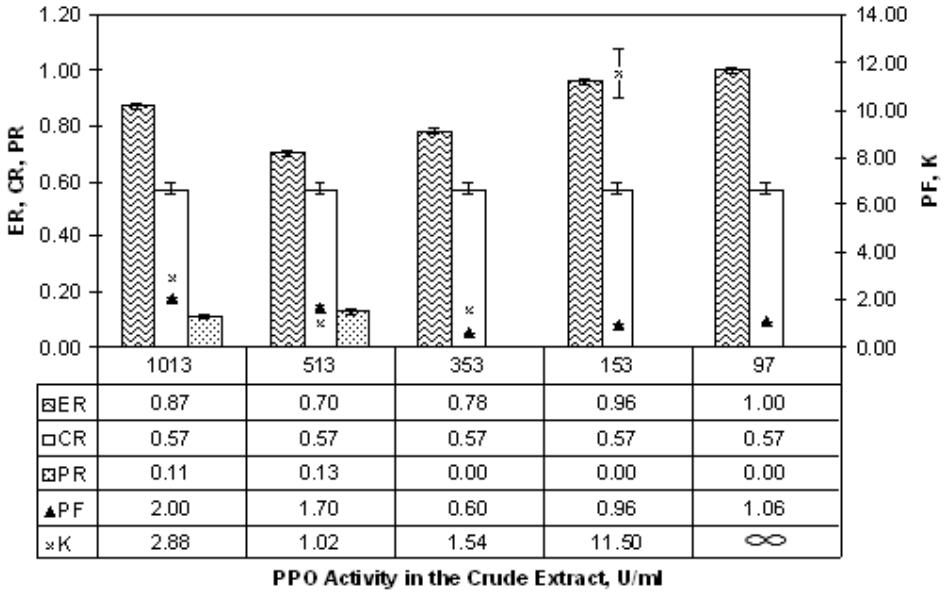
	0	1,5	3	6
ER	0,98	0,21	0,12	0,11
CR	0,67	0,53	0,53	0,53
PR	0,31	0,47	0,63	0,41
PF	15,50	0,84	0,76	0,76
K	36,70	0,09	0,05	0,04

Figure 3.11 Effect of NaCl addition on partitioning. System composition: 8% TX-114, pH: 6.0; T: 37ºC.

3.8 Effect of Crude Enzyme Concentration on Partitioning

Crude extract was prepared by using PVPP at pH 6.0 and then concentrated 9 fold by ultrafiltration. Afterwards crude extracts at various concentrations were prepared by dilution down to 18 fold by adding TX-114 to perform ATPS. So that five different crude extract concentrations in the range of 9 fold to 0.5 fold of the original crude extract were prepared. However their low molecular weight component compositions are different from the original crude extract because of the effect of ultrafiltration. As the concentration decreases, ER increases and PR decreases. PF and *K* values decrease as the concentration decreases from 9-fold up

to 3-fold (1013 U/ml to 353 U/ml). Further drop in concentration to (1 fold and 0.5 fold) causes PF and *K* values to increase (Figure 3.12). These results show that enzyme and other solute concentrations affect partitioning significantly.



	1013	513	353	153	97
⊠ER	0.87	0.70	0.78	0.96	1.00
□CR	0.57	0.57	0.57	0.57	0.57
⊠PR	0.11	0.13	0.00	0.00	0.00
▲PF	2.00	1.70	0.60	0.96	1.06
×K	2.88	1.02	1.54	11.50	∞

PPO Activity in the Crude Extract, U/ml

Figure 3.12 Effect of crude enzyme concentration on partitioning. System composition: 8% TX-114, pH 6.0, T: 37ºC.

3.9 Effect of Temperature on Partitioning

Phase volumes were sensitive both to surfactant composition and to temperature. At lower temperatures with the same surfactant concentration, and at the same temperature with a higher cloud point surfactant, the lower phase volume was greater. Since most of the surfactant partitions into the lower phase in all cases,

the increased volume must represent greater water content. This is consistent with greater hydration of oxyethylene chains at lower temperatures and greater hydration of longer chains at constant temperature (Ganong and Delmore, 1991). When ATPS was performed at room temperature instead of 37ºC, for the first time PPO was obtained in a more concentrated form. Concentration ratios increased 4 fold and 3.5 fold at pH 6 and pH 7, respectively. Enzyme recovery, phenolics removal, partition coefficient values increased with increase in temperature. Purification factor values remain almost constant with change in temperature (Figures 3.13 and 3.14). Therefore, aqueous two-phase separations can be performed at room temperature to concentrate the enzyme in the expense of reducing enzyme recovery.

