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LIQUID–LIQUID EXTRACTION OF PROTEINS WITH AOT/ISOOCTANE REVERSED MICELLES

Liquid–liquid extraction of proteins

As most proteins have a hydrophilic character onto their surface they are insoluble in organic solvents and consequently classical liquid–liquid extraction can not be used to protein bioseparation, although, reversed micelles can be involved in an equivalent process of liquid–liquid extraction of proteins as that one observed during the phase–transfer method for preparing reversed micelles. This first step (forward extraction) is followed by phase separation where the aqueous phase is transfer to the waste while the organic phase contacts with a new aqueous phase promoting the back transfer and consequent expulsion of the protein from the reversed micelles phase (Fig. 1).

Factors affecting protein extraction

The distribution of proteins between a micellar organic phase and an aqueous solution is largely determined by mechanisms based on:

Electrostatic and hydrophobic interactions which depends on the conditions in the aqueous bulk namely pH, ionic strength, type of salt and temperature.

The parameters related to the organic phase that also influence the partition of a protein, such as, concentration and type of surfactant, presence of co–surfactant, and type of solvent.

The phase–transfer depends on specific characteristics of the protein, namely the isoelectric point, size and shape, hydrophobicity and charge distribution.

MATERIALS AND METHODS

Cell growth and enzyme production: Penicillin acylase was produced by *Escherichia coli* ATCC 9637 strain. Enzyme biosynthesis was carried out in a growth medium containing 1% (w/v) yeast extract and 0.3% (w/v) phenylacetic acid as inducer at 24°C, initial pH 7.0. The *E. coli* cells were harvested during the exponential growth phase by centrifugation (Beckman J–21C, 4420xg for 10min at 5°C). The crude enzyme was extracted from the periplasmic space by osmotic shock [8] using 20% (w/v) sucrose and cold water (5°C).

Penicillin acylase activity and protein concentration assays: Penicillin acylase activity is based on the hydrolysis of a synthetic substrate 6–nitro–3–phenylacetamide benzoic acid (NIPAB) (Sigma) to 3–amino–6–nitrobenzoic acid (NABA) in aqueous medium. The same method was applied to organic enzyme solutions. The release of NABA in the assay mixtures was monitored via the increase in absorbance at 405 nm at 37°C. The NABA concentration was determined from extinction coefficient (ε_{405} = 8980 mol^{-1} l cm^{-1}). One unit of activity (U) was defined as the amount of enzyme required to produce 1 μmol of NABA per minute at 37°C.

Protein in aqueous and micellar solutions was assayed by absorbance measurements at 280 nm. The calibration curve was obtained from standard bovine serum albumin solutions.

Enzyme extraction and back–extraction (stripping): The periplasmic extract from *E. coli* cells subjected to osmotic shock at pH 5.6 was diluted 1:10 (v/v) in 0.10 mol l^{-1} NaCl. The enzyme was extracted into the micellar phase by stirring equal volumes (3.3 ml) of the aqueous solution and 0.05 mol l^{-1} of sodium bis–(2–ethylhexyl) sulfosuccinate (AOT) (99%) (Sigma) in isooctane (ACS grade) was from Merck, using a vessel of 10 ml with magnetic agitation at 100 rpm. Phase separation was performed by centrifugation (Sigma, model 2–15) at 480xg for 10 min at room temperature. Stripping of the enzyme–containing reversed micellar phase was routinely performed by stirring the organic solution with an equal volume of 1 mol l^{-1} KCl in 0.05 mol l^{-1} phosphate buffer pH 7.5, at 300 rpm for 5 min. Phase separation was achieved as described for the extraction step.

Water content: The surfactant's hydration degree (Wo = $[H_2O]/[AOT]$ in the organic phases was determined by Karl Fischer titration using a Mettler DL 18 titrator.

