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Review

Triton X-114 phase partitioning in plant protein purification

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Abstract

A brief overview is given of how Triton X-114 can be used not only to solubilize plant membranes but also as an excellent reagent in a bulk fractionation method to purify enzymes compared with the classical drastic methods using acetone powder or ammonium sulphate fractionation. Triton X-114 removes the tenacious phenols and chlorophylls on centrifugation. There is no need to use insoluble synthetic resins or organic solvents as Triton X-114 is so mild that the enzymes can be extracted in their natural form without activating them. The methods developed with Triton X-114 are easily reproducible and sufficiently cheap to be used in large-scale purification procedures. The classical topological use of Triton X-114 in plant membranes is also discussed.

Contents

1. Introduction

Protein purification from a complex biological crude extract containing thousands of different proteins and biomolecules is often a difficult multi-step process [1]. The time needed and the steps involved in developing a protein purification procedure depend on the protein, the starting material and the final use for which it is intended and the starting material. In plants, the isolation of enzymes is complicated by the existence of an important secondary metabolism [2], which produces highly reactive phenols and tannins that are *in vivo* confined to vacuoles.

During purification, these reactive compounds interact with proteins to produce both covalent and non-covalent complexes. To overcome this problem, three different methods have been used traditionally: the use of reducing agents (ascorbic acid, metabisulphite, etc.) to avoid the oxygen-driven oxidation of phenols to quinones, insoluble hydrophobic phenol-binding resins

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(*e.g.*, polyvinylpyrrolidone, Amberlite XAD-2) [3] and organic solvents (acetone, ethanol, etc.). However, in many instances their use is ineffective or can lead to partial enzyme inhibition or denaturation and/or the uncontrolled activation of the native inactive forms (latent enzymes).

An additional problem in acrial tissues is the release of chlorophylls, which occurs with the breakdown of chloroplasts during homogenization or during the digestion of their organelle membranes by detergents [4]. The resulting green extracts can be clarified by ammonium sulphate fractionation or the use of acetone powder, although these agents may alter the conformational structure of the protein and lead to a loss of certain activities in multifunctional enzymes.

Recent developments in the purification of plant proteins have been reviewed [5]. The topics discussed include immunoaffinity chromatography, subunit affinity chromatography, immobilized metal affinity chromatography (IMAC) and the use of detergents in protein isolation [6,7].

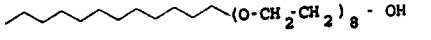
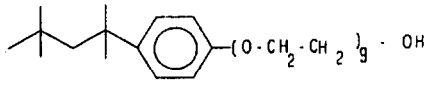
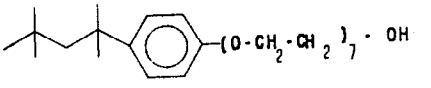
This paper presents a brief overview of the special characteristics of polyoxyethylene-type non-ionic detergents, especially Triton X-114 (TX-114), which can be used in the solubilization and purification of plant enzymes owing to their ability to produce aqueous two-phase systems in the biocompatible temperature range (4–30°C). TX-114 is particularly useful for purifying polyphenol oxidase (PPO) from both aerial and non-aerial plant tissues. This enzyme is a good example of a plant protein that is difficult to purify as it presents all the above-mentioned purification problems.

2. Physico-chemical characteristics of polyoxyethylene-type non-ionic detergents

Organic compounds that are insoluble or barely soluble in water can often be brought into aqueous solution by the addition of suitable surfactants. This phenomenon, called solubilization, has been the subject of much research involving ionic surfactants, although many fewer data are available concerning the use of non-ionic surfactants [8]. Surfactant molecules form micelles in aqueous solution 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. In our case, the solute is the protein that is to be solubilized from a particular membrane.

