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Hydroxynitrile lyase adsorption at liquid/liquid interfaces

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Abstract

The adsorption behavior of hydroxynitrile lyase from *Prunus amygdalus* (*p*-Hnl) at liquid/liquid interfaces has been investigated using dynamic-interfacial-tension measurements. Unfolding of proteins at organic/aqueous interfaces results in a decrease of interfacial tension due to an increasing number of interfacial contacts of hydrophobic parts of the protein molecule. Seven organic solvents with different physical properties, including hexadecane, heptane, diisopropyl ether (DIPE) and ethyl acetate, were studied. It is known that *p*-Hnl loses its activity very quickly in a variety of solvents, but not in DIPE and ethyl acetate. For these two organic solvents, dynamic interfacial tensions remain constant over a period of 20 h. However, slow decline in the dynamic interfacial tension is found for the less polar solvents suggesting that the protein adsorbs and denatures. Additionally, experiments have been carried out with the aqueous buffer phase at low pH values down to 3.5 with DIPE as the organic phase, simulating conditions that have been previously used for the synthesis of cyanohydrins. There, it is known that the enzyme loses its activity very quickly. Correspondingly, the interfacial tension decreases under these conditions indicating that the bulk enzyme denatures and adsorbs at the interface in an unfolded configuration.

Keywords: Hydroxynitrile lyase; Interfacial tension; Liquid/liquid interface; Protein adsorption

1. Introduction

The group of enzymes named hydroxynitrile lyases (Hnls) are of great interest for the production of enantiomerically pure cyanohydrins due to their acceptance of a wide range of aliphatic and aromatic substrates [1–3]. Nevertheless, these enzymes have not been applied on an industrial scale to date because they lose their activity quickly under many reaction conditions used to avoid the chemical background reaction, such as low pH aqueous buffer systems with high buffer salt concentrations [4]. Furthermore, these enzymes cannot be used in aqueous systems for the production of hydrophobic products, such as long chain aliphatic and aromatic cyanohydrins. One possible means of avoiding these problems is to use the enzyme in two-phase systems or in organic solvents.

Although several publications have discussed the use of Hnl in organic solvents or organic/aqueous two-phase systems [5,6], nothing is known about the factors that influence the enzyme activity and stability at the liquid/liquid interface. The aim of this investigation is to determine the extent of enzyme adsorption at liquid/liquid interfaces. The adsorption of pro-

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teins to a liquid/liquid interface is studied by determining the interfacial tension between two phases. As proteins adsorb to an oil/water interface, the reduction of interfacial tension gives an indication of the characteristic processes in the different stages of adsorption.

2. Experimental

Dynamic interfacial tension was measured using the pendant drop-technique [7–9]. This technique determines the interfacial tension of an oil/water boundary from the shape of a gravity-distorted liquid drop. An aqueous drop is formed and suspended from a vertical stainless-steel circular capillary immersed in a water-saturated organic solvent (oil) phase. The resulting axisymmetry of the drop allows the shape to be described completely in two dimensions. A collimated light source illuminates the drop and the surrounding oil, and the resulting image is digitized using a CCD camera and a Power Macintosh 7100 AV. The difference in indices of refraction of the oil and the water phase produces a digitized image of a dark drop silhouette (water) in a uniform, light background (organic solvent). This intensity difference defines the edge of the drop, and the planar drop edge coordinates are determined by the location of pixels of a minimum greyscale intensity. Sequential drop images are obtained at specific time intervals using the public-domain software NIH-Image 1.59. Programs written in NIH-Image also determine the locus of edge coordinates. These coordinates are subsequently fitted to the Young–Laplace equation, which gives the predicted profile of a pendant drop for a given interfacial tension. Results are reported as the reduction in interfacial tension from the initial value. This change is defined as the surface pressure Π:

$$\Pi(t) = \sigma_0 - \sigma(t)$$

where σ_0 is the interfacial tension of the pure liquid/liquid interface and $\sigma(t)$ is the dynamic interfacial tension at time t with enzyme present. The interfacial tension of pure organic solvent/aqueous buffer systems showed reductions of less than 2 mN m^{-1} over a period of 24 h. Impurities were assumed to not affect the dynamic tensions of the protein solution [9].

The enzyme used is the hydroxynitrile lyase from almonds (*Prunus amygdalus*; *p*-Hnl), obtained from Sigma (St. Louis). All measurements described were carried out with an enzyme concentration of 10 g m^{-3}. All experiments were carried out in 50 mM KH_2PO_4 buffer where the respective pH is adjusted by saturated KOH or 1 M H_3PO_4 solution.