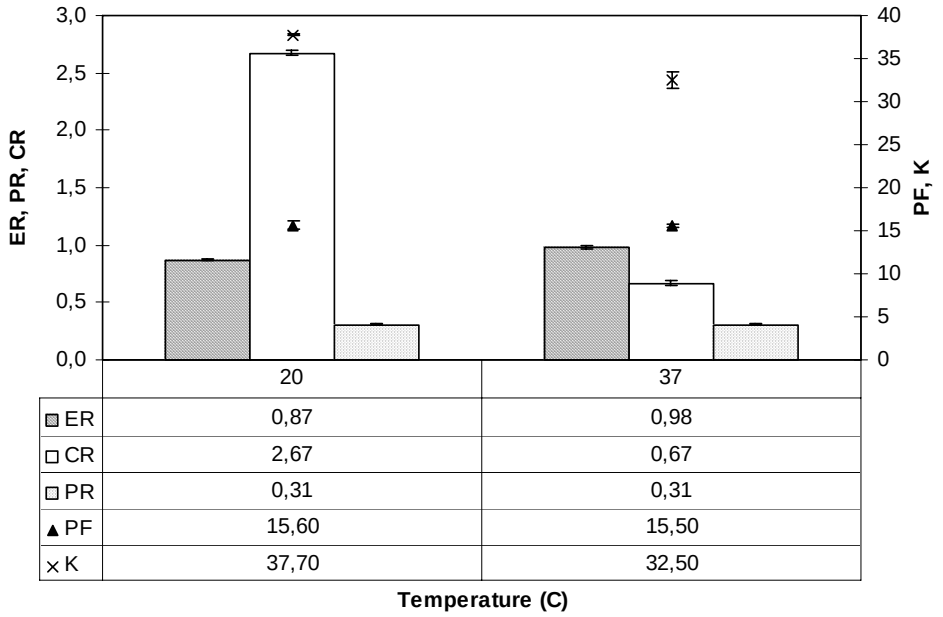


Figure 3.13 Effect of temperature on partitioning. System composition: 8% TX-114, pH 6.0.

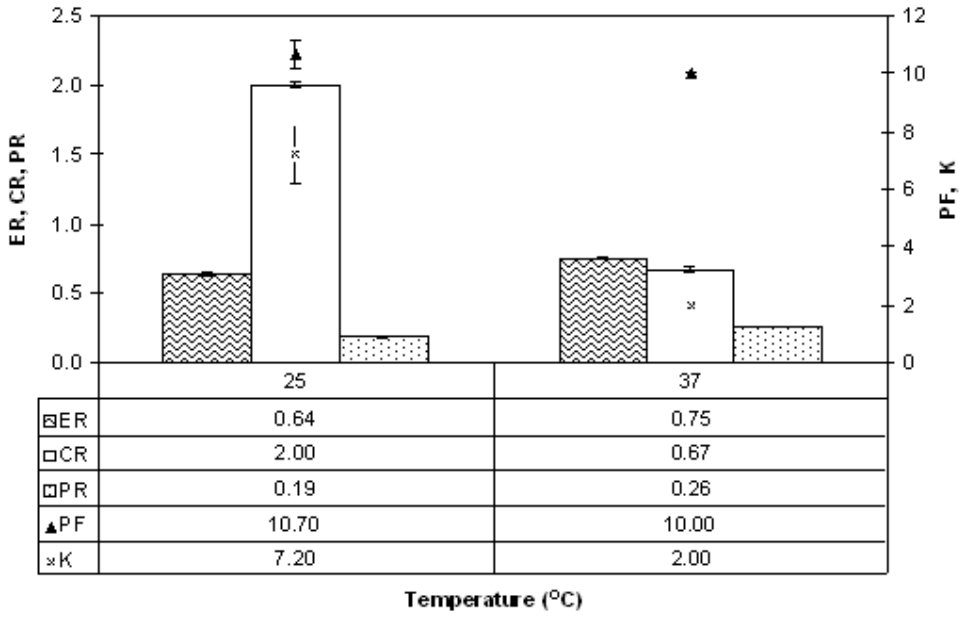


Figure 3.14 Effect of temperature on partitioning. System composition: 8% TX-114, pH 7.0