Among non-ionic surfactants, the polyoxyethylene type represents a large group whose formulae are shown in Table 1. 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 detergents

Table 1
Structures and properties of some polyoxyethylene-type non-ionic detergents [19]

Formula	Abbreviation	Trivial name	cmc (m*M*)	*N*[a]	HLB[a]	CP[a] (1%, w/v)
	$C_{12}E_8$		0.087	120	13.7	75
	t-$C_8\phi E_9$	Triton X-100	0.30	140	13.5	65
	t-$C_8\phi E_7$	Triton X-114	0.17	140	12.4	22

[a] *N* = aggregation number; HLB = hydrophilic–lipophilic balance; CP = cloud point (°C).

are solubilized in water by the hydration of the ether oxygens of the polyoxyethylene groups [9]. An increase in temperature leads to a decrease in the number of hydrogen bonds, which raises the micellar mass and decreases the cmc [10]. 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 [11,12]. This temperature is called the cloud point. A further rise in temperature causes the solution to begin to separate into two phases, one detergent-rich and the other detergent-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 22°C for TX-114 (7 units) to 67°C for Triton X-100 (TX-100) (9 units) [13]. Surfactant concentration is also important [14] because, for example, a 3% (w/v) concentration is necessary for C_6E_3 to partition at 37°C. However, surfactant concentration has less influence than the concentration of the additives, which are usually inorganic salts and hydrocarbons.

Normally, salts lower the cloud point owing to their salting-out effect. This effect is substantial with TX-100, especially when sulphate salts are added to the solution (Fig. 1) [14]. The salting-out effect with TX-114 is of biological importance as only low salt concentrations are needed to lower the cloud point to below 22°C [15]. However, high salt concentrations should be avoided in order to prevent enzyme inhibition by salts, mainly Cl^-, and to permit the use of ion-exchange chromatography in subsequent purification steps [16].

Saturated hydrocarbons do not lower the cloud point temperature to any great extent and can sometimes even raise it, whereas aliphatic alcohols, fatty acids and phenols lower the cloud point substantially [8].

Among the alcohols, glycerol has been used in conjunction with TX-114 in the isolation of the plant cytochromes P-450 and b_5, as its addition lowers the cloud point to 4°C [16]. Glycerol reduces TX-114 micelle solvation by decreasing

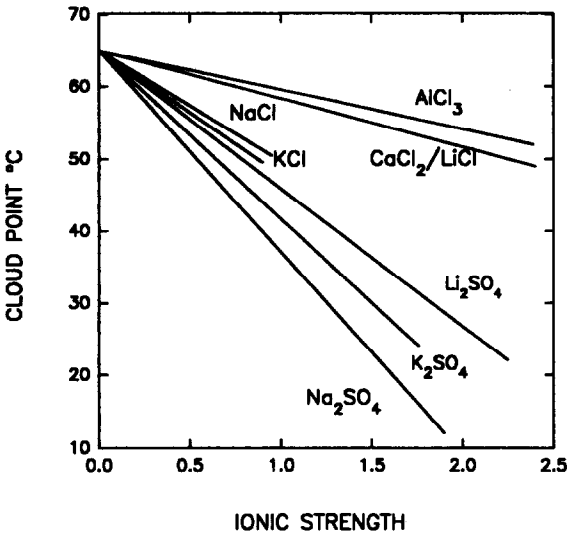


Fig. 1. Effect of added electrolytes on the cloud point of 2% TX-100 solutions [14].

the number of hydrogen bonds formed by the detergent's polyoxyethylene groups with water.

Other detergents that are sometimes added to digest biological membranes strongly influence the cloud point of polyoxyethylene-type detergents, the resulting cloud point being between those of the two individual surfactants. In fact, the cloud point of TX-114 can be decreased by adding Triton X-45 ($m = 5$), which has a lower cloud point. The combination of these two detergents (9:1) has been used to isolate phosphatidylinositol kinase at 9°C [17]. As is to be expected, the opposite occurs (*i.e.*, the cloud point is raised) when more hydrophilic detergents (deoxycholate, TX-100 and *n*-octyl glucoside) are used because of the increased solubilization of TX-114 [18].

Finally, phospholipids released from plant membranes also lower the cloud point to an extent that depends on the lipidic nature of the membrane. With plant microsome membranes, the temperature is lowered by 5°C [16].

3. Biomolecule phase separation in PEG non-ionic detergents

Non-ionic polyoxyethylene detergents have been used in various protein membrane studies.

TX-100 is probably the most widely used and best characterized of commercially available polydisperse compounds. It possesses an alkylaryl hydrophobic and a hydrophilic moiety composed of a polyoxyethylene chain with an average of 9–10 oxyethylene units. Its usefulness stems from its high solubility, low cost, mildness in solubilizing membrane proteins, low cmc and relatively large micelle size [19].

