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Mannanase Transfer into Hexane and Xylene by Liquid–Liquid Extraction

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Abstract The formation of noncovalent complexes between glycosidase, endo-1,4-β-D-mannanase, and ionic surfactant di(2-ethylhexyl) sodium sulfosuccinate (AOT) was shown to promote protein transfer into organic solvents such as xylene and hexane. It was found that mannanase can be solubilized in hexane and in xylene with concentration at least 2.5 and 2.0 mg/ml, respectively. The catalytic activity of the enzyme in hexane spontaneously increases with the concentration of AOT and is about 10% of the activity in aqueous system. In xylene, a catalytic activity higher than that in bulk aqueous conditions was found for the samples containing 0.1–0.3 mg/ml of mannanase, while for the samples with a higher concentration of enzyme, the activity was hardly detected.

Keywords Endo-1,4-β-D-mannanase · Liquid–liquid extraction · Hexane · Xylene · Organic solvent · Biocatalysis

Introduction

Microbial endo-1,4-β-D-mannanases have become key enzymes in industrial applications, such as paper and pulp industry [1], food and feed technology, coffee extraction [2], and in the detergent industry [3, 4]. Moreover, it has been shown that glycosidase, whose family mannanases belongs to, have interesting antifouling properties and have been utilized on surfaces for biofilm prevention [5–7]. The antifouling properties of enzymes have received increasing interest in the paint industry, especially for the marine coatings, due to the recently introduced total ban of tributyltin-based paints [8, 9]. However, since most paints

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are based on organic solvents, where enzymes may be inactivated [10, 11], the use of them as antifouling agents is currently still quite rare [8]. Identification of routes to the stabilization of enzymes in organic solvents relevant for coatings is thus an important issue for the paint industry [8]. The most straightforward approach to the transfer of enzymes into organic solvent is the formation of a reverse micelle-like system, based on noncovalent interaction between surfactant, water, and enzyme molecules [12–17]. However, for biotechnological purposes, more straightforward techniques such as liquid–liquid extraction can be used [18–20]. It has been shown that this method works well for several types of enzymes, which are produced in industry at large scale, such as chymotrypsin, subtilisin, peroxidases, and lipases [18–21]. However, despite the distinct importance of mannanases for nonaqueous applications, such adaptations of biocatalyst were not performed so far.

Here, we describe a successful method of extraction of mannanase into organic solvents, such as hexane and xylene. Since the ability of enzyme work in aqueous environment after solubilization in organic solvents is essential for marine application, the catalytic activity of mannanase in these solvents with aqueous solution of locust bean gum was studied.

Materials and Methods

The endo-1,4-β-D-mannanase from *Trichoderma reesei* (MW 53 kDa, pI 5.4 [22]) was obtained from Danisco A/S (Denmark). Di(2-ethylhexyl) sodium sulfosuccinate (AOT or sodium decussate), hexane, xylene, locust bean gum (LBG), and buffer components were obtained from Sigma-Aldrich (Steinheim, Germany). Bredford assay kit was obtained from Bio-Rad (USA).

Mannanase Solubilization

To transfer endo-1,4-β-D-mannanase into hexane solution, 1 ml of buffer (50 mM sodium citrate-phosphate, pH 4.6) containing 5 mg/ml of enzyme was mixed with 2 ml of hexane containing different concentrations of AOT. All solutions were stirred for 19 h at 500 rpm. Then the mixtures were centrifuged at 13,500 rpm for 10 min, and the transparent upper phase was used for further studies. The amount of enzyme solubilized in hexane was determined spectrophotometrically at 280 nm by using extinction coefficient for mannanase 0.73 $(mg/mL)^{-1}$ cm^{-1}, which was determined experimentally. Due to high absorbance of xylene at 280 nm, the protein transfer into xylene was measured indirectly using the Bradford assay [23]. In brief, the solvent from the enzyme-containing samples of xylene was allowed to evaporate, and the resulting powder was dissolved in de-ionized water for the assay.

