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Experimental Investigation of Laccase Purification Using Aqueous Two-Phase Extraction

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In this paper the partitioning behavior of laccase from a culture supernatant of a *Pleurotus sapidus* fermentation in an aqueous two-phase system (ATPS) of polyethylene glycol with a molecular weight of 3000 g/mol (PEG 3000), potassium and sodium phosphate and sodium chloride is experimentally investigated. Results show that enzyme activity is retained during aqueous two-phase extraction. In addition the enzyme activity partitioning strongly depends on the sodium chloride concentration. The partitioning of impurities is not influenced with varying amounts of sodium chloride.

1. Introduction

Biopharmaceutical products – usually proteins - are gaining increasing interest since they offer interesting opportunities for treatment of diabetes, cancer, and other diseases. While their titers in the fermentation broths could recently be increased from mg/L- to g/L, the downstream processing often remains the bottleneck in the production process because of limitation of chromatography, as the state of the art technology in terms of capacities and high titers. ATPS show a potential to overcome these drawbacks. One limitation for the industrial application of this process results from the uncertainty regarding process development and operating costs and lacking reliable process models.
ATPS form when two polymers, one polymer and a salt, two surfactants, an alcohol and a salt or an ionic liquid and a low molecular salt are mixed in aqueous solution (Salabat et al., 2010). The major advantage of ATPS lies within the aqueous character of both phases. Compared to aqueous/organic extraction ATPS offers mild and non-disruptive purification conditions for biomolecules such as proteins.
ATPS were firstly mentioned by Beijerinck (1910), who discovered mutual immiscibility of aqueous starch and aqueous gelatine solution in 1896. It took several decades until ATPS gained scientific attention, again. In the 1950-ies Albertsson used different ATPS to extract and thereby purify cell organelles and soluble biomolecules such as proteins and nucleic acids (Albertsson, 1985). Since these proofs of concept for the extraction capabilities of ATPS several groups focused on the establishment of ATPS for industrial application. Kroner et al. (1978) used a centrifugal extractor and showed that throughputs of 2000 mL/min are feasible and concluded that further scale-up is to any scale is possible. Several groups analysed the physical and thermodynamic properties of ATPS and published modelling results for these systems. Cabezas et al. (1996) reviewed and summarized these publications thoroughly. Later Salamanca et al. (1998) and Solano-Castillo and Rito-Palomares (2000) investigated phase separation kinetics for various ATPS. One limitation for the widespread application

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of the ATPS for downstream processes lies within the uncertainty regarding process development and operating costs. Although there are thermodynamic models for ATPS available, they have not been widely introduced into the process modelling of downstream processes within the biopharmaceutical industry in contrast to the chemical industry where reliable process models with correlations or thermodynamic models embedded enable early an efficient process design.
The scope of this research project is to develop a process model, utilizing data from single-stage extraction experiments and model the multistage purification of a protein. In the first step experimental partitioning data of the model enzyme laccase is acquired. The model protein system for purification is a culture supernatant of a *Pleurotus sapidus* fermentation containing the laccase as the product.

2. Experimental

The ATPS for the laccase purification consists of PEG 3000, phosphate, sodium chloride and culture supernatant and the pH is adjusted to 7 using dipotassium phosphate and sodium phosphate as a buffer and phase forming salt. In this work sodium chloride concentration is varied, as it shows the largest influence on the partitioning behaviour of laccase in previous experiments.

2.1 Materials

The polyethylene glycol (PEG) used for the experiments has a molecular weight of 3000 g/mol and it was purchased from Merck. The used phosphates salts are dipotassium phosphate trihydrate (>99 wt.-%) from AppliChem and sodium phosphate (>99.9 wt.-%) dihydrate from VWR Prolabo. Sodium chloride (>99 wt.-%) was purchased from Roth.

Table 1: Stock solutions for the experiments.