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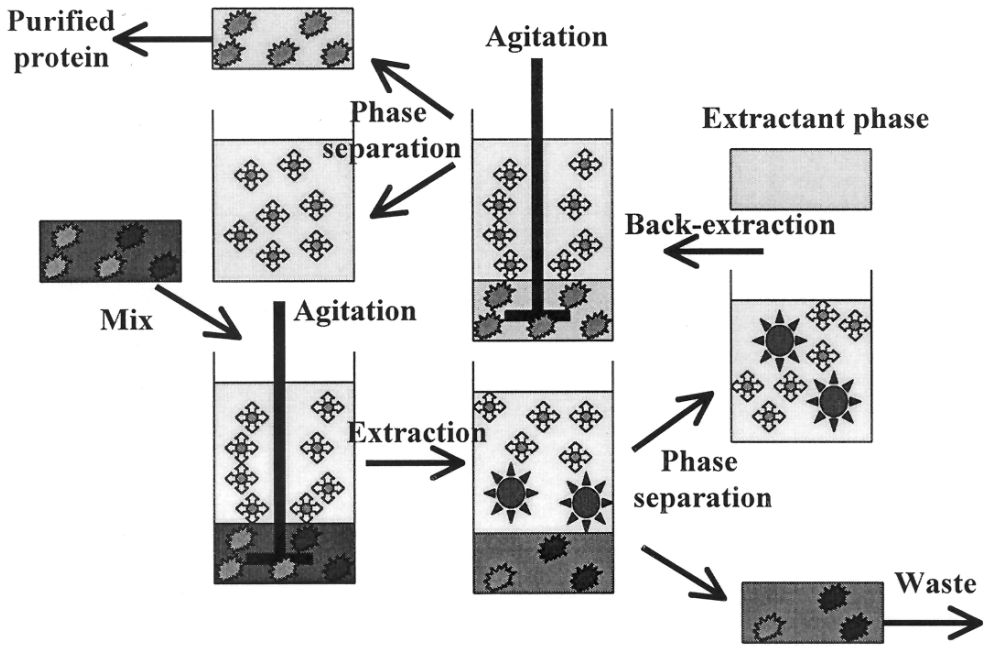

Figure 3. Enzyme activity in the organic phase (❐), in the aqueous phase (○) and Wo (■) in the phase–transfer process for different AOT concentrations. Initial activity (100%) 0.21U.

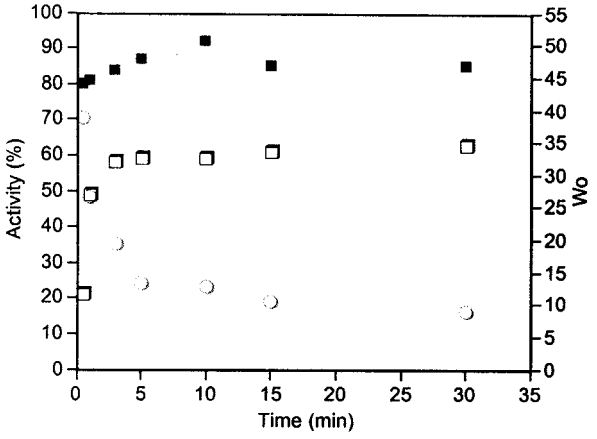

Figure 1. Liquid–liquid extraction of proteins with reversed micelles based on an initial phase–transfer process.

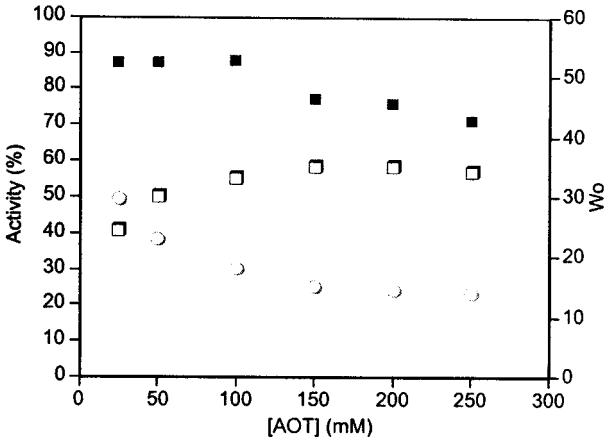

Figure 2. Enzyme activity in the organic phase (❐), in the aqueous phase (○) and Wo (■) in the phase–transfer process for different time of extractions. Initial activity (100%) 0.21U.