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

Mushroom polyphenoloxidase was separated by using TX-114/water aqueous-two phase system and PPO generally partitioned to the surfactant depleted top phase. The effects of surfactant concentration, pH, temperature, ionic strength, crude extract preparation and concentration on PPO partitioning were studied. Slightly acidic pH increased partitioning and purification of PPO to the surfactant-depleted top phase. PPO had a preference to the surfactant-depleted top phase in most of the studies except high surfactant concentrations and salt addition. Increasing ionic strength by adding potassium or sodium chloride changed the direction of PPO partitioning to the surfactant-enriched bottom phase. Change in surfactant concentration affected PPO partitioning in a different way. PPO partitioned more into the bottom phase by increasing surfactant concentrations over 16%. The optimum concentration for PPO partitioning was 8% (w/v) TX-114. Complete enzyme recoveries were achieved either by using ultrafiltration as a pretreatment

of crude extract or using PVPP together with reduction of pH to 6.0. Purification was also enhanced by using PVPP together with pH reduction. Enzyme and other solute concentrations affect the partitioning significantly. In general, recovery and purification decreased with decreased crude enzyme concentration. PPO could only be concentrated by reducing the temperature to a few degrees above the cloud point. So, aqueous two-phase partitioning can be performed at room temperature to concentrate the enzyme in the expense of reducing enzyme recovery.

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APPENDIX A

PREPARATION OF BRADFORD REAGENT

Chemicals listed below are used for the preparation of (5x stock) concentrated stock solution

- 500 mg Coomassie Brilliant Blue G-250 dye
- 250 ml 95% ethanol (spectroscopic)
- 500 ml 85% orthophosphoric acid)

500 mg Coomassie Brilliant Blue G-250 is dissolved in 250 ml 95% ethanol. To this solution, 500 ml 85% (w/v) orthophosphoric acid is added. The resulting solution is diluted to a final volume of 1L. This concentrated stock solution is diluted by mixing 1 volume concentrate with 4 volumes distilled water. The solution is mixed well and filtered through Whatman # 1 paper. Bradford reagent is ready to be stored at refrigeration temperature.

APPENDIX B

PREPARATION OF PROTEIN STANDARD

In 1976 Bradford published a rapid and sensitive method for determining the amount of protein in a sample (Bradford, 1976). The method has several advantages over previous methods of protein quantitation. The Bradford method is quite fast and convenient and has few of the interferences that many of the older methods were subjected to. The Bradford method utilizes the binding of Coomasie Brilliant Blue G 250 dye to proteins. The dye has both a blue and red form. When this dye binds to a protein, the red form is converted to blue form and the absorption maximum of the dye shifts from 465 nm to 595 nm. The binding is very rapid and reproducible. The protein-dye complex remains stable in solution for one hour. Protein samples are mixed with an excess amount of dye and allowed to react for at least two minutes, and then the absorption at 595 nm is measured in an hour. By comparison to protein standards, the amount of protein in an unknown sample can be easily determined.

Bovine serum albumin (BSA) is used as a standard. BSA is diluted in phosphate buffer (pH 7) to obtain a stock solution of 1mg/ml concentration.

After preparation of the samples 5ml Bradford reagent is added in the tube and the optical density is measured at 595nm after 5 minutes

Table B1 Preparation of protein standard curve

Protein (mg/ml)	0	0.02	0.04	0.06	0.08	0.10
BSA stock (µl)	0	10	20	30	40	50
Buffer (µl)	500	490	480	470	460	450

Table B2 Preparation of protein standard curve when 1% TX-114 was added

Protein (mg/ml)	0	0.02	0.04	0.06	0.08	0.10
TX-114 (w/v) %	0.25	0.25	0.25	0.25	0.25	0.25
TX-114 stock(µl)	125	125	125	125	125	125
BSA stock (µl)	0	10	20	30	40	50
Buffer (µl)	375	365	355	345	335	325

BSA stock: 1 µg/µl

TX-114 stock: 10 µg/µl

Table B3 Preparation of protein standard curve when 0.15% TX-114 was added

Protein (mg/ml)	0	0.02	0.04	0.06	0.08	0.10
TX-114 (w/v) %	0.15	0.15	0.15	0.15	0.15	0.15
TX-114 conc. (µl)	75	75	75	75	75	75
BSA stock conc. (µl)	0	10	20	30	40	50
Buffer (µl)	425	415	405	395	385	375