TX-114 shares the same properties as TX-100 but is insoluble in buffers at room temperature because of its low cloud point. This explains why TX-114 was not used as a biological detergent until Bordier [18] used it in 1981 to separate membrane proteins according to their different partitioning behaviour after phase separation. Bordier demonstrated that proteins which had been dissolved in TX-114 solution at 4°C (below the cloud point) partition between the lower (detergent-rich) and the upper (detergent-depleted) phase when the solution is warmed above the cloud point. The distribution appeared to depend on the protein's relative hydrophobicity: hydrophobic integral membrane proteins partitioned into the lower phase, whereas hydrophilic globular proteins partitioned into the upper phase. Thermal phase partitioning has since been employed as an analytical procedure is cellular and molecular biology [20]. Their distribution during phase separation has been used to indicate the cellular location of various proteins in membranes and cytoplasm. However, such a correlation is not always accurate, especially with integral membrane proteins with very large hydrophilic regions (glycoproteins), such as *Torpedo* nicotinic acetylcholine receptor (AchR) and the α subunit of the Na^+,K^+-ATPase of kidney microsome membranes, both of which partition into the aqueous phase [21].

4. Purification of plant enzymes by temperature-induced phase partitioning in Triton X-114

TX-114 was first used in plants by Bricker and Sherman [22] to assist in the investigation of the topological orientation of maize chloroplast thylakoid membranes. These membranes were first enzymatically labelled with ^{125}I or treated with trypsin and then phase fractionated with TX-114. The integral polypeptides which resolved in the detergent-rich phase were CP1 apoprotein, the PSII reaction centre polypeptides I and II, cytochrome *f* and b_6 and the major LHC apoprotein. Among peripheral polypeptides retained in the aqueous phase, the α and β subunits of CF1 were predominant.

These topological studies were extended to the other plant membranes and species. For example, the hydrophobicity of protochlorophyllide oxidoreductase from wheat etioplast membrane fractions was clearly demonstrated to be higher than that of ATP synthase polypeptides and ribulose bisphosphate carboxylase oxygenase and so this oxidoreductase was classified as an integral membrane protein with a hydrophilic domain that binds its water-soluble cofactor, NADPH [23].

Plasma membrane vesicles were also studied using TX-114. In spinach leaf, 80% of the protein was recovered in the hydrophobic phase of TX-114 and the remaining 20% in the aqueous phase [24]. Peripheral proteins were largely located in the inner, cytoplasmic surface of the plasma membrane.

Bulk protein fractionation in TX-114 was first used by our group in the purification of grape PPO [25] to avoid the difficulties normally associated with the purification of the phenol-oxidizing enzyme, as its self-generated *o*-quinones can sometimes modify or inactivate the enzyme. In addition, grape berry polyphenol oxidase (GB-PPO) was discovered to be a thylakoid-bound enzyme, as only detergents were able to extract from 1 to 100% of activity depending on the characteristics of the detergent used, the non-ionic detergents (TX-100, TX-114) being the most effective [25]. Ionic strength and typsin alone extracted only 2% and 10%, respectively.

Among non-ionic detergents, TX-114 solubilized the protein and chlorophylls with the same efficiency as TX-100. However, unlike TX-100, TX-114 failed to maintain all of the proteins and chlorophylls in solution at 4°C. This was used to

advantage, because after a few minutes a dark precipitate was formed due to the aggregation of large mixed micelles of TX-114, which contained membrane proteins, phospholipids, phenols and chlorophylls. After high-speed centrifugation, the supernatant was slightly green, clearly indicating that chlorophylls and phenols had been eliminated from the original extract.

This removal of non-protein material by TX-114 at 4°C had never before been described. The total removal of unwanted compounds was achieved by subjecting the clear green supernatant to a classical temperature-induced phase partitioning [18], adding an additional 4% of TX-114 to the mixture and warming it at 37°C for 15 min [25]. After this phase partitioning, GB-PPO remained in the aqueous phase and, for the first time, as an inactive (latent) form similar to that previously described for other leaf thylakoid-bound PPO (spinach, broad bean).

Table 2 shows the results obtained when the TX-114 method was compared with the well established method for purifying GB-PPO involving ammonium sulphate fractionation [25]. The degree of purification was the same in both methods, although the recovery was greater in the TX-114 method. The enzyme purified by TX-114 was latent, and was activated 64-fold with trypsin compared with the two times it could be activated with the ammonium sulphate fractionation method. As much as 75–80% of phenols and chlorophylls were removed by TX-114 by ultracentrifugation without the need for any synthetic resin such as Amberlite XAD-2 or organic solvents. Complete removal was attained in the next step by temperature-induced phase partitioning in 4% TX-114.

