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Separation and purification of laccases from two different fungi using aqueous two-phase extraction

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ABSTRACT

Selective purification still poses a challenge in the downstream processing of biomolecules such as proteins and especially enzymes. In this study a polyethylene glycol 3000 (PEG 3000)–phosphate aqueous two-phase system at 25 °C and pH 7 was successfully used for laccase purification and separation. Initially, the effect of phase forming components on enzyme activities in homogenous systems was studied. In the course of the extraction experiments tie lines, enzyme source, initial enzyme activities, phase ratio and sodium chloride concentrations were varied and their influence on the activity partitioning was determined. Partitioning results were validated using clear-native-PAGE and isoelectric focusing. Based on these results, the separation of laccases from *Trametes versicolor* and *Pleurotus sapidus* was investigated using the principle of superposition. Sodium chloride was used to adjust laccase partitioning in the applied aqueous two-phase system (ATPS). Finally, two modes of operation are proposed depending on the aim of the purification task. One mode with 0.133 g g^{-1} of PEG3000, 0.063 g g^{-1} of phosphate and without sodium chloride separates *P. sapidus* laccases from *T. versicolor* laccases with clearance factors of 5.23 and 6.45, respectively. The other mode of operation with 0.124 g g^{-1} of PEG3000, 0.063 g g^{-1} of phosphate and 0.013 g g^{-1} of sodium chloride enables a partitioning of both laccases into the bottom phase of the ATPS resulting in a purification factor of 2.74 and 96% activity recovery.

1. Introduction

While titers of proteins in fermentation broths could recently be increased from scales of milligram to grams per liter, downstream processing often remains the bottleneck in the production process of such biomolecules [1]. Therefore, purification and separation of enzymes and other proteins is of common interest in science and industry. In the field of white biotechnology, industrial biocatalysis using enzymes is expected to become even more important in future [2]. Depending on the application, enzyme purity can be of crucial importance. Rizwan [3] summarizes that precipitation, flocculation, extraction, immobilization, gel filtration, chromatography and/or three phase partitioning (TPP) are applied in preparative protein purification. Of these methods extraction combines high capacity with reasonable selectivity. Extraction's large capacity is well known from chemical downstream processing, where extraction columns with up to 5 m diameter and 1200 m^3 h^{-1} throughput are common practice [4]. Nevertheless, aqueous–organic solvent extraction may lead to enzyme denaturation due to the non-polar character of the solvents. Aqueous two-phase systems (ATPS) circumvent this drawback, since both phases mainly consist of water. They can be formed by mixing two hydrophilic components, such as polyethylene glycol (PEG) and phosphate, with water. In this specific case, the top phase (TP) contains most of the polyethylene glycol, while the bottom phase (BP) contains the majority of phosphate. The partitioning of solutes between the two phases strongly depends on the nature of the solute and the ATPS. Albertsson, who has applied firstly ATPS for separation purposes, generally distinguishes between size-dependent, electrochemical, hydrophobic affinity, biospecific affinity, conformation-dependent and chiral partitioning in ATPS [5]. Today, ATPS are well known as a method for the separation of proteins and enzymes. In general, the key to successful purification is utilizing the physical and biochemical differences between target molecule and the contaminants [6,7]. It can be stated that the similarity of the product and the contaminants determines the separation and purification challenge. The larger the similarity of the enzymes the more difficult is the separation [8]. In this study, laccases served as representative enzymes to be purified and separated via aqueous two-phase extraction under variation of partitioning influencing parameters.

Laccases were discovered in 1883 by Yoshida in the exudate of the Japanese lacquer tree *Rhus vernicifera* [9]. They are

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oxidoreductases (E.C. 1.10.3.2) which oxidize many aromatic and nonaromatic compounds via a radical-catalyzed reaction mechanism using molecular oxygen [10]. In nature they are involved in various processes including delignification, lignification, pathogenicity, detoxification, sporulation and morphogenesis [11]. In biotechnological and industrial processes laccases have a high application potential taking into account their broad substrate spectrum, the use of oxygen as final electron acceptor, and the independence from cofactors [11]. Besides their occurrence in plants, they are also found in bacteria and fungi [12]. The fungus *Pleurotus sapidus* belongs to the oyster mushroom family. This basidiomycete is known for laccase production and secretion [13] and served as a laccase producing microorganism for this work. *Trametes versicolor* is another basidiomycete, well-known for its laccase expression. Laccase from *T. versicolor* was added to the supernatant of *P. sapidus* to investigate the separation potential of ATPS.

In literature, several approaches to purify laccases from different microorganisms have been reported. Table 1 summarizes and compares these approaches. In these publications only recoveries and purification factors are reported.

Since the focus of this work is on developing process alternatives and showing separation potential rather than screening for new ATPS, a well-known system (PEG-phosphate) was chosen for the separation task. Initially, the phase system and its influence of the phase forming components on the enzyme stability were measured. In contrast to the majority of reports dealing with laccase purification using ATPS, the separation of *P. sapidus*' from *T. versicolor*'s laccases was investigated to prove selective enzyme purification. Initial enzyme activities, enzyme source, sodium chloride concentration as well as the different tie lines and the phase ratio of the ATPS were varied and the influences on the enzyme partitioning and purification were studied.