Table B4 Preparation of protein standard curve when 0.10% TX-114 was added

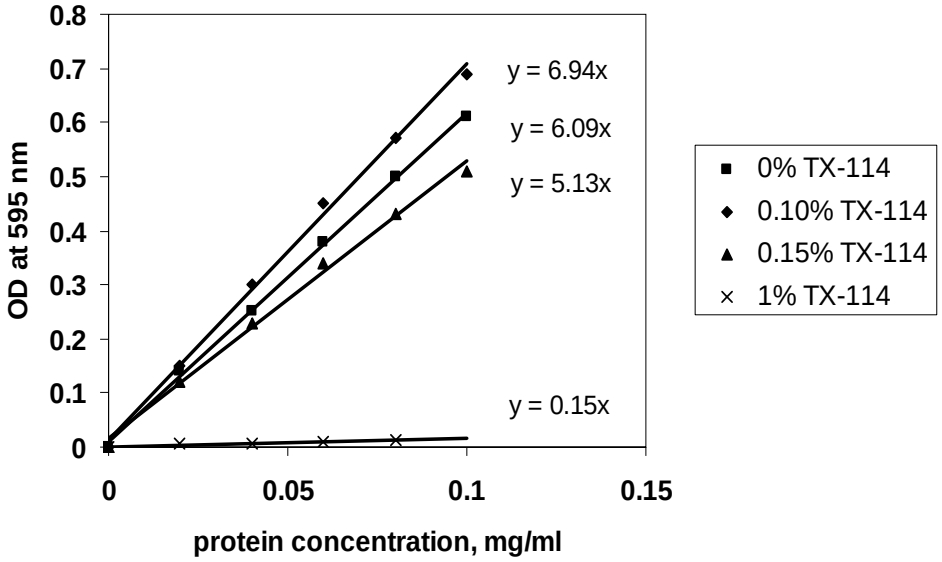
Protein (mg/ml)	0	0.02	0.04	0.06	0.08	0.10
TX-114 (w/v) %	0.1	0.1	0.1	0.1	0.1	0.1
TX-114 conc. (µl)	50	50	50	50	50	50
BSA stock conc. (µl)	0	10	20	30	40	50
Buffer (µl)	450	440	430	420	410	400

Aqueous two phase system was formed with TX-114 so protein concentration must be determined correctly in the presence of dilute concentrations of TX-114. Experiments were done to investigate the TX-114 concentration having no

interference with protein measurement. It was observed that the addition of 0.15% TX-114 (w/v) did not effect the protein measurement. Note that TX-114 was diluted in phosphate buffer (pH=7) to obtain a stock solution of 0.01 g/ml.

APPENDIX C

STANDARD CURVE FOR BRADFORD METHOD



APPENDIX D

PREPARATION OF PHENOLICS STANDARD

The Folin-Ciocalteau reagent contains phosphomolybdic and phosphotungstic acids which are reduced to heteropoly blue in alkaline solution. The reagent has been employed to determine humic substances in soils and freshwaters, polyphenolic compounds in lake sediments, and a wide range of phenolic substances Reagent is used in standard methods for measurement of tannins and lignins (APHA, 1989).

The method used the Folin-Ciocalteau reagent (Sigma). The reagent was stored in dark bottle at room temperature and renewed approximately every 6 months.

The general method involved the successive addition of following chemicals to 10 ml sample.

- 1.5 ml sodium carbonate (200 gl^{-1})
- 0.5 ml Folin- Ciocalteau reagent

After 60 min at 20ºC, the absorbance was measured at 750 nm in 4 ml cuvettes against distilled water and corrected for the absorbance of a distilled water reagent blank.

Catechol was used as a standard. Catechol was diluted in phosphate buffer to obtain a stock solution of 0.1 mg/ml concentration. The effect of only protein and only surfactant addition or protein and surfactant addition on catechol quantitation was studied. BSA was diluted in phosphate buffer to obtain a stock solution of 1 mg/ml concentration. TX-114 was used directly (TX-114 (+4 C) density = 1.05 g/ml). Addition of BSA to a final concentration of 0.1 mg/ml and addition of TX-114 to a final concentration of 6% (w/v) or addition of both at the same time didn't affect the determination of phenolics content.

Table D1 Preparation of phenolics standard curve

Final phenolics conc. (mg/ml)	0	0.002	0.004	0.006	0.008	0.010
Catechol stock(µl)	0	100	200	300	400	500
Buffer(µl)	5000	4900	4800	4700	4600	4500

Table D2 Preparation of phenolics standard curve

Final phenolics conc. (mg/ml)	0	0.002	0.004	0.006	0.008	0.010
Final protein conc. (mg/ml)	0.1	0.1	0.1	0.1	0.1	0.1
Phenol stock(µl) (catechol)	0	100	200	300	400	500
Protein stock(µl) (BSA)	500	500	500	500	500	500
Buffer(µl) (sodium phosphate)	4500	4400	4300	4200	4100	4000