The TX-114 method also preserved the two activities of PPO, the cresolase activity that oxidizes monophenols to *o*-diphenols and the catecholase activity that oxidizes diphenols to their corresponding quinones (Fig. 2) [26]. The first activity is very low compared with the catecholase activity and it is necessary to activate the latent enzyme to obtain any measurable activity. It should be noted that this activity presents a lag period, which is a consequence of the dynamic equilibrium between the enzymatic and chemical steps involved in the complex kinetic mechanism of this enzyme [27–30].

In addition, TX-114 avoids one of the problems normally encountered when plant PPO extracts are subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), namely the multiplicity of activity bands arising from the formation of artefactual bands caused by the covalent interaction of *o*-quinones and phenols with the enzyme [31].

Another characteristic of GB-PPO purification was its unexpected behaviour, as it was retained in the aqueous phase after phase partitioning. This is explained by the presence of a short hydrophobic tail, which anchors it to the membrane but which is not long enough to bind a sufficient number of detergent molecules to be precipitated with the larger micelles of TX-114 during temperature-induced phase partitioning [25]. This hypothesis is based on the different activations found in the latent enzyme with trypsin, detergents and lectins.

The TX-114 method was also used to purify previously described latent leaf thylakoid-bound PPO. With spinach [32] and broad bean leaves [33], 1.5% TX-114 partially removed the undesirable phenol and chlorophyll components on centrifugation at 4°C, when osmotically shocked chloroplast membranes were digested by the detergent. The remaining chlorophylls and phenols were removed after phase partitioning at 37°C in 8% TX-114.

Latent PPO remained in the aqueous phase in both instances with fivefold purification and recoveries of 86% and 43% for spinach and broad bean PPO, respectively [32,33]. The activation found in both latent enzymes was 5–10 times higher than that obtained in previously published methods (acetone powder and ammonium sulphate fractionation) [34], clearly demonstrating the mild purification in the TX-114 method compared with the classical methods.

The final example of leaf thylakoid-bound enzyme purified by the TX-114 method is potato leaf PPO, which had never been purified previously. The method used differed from the above TX-114 methods in that the second phase partitioning step must be repeated as the level of

Table 2
Partial purification of GB-PPO using both ammonium sulphate and TX-114 methods [35]

Method	Material	Total protein (mg)	Total activity (units)[a]		Specific activity (units/mg)	Purification (-fold)	Recovery (%)	Activation (%)	Chl (μg/ml)	Phenolic compounds (mg/ml)
			−Trypsin	+Trypsin						
Ammonium sulphate	Crude extract	15	500	500	33	1	100	100	11	42.5
	TX-100 extract	7.8	30	370	47	1.5	74	1233	16	14.5
	Supernatant of TX-100 extract	5.7	5.7	324	57	1.7	65	5684	13	12.1
	45–95% ammonium sulphate	0.6	50	101	168	5.1	20	200	ND[b]	0.5
TX-114	Crude extract	15	500	500	33	1	100	100	11	42.2
	TX-114 extract	7.8	10	270	47	1.4	74	3700	19	14.5
	Supernatant of TX-114 extract	4.3	5.5	322	75	2.3	65	5854	4	3.5
	Supernatant 4% of TX-114	1.9	5.0	320	168	5.1	64	6400	ND[b]	0.6

[a] Assayed with 4-methylcatechol as substrate.
[b] Not detected.