2. Materials and methods

Two different sources of laccases were used in the course of this work. In one case, laccase was taken from a *P. sapidus* (*P.s.*) fermentation. The fermentation was done according to Linke et al. [14]. After fermentation, the culture supernatant was frozen in aliquots of 50 mL at −20 °C. For extraction experiments, aliquots were defrosted and filtered (5–13 μm). After this procedure, laccase activity was around 750 U L^{-1} and the protein content at about 130 mg L^{-1}. The second laccase was taken from *T. versicolor* (*T.v.*) (Sigma–Aldrich; cat. #38429; ≥0.5 U mg^{-1}). For the experiments, the powder was solubilized in water at room temperature and filtered (5–13 μm) afterwards. The amount of powder was varied to adjust the initial *T. versicolor* laccase activity to the laccase activity of the culture supernatant. For the experiments with varying initial activity, culture supernatant and enzyme solution was diluted with water to adjust the initial laccase activity in the ATPS.

The water used for all experiments and dilutions was purified by a Milli-Q Synthesis A10 system (Millipore, conductivity $K \leq 0.06$ μS cm^{-1}). 2,2′-Azino-bis-(3-ethylbezothiazoline-6-sulphonic acid (ABTS) with a purity of ≥98% was purchased from Sigma–Aldrich (cat. #A1888). Sodium chloride (NaCl) was also purchased from Sigma–Aldrich (cat. #71379) and had a purity of ≥99.5%. PEG with a molecular weight of 3000 g mol^{-1} Ph. Eur. grade (PEG3000) was purchased from Merck (cat. #34042000). Di-potassium hydrogen phosphate trihydrate was purchased from AppliChem (cat. #A4001) with a purity of ≥99%. Sodium dihydrogen phosphate dihydrate Ph. Eur. grade (≥99.8%) was purchased from VWR Prolabo (cat. #28014.360). For the experiments a PEG3000 stock solution with a concentration of 0.50 g g^{-1} and a phosphate stock solution of 0.29 g g^{-1} of PO_4^{3-} were used. The pH of the phosphate solution was adjusted to pH 7 by varying the amount of sodium and di-potassium phosphate. Sodium/di-potassium phosphate buffer solution with a pH of 7 will be referred to as "phosphate" and considers only the anions.

2.1. Measurement of laccase activity

Laccase activity was measured using the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay according to Majcherczyk et al. [15] and was calculated using Eq. (1):

$$\text{act}\left[\frac{U}{F}\right] = \frac{\Delta E \times \text{DF} \times V_t}{\Delta t \times V_s \times d \times \varepsilon_{420}} \quad (1)$$

The pH of the 50 μL sample V_s was adjusted to 4.5 with 150 μL of 80 mM sodium acetic acid buffer. All samples were diluted (dilution factor DF = 20) volumetrically to avoid previously observed interferences of the phosphate buffer in the ATPS with the acetic acid buffer of the assay. After the addition of 50 μL 5 mM ABTS solution the change of absorbance ΔE was measured for three minutes (Δt) at 420 nm and 37 °C. Laccase activity was calculated using an extinction coefficient of $\varepsilon_{420} = 0.04321$ L μmol^{-1} cm^{-1}, a layer height $d = 0.63$ cm and a total liquid volume of $V_t = 250$ μL. The assay was done in a Multiskan® FC well plate reader from Thermo Scientific.

Enzyme activity U is defined as the amount of enzyme catalyzing the oxidation of 1 μmol ABTS per minute. All samples were measured in triplicates and their standard deviation was calculated. Dilution error and sample standard deviation were used for Gaussian error propagation. Resulting errors are indicated in diagrams using error bars. The relative average deviation of all measured activities was 7.1%.

2.2. Measurement of total protein content

The total protein content was measured on a Multiskan® FC well plate reader from Thermo Scientific using the bicinchoninic acid (BCA) assay introduced by Smith et al. [16] with bovine serum albumin as standard. In order to prevent interference of impurities from the supernatant, mainly PEG3000 containing top and mainly phosphate containing bottom phase, samples were filtered using centrifugal filters (VWR Centrifugal Filter PES 10K) with a molecular weight cut-off of 10 kDa in a MiniSpin® Plus Centrifuge from Eppendorf at 12,100 × g. The retentate was filled up with millipore water to the initial volume of 500 μL. For the assay, 25 μL of sample were mixed with 225 μL mixture of reagent A and B according to the manual of the assay provider Uptima Interchim (product number: UP40840A). After 30 min of incubation in the well plate reader at 37 °C the well plate was cooled at ambient temperature for 10 min. Then the absorbance was measured at 562 nm Protein concentrations were calculated from the slope of the standard curve for the concentration range of 0–500 mg L^{-1} after blank subtraction. All samples were measured in triplicate and their standard deviation was calculated. The dilution error, the standard error of the calibration curve and the sample standard deviation were used for Gaussian error propagation. The relative average deviation of all measured protein concentrations was 5.3 %.

2.3. Measurement of PEG concentration

PEG concentration (wt_{PEG}) was measured using high performance liquid chromatography (HPLC) from Merck Hitachi (L-6200 Intelligent Pump, AS-4000 Autosampler, D-6000A Interface, Shimadzu HIC-GA column oven) with a size exclusion column (Suprema 100 Å from Polymer Standard Services (300 × 8 mm particle size 10 μm) and the corresponding pre-column (50 × 8 mm)) at 40 °C with millipore water as eluent. Similar to other polymers, PEG was detected by a refractive index (RI) detector using