	Component	Mass [g]	Weight fraction [g/g]
PEG-Stock solution	PEG 3000	400	0.4997
	Water	400.5	0.5003
PO_4-Stock solution	K_2HPO_4 [$3H_2O$]	414.5	0.3135
	NaH_2PO_4 [$2H_2O$]	195.8	0.1492
	Water	398.9	0.5373

For the experiments stock solutions (see Table 1) were prepared to assure constant systems compositions and to simplify the experimental setup avoiding solids handling.
Cultivation of the fungus *Pleurotus sapidus* was conducted by the Institute of Food Chemistry and Food Biotechnology in Giessen, Germany according to Linke (2005). The culture supernatant was split into aliquots and stored in 50 mL falcon tubes at -4 °C. Previous to the extraction experiments the culture supernatant was thawed, centrifuged at 3000 rpm for 30 min and the filtrated (0.45 µm) in order to remove any unsolved particles from the culture supernatant. The laccase activity of the culture supernatant was artificially increased by adding 0.5 g/L purified laccase from Sigma Aldrich (5 U/mg).

2.2 Methods

The chloride concentration was determined using the ion chromatography ICS-2100 from Dionex with a RFIC IonPac 22 4x30 mm column. To determine the chloride concentration in the top phase, the samples were diluted gravimetrically in two steps to 1:200 and the samples from the bottom phase 1:500 respectively. The measurements were performed at 35 °C, a flow rate of 1.2 mL/min and an injection volume of 25 µL. A carbonate buffer (4.5 mmol Na_2CO_3 / 1.4 mmol $NaHCO_3$) was used as the eluent and the suppressor was set to 37 mA. The relative error (< 1 %) was calculated from standard deviation of the triplicate measurements and the error of the calibration curve.
In addition to the ATPS properties, the laccase activity was determined with well plate reader Multiskan FC from Thermo Scientific according to Majcherczyk et al. (1999) using 2,2'-azino-bis- (3-

ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a substrate. Each well contained 50 µL of the sample, 200 µL of a sodium acetate acetic acid buffer at pH 4.5 and 50 µL of a 5 mmol ABTS solution. The activity was determined at a wavelength of 420 nm and at a temperature of 37 °C. The extinction coefficient for ABTS is 0.0432 L/µmol/cm.
Total protein concentration was determined by the colorimetric bicinchoninic (BC) assay introduced by Smith et al. (1985), using the BCA-kit provided by Uptima Interchim and bovine serum albumin (BSA) as standard. 25 µL of sample were mixed with 200 µL of a solution containing BC assay reagent A and B by Uptima in a volume ratio of 50:1 according to the manual of the provider. Before measuring the absorption in the well plate reader Multiskan FC by Thermo Scientific at 37 °C, the samples were incubated for 30 min and afterwards cooled down to room temperature for 10 min. Three samples of each top and bottom phase were measured. The absorption was measured at 560 nm. For each system a blank system without protein was set up. These blanks served as references and their absorption values were subtracted from the corresponding phase with protein. The absorption was evaluated by a calibration line of BSA between concentrations of 2 to 500 mg/L. For this calibration line, water served as reference. Standard deviations were calculated for each triplicate measurement. The deviation of the mean was 5 %.

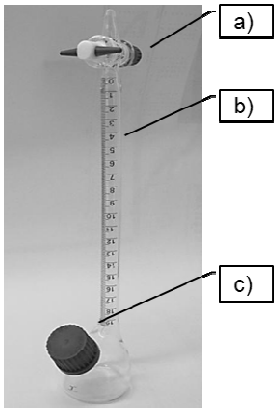

Figure 1: Extraction vessel consisting of burette valve a), burette b) and flask c).

The experiments were carried out in self-designed extraction vessels of about 15 mL working volume (see Figure 1). These vessels consist of a flask with a burette on top of it for measurement of phase volumes and precise phase separation using the valve on top of it. To switch between mixing and settling application the vessels turned upside down. First, PEG and phosphate stock solutions were prepared and the desired amount was filled into the flask part of the vessel. Then sodium chloride was added as a solid and the vessel filled up with culture supernatant and a certain amount of Millipore water. With the amount of Millipore water the concentration of phase forming components (PEG and phosphate) were adjusted. The extraction vessels were stored in a tempered glass tank with 25 °C. The phases were mixed using a magnetic stirrer at 300 rpm for one hour to reach equilibrium conditions. After mixing the dispersion was settled for 1 h. After that the vessel was slowly turned upside down and the liquids filled thereby into the burette. Due to the low interfacial tension of ATPS (Kim and Rha, 2000) and low density differences settling is a crucial part of these experiments. To achieve a complete phase separation the system was stored overnight in the vessel. Clear bottom and top phases indicated that the phases were in equilibrium. For phase separation the vessel was removed from the tank. The phases were separated using valve of the burette and the masses balanced using lab scale and diluted with water to avoid the formation of a second phase. Samples of each phase were taken and analysed as described in the previous chapter.