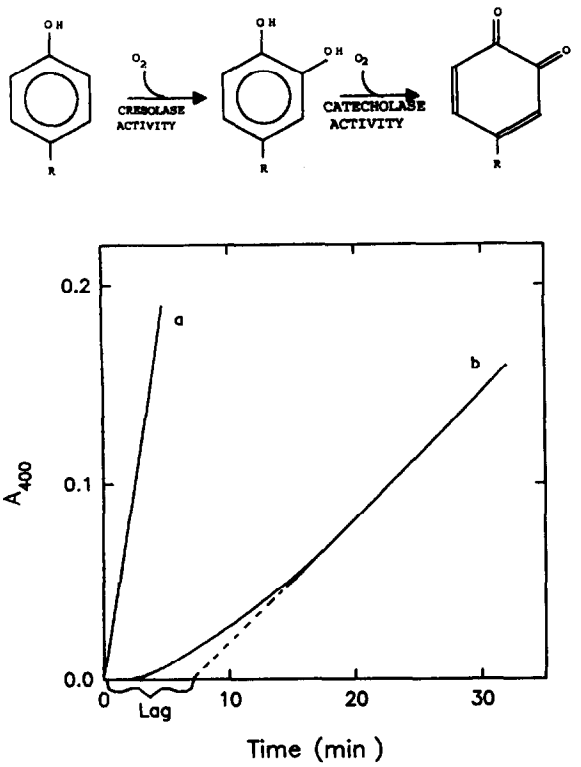


Fig. 2. Enzyme activities of trypsin-activated latent GB-PPO. (a) Catecholase activity (1 μg/ml of enzyme) using 24 m*M* 4-methylcatechol as diphenolic substrate; (b) cresolase activity (100 μg/ml of enzyme) using 2.1 m*M* *p*-cresol as monophenol.

phenols was higher than in the other leaves studied. This additional step is necessary because, at high phenol concentrations, the phenol-binding sites in the TX-114 micelles are saturated [35].

Recently, TX-114 was extended to non-aerial tissues, such as potato tubers [36]. The purification of potato tuber PPO is completely different from that of the leaves as the level of phenols, mostly as chlorogenic acid, is so high that the extract becomes black only a few seconds after homogenization. To avoid this, ascorbic acid, metabisulphite and insoluble phenol scavengers were used at high concentrations in previously published methods [37–39]. However, all of these are inhibitors of potato PPO and so the new strategy was to keep phenols away from the enzyme by adding 6% TX-114 to the homogenization buffer without any other phenol scavenger and to insolubilize the starch by keeping the homogenate at 4°C for 90 min. The extract remained light yellow during this time owing to the presence of TX-114. After centrifugation, the starch was removed and the supernatant subjected to phase partitioning by increasing the TX-114 concentration by a further 4% and warming it for 15 min.

An attempt was made to activate the PPO thus obtained in the aqueous phase with trypsin or detergents, but no activation was found [36]. As potato tuber PPO was present in its active form, ammonium sulphate fractionation could be used to purify it further. The degree of purification finally achieved was fivefold, similar to that obtained with the previously described methods [37,38], although the recovery was lower, owing to the transfer of particulate PPO to the detergent-rich phase. This particulate enzyme has been evaluated and represents over half of the enzyme activity [40].

Phenols were almost totally removed, only 3% remaining. This prevented the enzyme solution from browning and enabled enzyme stability to be preserved for months at −20°C. This removal of phenols also avoids the multiplicity of enzyme bands found with SDS-PAGE for potato PPO (up to eighteen bands) when it is purified with ammonium sulphate alone, without TX-114 [41].

Finally, the purification of cinnamic acid 4-hydrolase, a plant cytochrome P_{450}, is worth mentioning as it is the first example of a plant enzyme, which remains in the detergent rich phase, being purified to electrophoretic homogeneity using chromatography with DEAE-Trisacryl and hydroxyapatite [42]. This was achieved by adding 0.5% (w/v) of Emulgen 911 (Kao, Atlas, Tokyo, Japan) and 0.5% (w/v) of TX-114 to the eluting buffers. The presence of Emulgen 911 during the chromatographic steps was essential to avoid aggregation of TX-114 micelles.

5. Conclusion

The use of TX-114 not only for solubilization but also as a phase partitioning reagent is a

useful tool in plant biochemistry as it overcomes the main problems associated with plant protein purification. It removes phenols and chlorophylls by simple centrifugation; it is mild, and so does not alter the structure of inactive native enzymes (latent enzymes); it is rapid, reducing purification times from days to hours [25]; it is cheap and reproducible, and can easily be used in practical classes [43,44] or in large-scale purification processes. However, method has to be optimized according to the starting material and the enzyme to be purified.

Future challenges in purifying plant enzymes with detergents appear to be strongly linked to interdisciplinary collaboration between biochemistry and synthetic organic chemistry [5] to develop new detergents with a wide range of cloud points and which are transparent in the UV region and easily removed by dialysis.

6. Acknowledgements

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