Determination of Catalytic Activity of Mannanase

The catalytic activity of the enzyme was determined by the ability to hydrolyze LBG as a substrate—the endo-(1-4)-β-D-mannanase activity. Six hundred seventy microliters LBG solution (2.8 mg/ml) was mixed with 170 μl of enzyme solution either in aqueous buffer or in organic solvent and incubated for 10 min at 40°C. Then 1,000 μl of quench solution, containing 10 g/l 3,5-dinitrosalicylic acid in tartrate buffer, was added. The final mixture was boiled in a water bath for 15 min with subsequent cooling with ice water for 50 min. After equilibrating the system at 20°C, the absorbance of the solution was either measured at 540 nm directly or in the case of enzyme in organic solvent with preliminary centrifugation for 5 min at 13,500 rpm.

In order to estimate the relative catalytic activity of mannanase in organic solvents, the obtained absorbance was correlated with the extracted amount of enzyme and extrapolated to a calibration curve obtained in an aqueous system. The calibration curve was linear over the whole range of experimental values, and a linear fit was used for extrapolation.

All experiments were performed at 22°C.

Results and Discussions

Mannanase in Hexane Solutions The efficiency of the AOT-solubilized mannanase transfer into hexane as a function of the AOT concentration in hexane is presented in Fig. 1. The transfer of the protein was measured spectrometrically, and the dependence of transfer efficiency of mannanase into hexane is presented in Fig. 1a. It can be seen that the concentration of mannanase was almost 2.0 mg/ml (80% of theoretical), already at 40 mM AOT concentration in hexane. The catalytic activity of mannanase per milligram of enzyme was seen to increase continuously with the AOT concentration and correlated with the solubilization efficiency (Fig. 1b) The highest activity was measured for samples with the concentration of AOT above 40 mM. Although even for these samples the activity did not exceed 10% of the bulk aqueous activity, in the absence of AOT, mannanase is completely inactive in hexane.

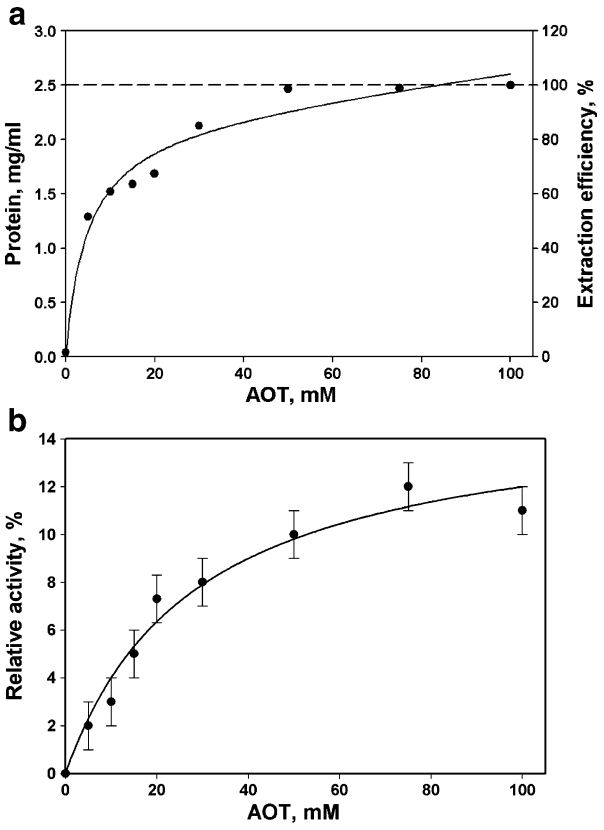

Fig. 1 Mannanase in hexane containing AOT systems. **a** Dependence of protein transfer on concentration of AOT in the system. *Dashed line* corresponds to the initial amount of protein—theoretical maximal solubility. **b** Relative catalytic activity of mannanase in hexane related to the activity in aqueous buffer per milligram of enzyme

Mannanase in Xylene Solutions The obtained results of enzyme transfer into xylene are presented in Fig. 2a. As can be seen from the figure, although the maximal solubility of enzyme was reached at the concentration of AOT in xylene of 100 mM (ca. 2.0 mg/ml), for the other studied systems, the concentration of solubilized enzyme did not exceed 0.5 mg/ml or 20% of the theoretical maximum. Moreover, the level of protein transfer at concentrations of AOT below 20 mM was negligible. In contrast to the protein transfer data, catalytic activity of mannanase in xylene was drastically decreased with increase of the concentration of AOT in the system. For samples containing 75 and 100 mM of AOT, activity was not detected at all. The results for all samples are presented in Fig. 2b, and it can be seen that the highest activity was obtained for samples with low concentration of the protein and low concentration of AOT. For samples containing 20–30 mM of AOT, the activity was higher than the aqueous control, while for samples with higher concentration of the protein, activity was not detected. The high level of activity measured in xylene, being significantly above the aqueous catalytic activity of mananase, is an important scientific observation, but the low levels of transferred enzyme make it of limited applicability.