RESULTS AND DISCUSSION

Effect of time and kinetic extraction

The extraction time and kinetic extraction during phase–transfer method was established by monitoring extracted and non extracted penicillin acylase activities and Wo at various time intervals (Fig. 2). Equilibrium is to be established in a short time as the enzyme transfer to the organic phase reached 90% after about 5–10 min. This fast extraction process of the penicillin acylase minimizes enzyme inactivation that can be seen by the accumulation of protein precipitate at the interface. Another advantage is that the Wo is maintained almost constant, i.e., a short range variation between 45 and 50 (Fig. 1), which shows that the extraction did not affect the size of the reversed micelles under these experimental conditions (0.10 mol l^{-1} NaCl, pH 5.6 and 0.05 mol l^{-1} AOT in isooctane).

Surfactant nature and concentration

The most often used surfactant in protein extraction by reversed micelles is the AOT, primarily due to the fact that a co–surfactant is not required for protein solubilization. The protein extraction appears to be governed by the nature of interfacial complex formed between the protein and the surfactant, the solubilization being favoured when the interfacial complex has high hydrophobicity. Also, surfactants that can form a close–packed complex with the protein are excellent protein–solubilizing agents. Increasing the surfactant

concentration favours protein solubilization in the organic phase (Fig. 3).

Optimisation of the surfactant concentration is not only important in terms of achieving high extraction yields, but also the surfactant partitioning to the aqueous phase, which may limit the applicability of reversed micelle to protein extraction. However, low surfactant concentrations are beneficial for protein stripping from the reversed micelle phase as well as for accelerating the process as a consequence of improved phase separation characteristics.

Effect of pH and ionic strength

The pH of the aqueous solution determines the net charge of proteins. Electrostatic interactions between the penicillin acylase and the AOT head groups favour the transfer of enzyme into the organic phase.

A sharp extractable activity maximum is observed at pH 5.5, c.a 60% of the loaded activity (Fig. 3). A deviation of 0.5 pH units on either side decreases shiftly the extraction yields to values close to 10%, which is a consequence, on the acidic side, of severe enzyme inactivation as can be seen in Fig. 3 by the balance of enzyme activity between the two phases. Electrostatic interactions are clearly the dominant factor, since at pH values higher than pI (6.6) enzyme solubilisation does not occur at all.

For higher ionic strength, the interactions between hydrophilic biomolecules and the surfactant polar groups are reduced as a result of the Debye screening effect and smaller micelles being formed. Consequently, it is possible to promote an inversion of the partition coefficients of a protein by simple increase the ionic strength in a very narrow range of pH, which depends on the protein species to extract. The explanation for this general observation is based on the competition of the proteins with ionic species for transfer into the reversed micelles and changes in the electrostatic state of the micelles and/or proteins.

NaCl concentrations lower than 0.10 mol l^{-1} lead to microemulsions, without phase separation, and could therefore not be used. A transparent single phasic microemulsion was obtained for the lowest possible NaCl concentration (0.10 mol l^{-1}) which corresponds to a WO value of about 50, allowing 60% enzyme recovery. Higher salt concentrations, however, lead to a drastic decrease in enzyme transfer (10% at 0.125 mol l^{-1} NaCl and only 5% for NaCl concentrations above 0.15 mol l^{-1} (Fig. 2). This could lead to a molecular exclusion effect. However, an increase in NaCl concentration from 0.10 to 0.125 mol l^{-1} leads to a drastic decrease in the extracted activity and only to a comparatively small decrease in micellar size (Fig. 5).

