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Pressure effects on activity and selectivity of *Candida rugosa* lipase in organic solvents

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ABSTRACT

Even though a lot of high pressure studies on enzyme structure, stability and activity are published in the last years, just a few works deal with the influence of pressure on the enantioselectivity of an enzymatic reaction. Furthermore, a change of the reaction medium from buffer to organic solvents for high pressure studies offers some interesting advantages, like pH independency and higher sensitivity towards hydration changes. From this point of view, in the present paper the influence of high pressure on the activity and selectivity of a *Candida rugosa* Lipase catalyzed reaction in organic solvents was examined. The transesterification of 1-phenylpropan-2-ol with vinyl acetate was chosen as a model reaction. The reactions carried out at 50 MPa showed an increased specific activity of the lipase, independent of solvent composition, reaction temperature and water content of the solvent. An activity maximum, without deactivation, was observed in hexane at 45 °C and 200 MPa. Between 50 MPa and 200 MPa a linear increase in the enantiomeric excess (ee_R) could be detected, also independent of the solvent composition, reaction temperature and water content of the reaction medium. Furthermore, if additional water was added to the reaction solvent no change of the ee_R at high pressures could be observed. This leads to the conclusion that the ee_R under pressure is probably mainly influenced by the compression state of the enzyme or by structural changes of the active center rather than by the water content of the enzyme, as it is the case at ambient pressure.

1. Introduction

High pressure as a tool in biotechnology is becoming more and more important in the last few years. Common applications are found in food industry for inactivation of microorganisms [e.g., 1–3] or spores [e.g., 4,5] and in preservation due to pressure-induced proteolysis [6,7]. Additional applications include the production of fine chemicals and pharmaceuticals [8]. Since high pressure not inevitably leads to protein denaturation but rather causes only local changes in protein structure, it can be used to analyze changes in structure and function during the catalytic reaction. These changes could affect the activity, selectivity and stability of the enzyme, which was intensively discussed and reviewed over the past years [e.g., 9–15].

In order to describe the influence of high pressure on the enzyme structure or more specific on the chemical interactions within the enzyme, it is important to look at the different pressure dependencies of chemical bonds and interactions, because these binding types differ in their sensitivity to pressure [16,17]. In general, negative reaction volumes are preferred under high pressure (Le Chatelier principle). That means that covalent bonds are built under high pressure because their formation leads to a volume decrease. Furthermore, they are stabilized by the pressure. Hydrogen bonds are likewise stabilized because their formation is associated with a small volume reduction. Besides, the interatomic distances are reduced which leads to shorter, more stabilized hydrogen bonds [18]. In contrast, electrostatic interactions are destabilized. This is due to the electrostriction which describes the hydration of polar and charged groups as well as their dissociation followed by hydration. Both of these effects result in a volume decrease [19]. This effect causes higher hydration states of the enzyme which leads to higher local flexibilities [15]. Furthermore, hydrophobic interactions are destabilized because their formation is associated with a positive reaction volume. In contrast, the stacking of aromatic rings results in a volume decrease which means that they are stabilized under pressure.

Because the enzyme structure is characterized by all of these different kinds of bonds it can be concluded that the primary

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structure is pressure resistant, because it is linked by covalent bonds. The secondary structure is stabilized by hydrogen bonds, likewise resulting in a pressure resistance. At very high pressures between 300 and 700 MPa denaturation effects of the secondary structure can be observed [20]. The tertiary structure is predominantly stabilized by salt bridges and disulfide bonds and denaturation effects upon them can be detected at around 200 MPa. For smaller proteins denaturation arises just at 400 MPa [21]. Last, quaternary structure is stabilized by intermolecular electrostatic and hydrophobic interactions, which are destabilized at pressures from 50 to 200 MPa. Furthermore, oligomers dissociate into their subunits [22].

Besides the pressure influence on the enzyme structure, the chemical reaction exhibits a pressure dependency as well. The dependence of the equilibrium constant (K) is displayed in Eq. (1):

$$\left(\frac{\partial \ln K}{\partial p}\right)_T = -\frac{\Delta V}{RT} \quad (1)$$

with the pressure p, the temperature T, the universal gas constant R and the difference of the reaction volumes of products and educts ΔV. If the difference in the reaction volume is negative, the product formation is favored with increasing pressure. On the other hand, if the reaction volume is positive, the formation of the educt is preferred.

Besides the state of equilibrium, high pressure also influences the reaction rate (k) to accommodate the reaction equilibrium (Eq. (2)):

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^{\neq}}{RT} \quad (2)$$

with $\Delta V^{\neq}$ as the difference in the activation volume. Here the molar volume difference between the transition state and the ground state is taken into account. The reaction is accelerated when having a negative reaction volume under high pressure [23].