3. Results and Discussion

In all experiments the lighter top phase consisted mainly of PEG and the bottom phase of phosphate. For the description of the experimental results three entities besides the measured enzymatic activity and protein content are used. At first, the activity partitioning coefficient ($K_{Activity}$) is defined as the ratio of activity in the top phase (act_{TP}) over the activity in the bottom phase (act_{BP}).

$$K_{Activity} = act_{TP}\ [kU/L]/\ act_{TP}\ [kU/L] \quad (1)$$

Accordingly, the protein partitioning coefficient ($K_{Protein}$) is defined as the ratio of protein content $c_{Prot,TP}$ in the top phase over the bottom phase $c_{Prot,BP}$.

$$K_{Protein} = c_{Prot,TP}\ [mg/L]/\ c_{Prot,BP}\ [mg/L] \quad (2)$$

The third entity is the recovery of activity (Recovery), which compares the initial enzymatic activity (act_{ini}) of the culture supernatant with the activity in the top or the bottom phase.

$$Recovery = act_{TP/BP}\ [kU/L]/\ act_{ini}\ [kU/L] \quad (3)$$

In contrast to the classical understanding of the recovery of activity can be larger than 100 % since enzyme activity is dependent various physiological conditions such as acidity, temperature and the concentration of other components (e.g. PEG, phosphate and other salts). It has been shown by Yoon and Robyt (2005) that PEG can have a stabilizing and activating effect on enzymes and increases their activity; therefore, the recovery of activity is not limited to 100 %.

In Figure 2 the laccase activity partitioning for varying amounts of sodium chloride is presented. The enzymatic activity of the laccase in the top phase is between 1 and 3 kU/L and shows only limited change for different concentrations of sodium chloride. In contrast the activity in the bottom phase first increases from 7 to almost 10 kU/L and then decreases to about 3 kU/L. Since the activity remains mainly in the top phase the activity partitioning coefficient is always lower than unity.

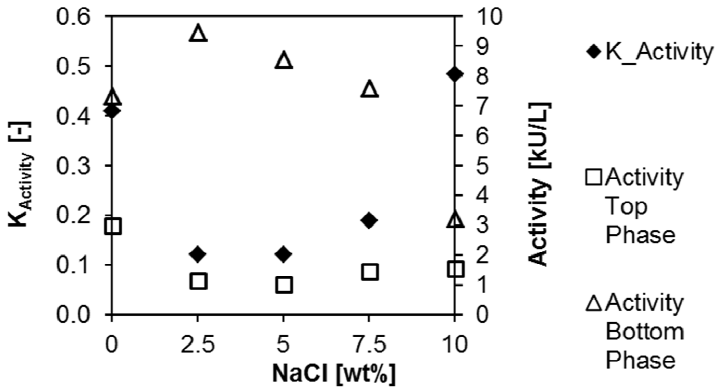

Figure 2: Activity partitioning coefficient and enzymatic activity of laccase from Pleurotus sapidus in an ATPS containing 12 wt.- % PEG 3000, 7 wt.- % phosphate and pH 7 with varying concentrations of sodium chloride

For increasing amount of sodium chloride activity partitioning coefficient shows a U-shaped curve with a minimum between 2.5 and 5 wt. - %. Still, the increase of the activity partitioning coefficient from 5 wt. - % of sodium chloride onwards is mainly due to the decreasing activity in the bottom phase. It shows that the partitioning coefficient can be influenced by sodium chloride.

In contrast to the partitioning of the laccase activity the partitioning of proteins (see Figure 3) is much less influenced by sodium chloride. The concentration of proteins in the top phase decreases slightly to 2.5 wt. - % of sodium chloride and then increases again, whereas the opposite is true for the bottom phase. Since the volume ratio of bottom to top phase is always between 1.2 and 1.7 the changes in

protein content are larger in the top phase than in the bottom phase. Nevertheless, since the changes of protein content are rather low the change in the protein partitioning coefficient is low as well.