The enzyme recovery decreased by increasing the ionic strength of aqueous pools due to hiding of electrostatic interactions between the oppositely

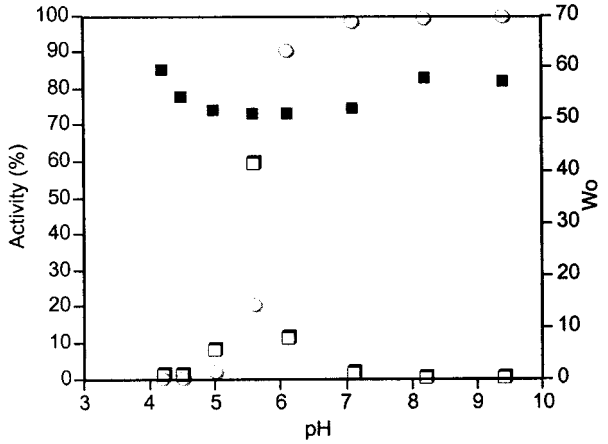

Figure 4. Enzyme activity in the organic phase (□), in the aqueous phase (○) and Wo (■) in the phase–transfer process for different values of pH. Initial activity (100%) 0.21U.

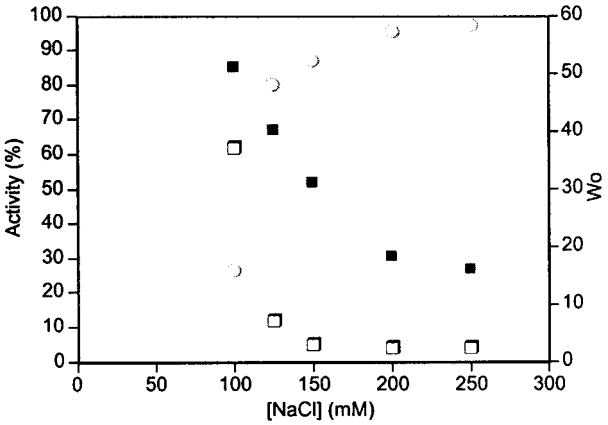

Figure 5. Enzyme activity in the organic phase (□), in the aqueous phase (○) and Wo (■) in thephase–transfer process for different NaCl concentrations. Initial activity (100%) 0.21U.

charged enzyme surface (positive, pI of 6.6) and surfactant negative head group by sodium ions. It is also important to verify that simultaneously there was not significant loss of enzyme activity at these experimental conditions as almost 100% of penicillin acylase activity was present in aqueous phase for the higher NaCl concentrations tested. It is therefore likely that salting–out effect is playing a dominant role excluding completely the possibility of hydrophobic interactions between the penicillin acylase and the surfactant tails which are unaffected by the presence of NaCl ions.

Penicillin acylase back–extraction from AOT/isooctane reversed micelles

The back–extraction of the enzyme, was easily achieved, inclusively for higher surfactant (AOT) concentration. The percentage of re–extracted enzyme was always close to 100% with substantial purification factors of 64 and 8 in relation to the enzyme extracts obtained by sonication (data not shown) and osmotic

shock respectively. The penicillin acylase is back extracted by a simple salt concentration change (1 mol l^{-1} KCl) and then the organic phase is characterized by a low hydration degree (Wo = 3.9) that is almost equal to that one measured in fresh reversed micelles of AOT/isooctane, Wo 1.9. This efficient back–extraction can ensure a complete removal of protein into the salt aqueous phase and reuse of the organic phase (reversed micellar system) for new extraction batch. This aspect is very important as in opposition with other proteins like lipases that could only be back extracted by the addition of polar ketones or alcohols to the organic phase that drastically reduce the solubility of the reversed micelles but demand solvent regeneration before organic phase reutilization.

The highest purity of the back–extracted enzyme obtained from a crude periplasmic extract was confirmed by FPLC chromatography and electrophoresis analysis.

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