A wide range of fundamental research has been performed in order to explain structural changes of the enzyme during high pressure treatment [16,17,24]. Thereby, most of the reactions were carried out in buffer solutions. Up to now, there are just a few publications on the impact of high pressure on enzyme activity in organic solvents [25–28]. One advantage of using organic solvents is their independency to the pH value. Buffer solutions exhibit a pressure addiction of the pH value which could affect the enzyme [29]. On the other hand, enzyme reactions in organic solvents are strongly influenced by the water content of the solvent, and by the hydration state of the enzyme, respectively [30]. Therefore, the impact of high pressure on the binding and dissociation of water molecules in relation with the hydration of polar charged amino acids could be more relevant in organic solvents. Thus, a higher sensitivity of the protein to pressure could be expected.

Previous works, carried out in buffer solutions with *Pseudomonas putida* Benzoylformate decarboxylase, could already show that the enantiomeric excess increases with increasing pressure [31,32]. The aim of this work was to investigate the influence of high pressure not only on the enzyme activity, but also on the enantiomeric excess of the reaction. As a model reaction the transesterification reaction of 1-phenylpropan-2-ol with vinyl acetate as acyl donor to (R/S)-1-phenylpropan-2-yl acetate catalyzed by *Candida rugosa* lipase was chosen. For the examined biocatalysis in organic solvents *Candida rugosa* lipase is an ideal candidate, because it is commercially available, there is no need of a cofactor and the lipase has a broad substrate specificity [33]. Different parameters were varied to characterize the reaction at ambient and high pressure. Therefore, to investigate the influence of different solvent compositions three solvents were tested at 35 °C. Furthermore, the temperature was varied from 20 to 55 °C to analyze the influence of the reaction temperature. At least water was added to the reaction mixture, to analyze the influence of the water content.

2. Materials and methods

2.1. Chemicals and enzymes

Racemic 1-phenylpropan-2-ol (PP), racemic 1-phenylethanol (PE), vinyl acetate (VA), anhydrous hexane (Hex) and anhydrous tetrahydrofurane (THF) were purchased from Sigma–Aldrich Munich, Germany. Hexane and THF were stored in septum bottles to avoid contact with the surrounding atmosphere. The water content, determined by coulometric Karl-Fischer titration, was 0.003% for hexane and 0.017% for tetrahydrofurane. A reliable determination of the water content of vinyl acetate was not possible because of the molecular double bond which causes a side reaction during the coulometric Karl-Fischer titration. Therefore vinyl acetate was stored and handled under argon atmosphere. The racemic product 1-phenylpropan-2-yl acetate (PPA) was prepared by chemical acetylation (pyridine, acetic anhydride, 5 mg scale) from the racemic alcohol 1-phenylpropan-2-ol as standard for gas chromatography (GC) analyses. All further chemicals and solvents were of analytical grade or higher and purchased from Sigma–Aldrich Munich, Germany or Fluka Munich, Germany. Lipase from *Candida rugosa* (activity in aqueous solution: 30,000 U/g; protein content: 4%) was donated from Amano Enzyme Inc. Nagoya, Japan.

2.2. Experimental procedures

The substrate solution consists of the solvents hex and THF as well as the substrates VA and PP. None of the components were pretreated. The mixture had a total volume of 25 mL. Because VA as the acyl donor has to be present in excess it was treated like a solvent.

To analyze the influence of different solvent compositions the following three different mixtures were created. Solution 1: 18.75 mL hex and 6.25 mL VA (ratio hex:THF:VA 3:0:1); solution 2: 12.5 mL hex, 6.25 mL THF and 6.25 mL VA (ratio 2:1:1); solution 3: 18.75 mL THF and 6.25 mL VA (ratio 0:3:1). All three solutions were staggered with 0.41 mol/L of the acyl acceptor PP and stirred for 5 min at 300 rpm to dissolve the viscous PP. The reactions were carried out at 35 °C.

The reactions to investigate the influence of different reaction temperatures were exclusively examined in pure hexane (solution 1 staggered with PP) at 20 °C, 35 °C, 45 °C, and 55 °C, respectively.

In order to investigate the role of the water content the reactions were exclusively examined in pure hexane as well. Therefore, water was added to solution 1 staggered with PP (1 μL water per mL solvent). The reactions were carried out at 35 °C.

Due to the low reaction rates in all cases the prepared solutions were mixed cold and after that added to the crude lipase powder (concentration CRL 6 g/L).

For the reactions at ambient pressure the lipase-substrate-mixture was transferred into sealed glass vials and accomplished in a drying oven to control the reaction temperature. To ensure a good dispersion of the lipase in the solvent, the solution was stirred over the whole reaction time with a stirrer bar on a magnetic stirrer (300 rpm).

For high-pressure reactions the lipase-substrate-mixture was directly transferred into a temperature controlled high pressure vessel with a volume of 25 mL. The pressure vessel is depicted in Fig. 1. The temperature of the vessel is regulated through a water jacket which is connected to a temperature control device. A magnetic stirrer is placed underneath the vessel to ensure a