Table D3 Preparation of phenol standard curve

Final phenolics conc. (mg/ml)	0	0.002	0.004	0.006	0.008	0.010
Final TX-114 conc. % (mg/ml)	6	6	6	6	6	6
Phenolics stock(µl) catechol	0	100	200	300	400	500
TX-114 (µl)	286	286	286	286	286	286
Buffer(µl) (sodium phosphate)	4714	4614	4514	4414	4314	4214

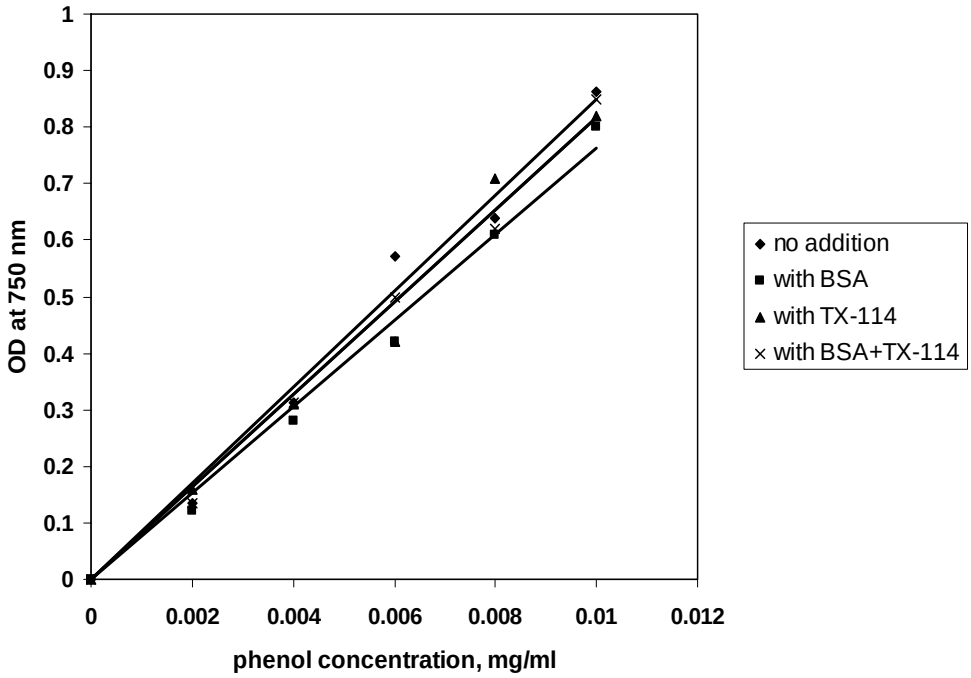
Table D4 Preparation of phenolics standard curve

Final phenolics conc. (mg/ml)	0	0.002	0.004	0.006	0.008	0.010
Final protein conc. (mg/ml)	0.1	0.1	0.1	0.1	0.1	0.1
Final TX-114 % (mg/ml)	6	6	6	6	6	6
Phenolics stock(µl) (catechol)	0	100	200	300	400	500
Protein stock(µl) (BSA)	500	500	500	500	500	500
TX-114 (µl)	286	286	286	286	286	286
Buffer (µl)	4214	4114	4014	3914	3814	3714

After preparation of samples 0.75 ml sodium carbonate (200 gl^{-1}) and 0.25 ml Folin- Ciocalteau reagent are added successively and after 1hour the optical density is measured at 750nm. It is observed that to add 0.1 mg/ml protein (BSA) to the sample doesn't effect the phenol content determination.