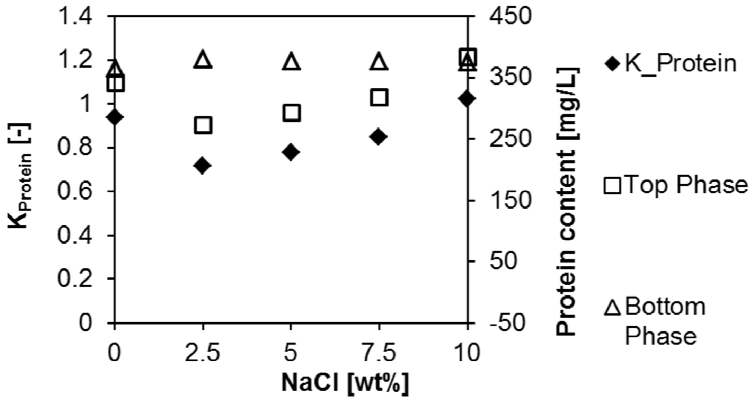

Figure 3: Protein partitioning coefficient and protein content of laccase from Pleurotus sapidus in an ATPS containing 12 wt.- % PEG 3000, 7 wt.-% phosphate and pH 7 with varying concentrations of sodium chloride.

Overall the influence of sodium chloride on the protein partitioning coefficient is a lot lower than the influence on the activity partitioning coefficient.
The recovery of the enzymatic activity for the top and bottom phase as well as the total recovery is plotted over the sodium chloride concentration in Figure 4. For systems without sodium chloride the recovery of the enzymatic activity is already higher than 100 % which suggests that the phase forming components PEG and phosphate have an activity increasing effect on laccase.

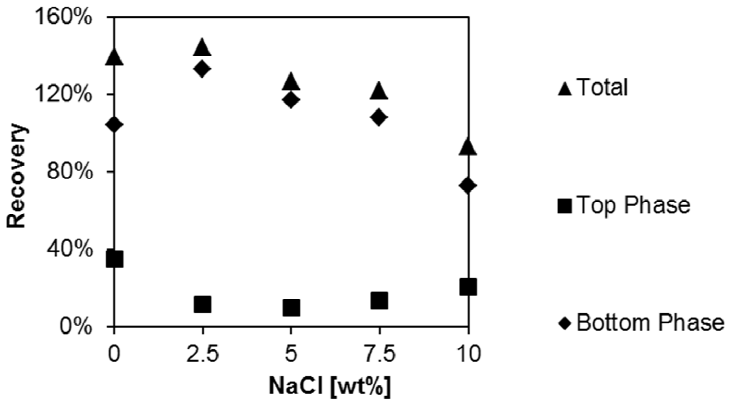

Figure 4: Activity recovery of laccase from Pleurotus sapidus in an ATPS containing 12 wt.- % PEG 3000, 7 wt.- % Phosphate and pH 7 with varying concentrations of sodium chloride.

With increasing sodium chloride content the top phase recovery first decreases from about 40 % to 10 % and then increases again to about 20 %. For the recovery of activity in the bottom phase the opposite behaviour was found. Initially it increases with increasing sodium chloride concentration from 100 to about 130 % but then decreases to 73 %. The total recovery decreases from about 140 % to 93 % at 10 % sodium chloride.

4. Conclusion

In this work a strong influence of sodium chloride on the partitioning behaviour of laccase activity in an ATPS consisting of PEG 3000 and phosphate could be shown. Depending on the sodium chloride concentration high recoveries in the bottom phase consisting mainly of phosphate are achieved. The maximum recovery of 130 % in the bottom phase is partly due to the preferred partitioning of laccase into the bottom phase and partly due to the stabilizing and activating effect of PEG on laccase. For higher sodium chloride concentration the recoveries decrease. This can be explained by salting-out and therefore deactivating influence of the salt. From these results the optimal concentration of sodium chloride for a multi-stage extraction process is 2.5 %, since proteins partition almost equally between the two phases; whereas laccase partitions mostly into the bottom phase and even increases its activity.

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