By comparing the results obtained in hexane and in xylene, it is clear that in both systems, the efficiency of protein transfer is enhanced with increase of the concentration of AOT. However, the final concentration of enzyme which can be obtained in the separate solvent systems is different, likely due to different solvation properties of hexane and xylene and the

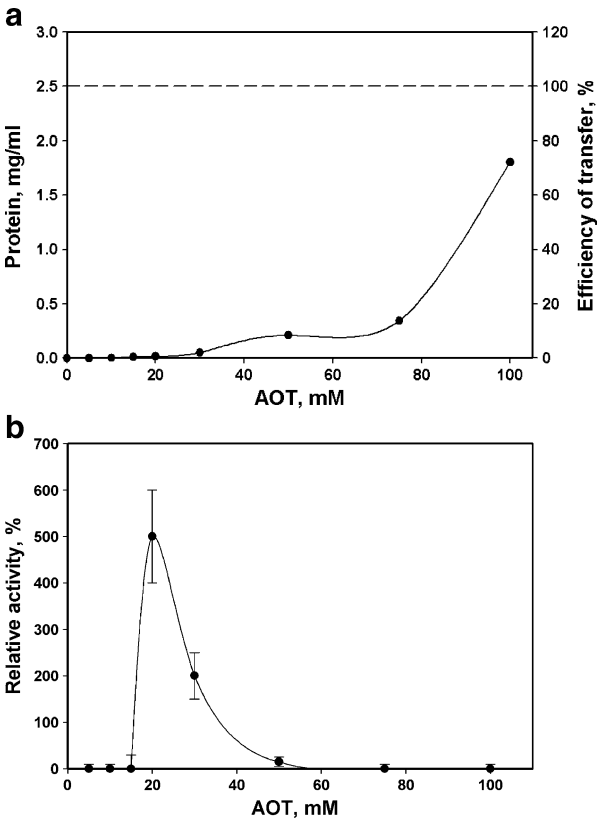

Fig. 2 Mannanase in xylene containing AOT systems. **a** Dependence of protein transfer on concentration of AOT in the system. *Dashed line* corresponds to the initial amount of protein—theoretical maximal solubility. **b** Relative catalytic activity of mannanase in xylene related to the activity in aqueous buffer per milligram of enzyme

differences in stability of AOT layer on water-solvent interface. In xylene–AOT–water system, there is a large area in the phase diagram where several types of assemblies are presented. However, in the same area of the phase diagram hexane–AOT–water, only one type of organelles is presented [24, 25]. Therefore, for solubilizing of the same amount of protein, the higher concentration of AOT is required in xylene than in hexane.

Despite the similarities in the protein transfer profile with AOT concentration, the catalytic activity profiles for mannanase in hexane and xylene are very different. The highest relative activity of the enzymes in hexane is about 10% and corresponds to the highest efficiency of protein transfer. For xylene, the system's catalytic profile has an opposite relationship with a maximum when the concentration of the protein in the system is low (0.1–0.2 mg/ml). Due to a higher polarity of xylene, the diffusion limitation between the substrate and enzyme can be different and might play a crucial role in the profile of the catalytic activity in both systems. For example, LBG can have a better access to the active site of the enzyme in xylene at low concentration of AOT. But the bell-shaped profile of the activity is probably due to interaction of LBG with AOT molecules at higher concentration of AOT in the system, which results in a decrease of substrate accessibility for enzyme. Such effects are likely to be more pronounced in xylene than in hexane, and the enzyme shows 0% and 10% of activity, respectively, at the highest amounts of transferred protein, probably due to enhanced AOT–LBG interaction in xylene.

In conclusion, solubilization of endo-1,4-β-D-mannanase in hexane and xylene can be achieved through liquid–liquid extraction with formation of noncovalent complexes of the anionic surfactant AOT with the enzyme. Although protein concentration in hexane of about 2.5 mg/ml can be achieved, the enzyme shows only 10% of the aqueous activity. In xylene, active enzyme can be transferred in concentrations of only 0.1–0.3 mg/ml, even though with a higher activity compared to aqueous conditions.

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