APPENDIX E

STANDARD CURVE FOR FOLIN CIOCELTEAU METHOD



APPENDIX F

DETERMINATION OF TRITON X-114 CONTENT

Table F1 Dilutions for TX-114 standard curve

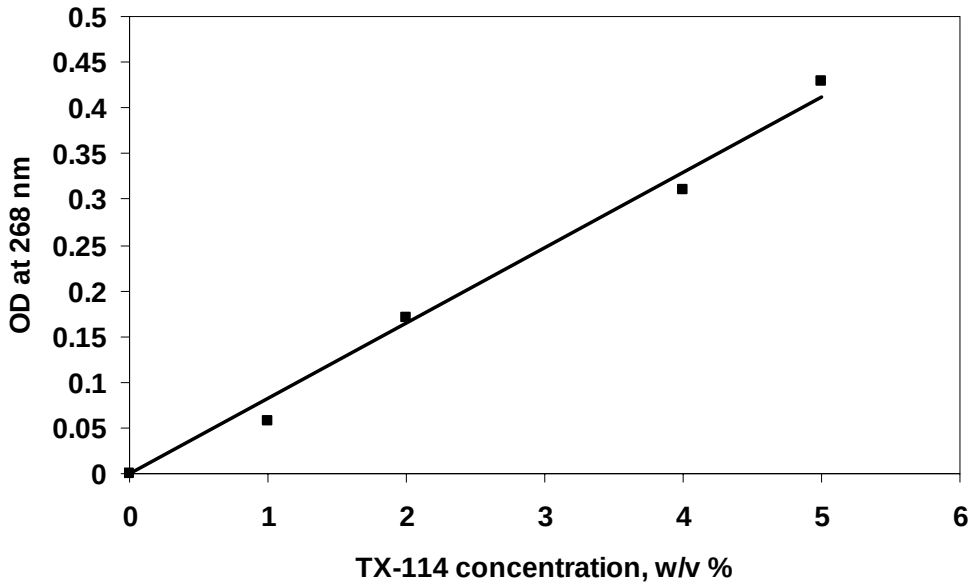
TX-114 %(w/v)	0	1	2	4	5
TX-114 (µl)	5000	4950	4905	4810	4762
Buffer (µl)	0	47.6	95	190	238

Density (TX-114): 1.05

After preparing the samples, to maintain the solubility at room temperature, samples are diluted in 0.5 % sodiumdodecylsulfate (SDS) (dil. 1:270). Then the optical density (O.D.) is measured as reference at 268 nm to determine TX-114 concentration using glass cuvettes.

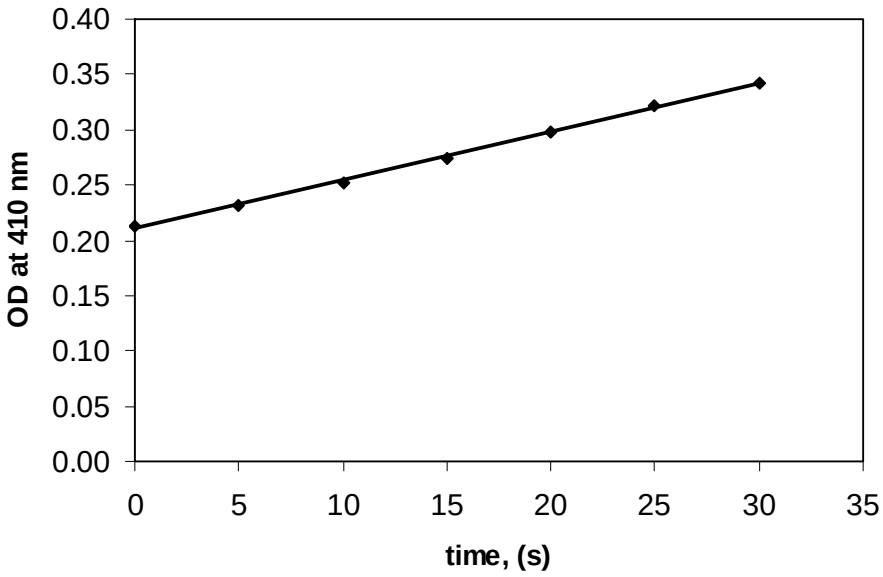
APPENDIX G

STANDARD CURVE FOR TRITON X-114



APPENDIX H

A SAMPLE ENZYME REACTION PROFILE



After preparing the samples, 0.2 M catechol solution is added into the samples. Then the optical density (O.D.) is measured at 410nm for every five seconds.

APPENDIX I

DATA RELATED TO CHAPTER 3

Table I.1 ATPS with 4% TX-114

	TA (U)	SA (U/mg)	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract*	322±14	805	0.4±0.14	0.096±0.01	_	_
Top phase	276±8	1255	0.22±0.04	0.094±0.009	4±0.1	1±0.1

* Total PPO activity used in the ATPS

Table I.2 ATPS with 8% TX-114

	TA (U)	SA (U/mg)	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract*	294±18	1972	0.146±0.004	0.1±0.008	_	_
Top phase	281±24	3863	0.073±0.003	0.1±0.010	3.7±0.2	1.3±0.2

* Total PPO activity used in the ATPS

Table I.3 ATPS with 6% TX-114 when there is dilution

	TA (U)	SA (U/mg)	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract*	100±18	2941	0.034±0.01	0.079±0.008	–	–
Top phase	101±12	2971	0.034±0.007	0.077±0.006	4.2±0.1	0.8±0.1

* Total PPO activity used in the ATPS

Table I.4 ATPS with 8% TX-114 when there is dilution

	TA (U)	SA (U/mg)	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract*	86±10	2263	0.038±0.001	0.0104±0.002	–	–
Top phase	60±12	1579	0.038±0.003	0.0100±0.003	4±0.2	1±0.2

* Total PPO activity used in the ATPS

Table I.5 Effect of TX-114 concentration on catechol separation. Total catechol added to the system was 0.1 mg/ml

TX-114 % (w/v)	phenol content in the top phase (mg)	PR
2	0.0989±0.0010	0.01
4	0.0588±0.0004	0.41
6	0.0546±0.0001	0.45
8	0.0494±0.0001	0.51
10	0.0432±0.0001	0.57

Table I.6 Effect of TX-114 concentration for phenolics removal

TX-114 % (w/v)	phenol content (mg)	PR
Crude extract	0.0902±0.00500	1.00
6	0.0902±0.00600	0.00
8	0.0807±0.00700	0.10
10	0.0805±0.00500	0.11
12	0.0740±0.00200	0.18
Crude extract	0.1050±0.00950	1.00
14	0.0847±0.00600	0.20
16	0.0693±0.00010	0.34
18	0.0513±0.00005	0.51
20	0.0530±0.00007	0.50

Table I.7 Effect of Triton X-114 concentration on PPO partitioning

	TA (U)	SA (U/mg)	Protein (mg)	V_t (ml)	V_b (ml)
Crude extract*	550±31	1375	0.40±0.100	_	_
Top phase (6%)	403±14	3358	0.12±0.010	4.2±0.1	0.8±0.1
Top phase (8%)	451±11	2910	0.16±0.040	3.7±0.1	1.3±0.1
Top phase (10%)	382±13	1910	0.20±0.020	3.5±0.1	1.5±0.1
Top phase (12%)	422±15	3246	0.13±0.040	3.3±0.1	1.7±0.1
Crude extract	866±8	1397	0.62±0.050	_	_
Top phase (14%)	594±4	1414	0.42±0.060	3.0±0.1	2.0±0.1
Top phase (16%)	426±5	722	0.59±0.004	2.8±0.2	2.2±0.2
Top phase (18%)	440±5	1100	0.40±0.004	2.0±0.1	3.0±0.1
Top phase (20%)	385±14	895	0.43±0.003	1.8±0.2	3.2±0.2

* Total PPO activity used in the ATPS

Table I.8 Effect of using ultrafiltration as a pretreatment on PPO partitioning

	TA (U)	SA (U/mg)	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract*	798±13	4534	0.176±0.0090	0.08±0.00600	_	_
After U.F	504±13	5600	0.090±0.0003	0.02±0.00003	_	_
Top phase (4%)	500±25	9434	0.053±0.0007	_	4.2±0.1	0.8±0.1
Top phase (6%)	500±10	11905	0.042±0.0006	_	4.0±0.1	1.0±0.1
Top phase (8%)	455±8	12297	0.037±0.0001	_	3.5±0.1	1.5±0.1
Top phase (10%)	408±25	7158	0.057±0.0001	_	3.0±0.1	2.0±0.1

* Total PPO activity used in the ATPS

Table I.9 Effect of using two step ultrafiltration as a pretreatment on PPO partitioning.

	TA (U)	SA (U/mg)	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract*	792±36	1980	0.400±0.0300	0.1078±0.0030	_	_
After U.F	110±12	2340	0.047±0.0003	0.00485±0.0001	_	_
Top phase (4%)	110±7	5789	0.019±0.0030	_	4.4±0.1	1.6±0.1
Top phase (6%)	110±6	9167	0.012±0.0006	_	4.0±0.1	2.0±0.1
Top phase (8%)	110±3	11000	0.010±0.0001	_	3.5±0.1	1.5±0.1
Top phase (10%)	110±4	11458	0.0096±0.0003	_	3.2±0.1	1.8±0.1

*Total PPO activity used in the ATPS

Table I.10 Effect of PVPP addition as a pretreatment on PPO partitioning

	TA (U)	SA (U/mg)	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract*	–	–	–	0.11±0.005	–	–
After PVPP ad	554±13	832	0.666±0.1	0.093±0.005	–	–
Top phase (4%)	361±7	4570	0.079±0.0007	0.092±0.0001	4.4±0.1	0.6±0.1
Top phase (6%)	356±11	5933	0.060±0.0006	0.083±0.0003	4.0±0.1	1.0±0.1
Top phase (8%)	396±19	8426	0.047±0.0010	0.077±0.001	3.5±0.1	1.5±0.1
Top phase (10%)	288±7	10286	0.028±0.0010	0.067±0.0007	3.0±0.1	2.0±0.1

* Total PPO activity used in the ATPS

Table I.11 Effect of pH on PPO partitioning. The concentration of TX-114 was 8% (w/v)

	TA (U)	SA (U/mg)	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract at pH=6 *	434±34	286	1.52±0.0012	0.108±0.00200	_	_
Crude extract at pH=7 *	564±24	320	1.76±0.0012	0.120±0.00300	_	_
Crude extract at pH=7.5 *	416±48	260	1.60±0.0021	0.086±0.00300	_	_
Crude extract at pH=8 0 *	444±24	404	1.10±0.0004	0.094±0.00200	_	_
Top phase						
pH=6	426±18	4427	0.096±0.0019	0.075±0.00040	3.0±0.1	2.0±0.1
pH=7	423±31	3209	0.130±0.0050	0.084±0.00450	3.5±0.1	1.5±0.1
pH=7.5	266±17	2038	0.130±0.0056	0.070±0.00150	3.5±0.1	1.5±0.1
pH=8	259±10	1898	0.137±0.0003	0.070±0.00150	3.5±0.1	1.5±0.1

***** Total PPO activity used in the ATPS

Table I.12 Effect of NaCl addition on partitioning.

	TA (U)	SA (U/mg)	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract*	336±12	1244	0.27±0.02	0.076±0.00700	–	–
Top phase NaCl %						
0	330±13	19412	0.017±0.0005	0.052±0.00040	3.00±0.1	2.00±0.1
1.5	72±3	655	0.110±0.0030	0.064±0.00015	3.75±0.1	1.25±0.1
3	41±3	456	0.090±0.0024	0.058±0.00012	3.75±0.1	1.25±0.1
6	36±3	735	0.049±0.0030	0.058±0.00012	3.75±0.1	1.25±0.1

* Total PPO activity used in the ATPS

Table I.13 Effect of KCl addition on partitioning.

	TA (U)	SA (U/MG)	Protein (MG)	V_t (ML)	V_b (ML)
Crude extract*	336±12	1244	0.27±0.02	–	–
Top phase KCl %					
0	330±13	19412	0.017±0.0005	3.00±0.1	2.00±0.1
1.5	98±3	803	0.122±0.0017	3.50±0.1	1.50±0.1
3	42±3	396	0.106±0.0018	3.50±0.1	1.50±0.1
6	13.3±28	200	0.066±0.0023	3.50±0.1	1.50±0.1

* Total PPO activity used in the ATPS

Table I.14 Effect of crude enzyme concentration on partitioning

	TA (U)	SA U/mg	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract*	528±36	2200	0.24±0.01	0.072±0.0070	_	_
X9	2026±10	3268	0.62±0.0030	0.113±0.0014	_	_
X6	1022±10	2174	0.47±0.0030	0.085±0.0011	_	_
X3	706±14	4584	0.154±0.0006	0.047±0.0007	_	_
X1	305±4	4621	0.066±0.0020	0.023±0.0005	_	_
X0.5	194±2	4409	0.044±0.0003	0.019±0.0002	_	_
Top phase						
X9	1764±42	6368	0.277±0.0010	0.1±0.0007	3.5±0.1	1.5±0.1
X6	720±46	3600	0.200±0.0006	0.074±0.0007	3.5±0.1	1.5±0.1
X3	552±10	3807	0.145±0.0003	0.048±0.0005	3.5±0.1	1.5±0.1
X1	294±25	4455	0.066±0.0003	0.023±0.0001	3.5±0.1	1.5±0.1
X0.5	195±38	4664	0.0416±0.0003	0.019±0.0001	3.5±0.1	1.5±0.1

* Total PPO activity used in the ATPS

Table I.15 Effect of temperature on partitioning

	TA (U)	SA (U/mg)	Protein (mg)	Phenol (mg)	V_t (ml)	V_b (ml)
Crude extract at pH=6*	398±11	2211	0.1800±0.03000	0.079±0.0030	–	–
Top phase (T=20ºC)	346±22	34600	0.0100±0.00006	0.078±0.0001	0.75±0.1	4.25±0.1
Top phase (T=37ºC)	390±36	34210	0.0114±0.00300	0.066±0.0008	3.00±0.1	2.00±0.1
Crude extract at pH=7 *	514±36	2570	0.2000±0.02000	0.080±0.004	–	–
Top phase (T=20ºC)	331±7	27583	0.0120±0.00010	0.045±0.0001	1.00±0.1	4.00±0.1
Top phase (T=37ºC)	385±19	25667	0.0150±0.00020	0.013±0.0014	3.00±0.1	2.00±0.1

* Total PPO activity used in the ATPS