Hexadecane and tetradecane were obtained from Sigma; heptane from EM Science (Gibbstown, NY); cyclohexane from Fischer Scientific (Pittsburgh, PA); toluene, diisopropylether and ethyl acetate from Aldrich (Milwaukee, USA). All solvents had the highest purity commercially available. If the initial interfacial tension of the organic solvent/buffer was found to be lower than the literature value, the solvent was purified by adsorption of the impurities on silica.

3. Results and discussions

Several different organic solvent/aqueous two-phase systems were investigated. Only three solvents have been previously found to be suitable for the production of cyanohydrins by Hnls, namely ethyl acetate, diisopropyl ether (DIPE) and *tert*-butyl methyl ether [1,2,10]. In many other organic solvents the Hnl activity decreases very quickly [2] (H. Griengl, Personal communication, 1997). This is in contrast to other reports where it is suggested that very 'polar' solvents, such as ether and ethyl acetate, interfere in the enzyme structure and lead to a very fast deactivation of the protein by denaturation [11–14]. As pointed out before, unfolding is thought to lead to a decrease in interfacial tension and can be followed by dynamic interfacial

tension measurements. Therefore, we determined the decrease of the dynamic interfacial tension over a period of 20 h. We tried to find correlations between the inactivation of the *p*-Hnl and organic phase polarity as well as hydrophobicity. Two physical properties were often used for comparison of organic solvents applied in biocatalysis, namely the interfacial tension of the pure solvent/water and the log *P* values (logarithm of the partition coefficient in octanol/water). As seen in Table 1, we examined seven organic solvents as the 'oil' phase to establish possible differences in the enzyme adsorption at the interface. Note in Table 1 that the organic solvent/water interfacial tension does not correlate strongly with the corresponding log *P* value.

Fig. 1 shows the changes in surface pressure for the seven different solvents with the dilute pH 6.5 *p*-Hnl buffer solutions. Large increases in interfacial pressure are found for very hydrophobic solvents such as hexadecane, tetradecane, heptane, and cyclohexane. The changes over time are very similar for all of these solvents, and, therefore, the mechanism of adsorption seems to be analogous. Furthermore, the initial interfacial tensions of these solvents are comparable (see Table 1). From previous studies, the dynamic interfacial tension can be divided into three time regimes that are typical for many proteins [9,15]. The first is an induction regime (lag-time or regime I), where the interfacial tension remains relatively constant at the pure fluid values. At early times and low protein concentrations, protein molecules are present at the interface, but do not appreciably reduce the interfacial tension. The second regime is characterized by a sharp decline in tension from this initial value. As the interface becomes more saturated with protein by diffusion of new protein from the bulk aqueous phase and by adsorbed protein relaxation, changes in the interfacial tension are seen. Enzyme unfolding allows new side chains from the interior of the protein to adsorb, increasing the number of contacts between hydrophobic protein residues and the organic solvent phase and with each other. These multiple contacts eventually lead to irreversible adsorption. The final regime is characterized by a steady decline in interfacial tension, at a less negative slope on a semi-log plot than in regime II. The slow decline is attributed to slower conformational changes of the adsorbed layer and development of a protein network. These processes can take place over several days, as illustrated for various enzymes [15]. All these regimes can be found for hexadecane, tetradecane, heptane, and cyclohexane. There, the lag-time is about 20 s and a change in surface pressure is still determinable after more than 20 h.

Table 1
Comparison of interfacial tension (solvent/water) and log *P* values of various organic solvents used for dynamic interfacial tension measurements

Solvent	Interfacial tension, σ_0 (mN m^{-1})	Log *P*
Hexadecane	51	8.8
Tetradecane	51	7.6
Heptane	50.2	4.0
Cyclohexane	50.0	3.2
Toluene	36.1	2.5
Diisopropyl ether	17.8	1.9
Ethyl acetate	6.8	0.64

No change in surface pressure is determinable with ethyl acetate and DIPE as the organic phase over a period of 20 h. It is very likely that the protein molecules adsorb at the interface but, as there are insufficient contacts between the enzyme and the organic phase, the interfacial tension is not influenced. This leads to the assumption that the native structure of the *p*-Hnl is not destroyed by adsorption at the interface and that adsorption is reversible there. Moreover, this result is also in agreement with the fact that the *p*-Hnl can be used in these solvents for the synthesis of cyanohydrins without loss of activity.

The increase in surface pressure with toluene as the organic phase was less than with the more apolar solvents but more than with DIPE and ethyl acetate. These changes in surface

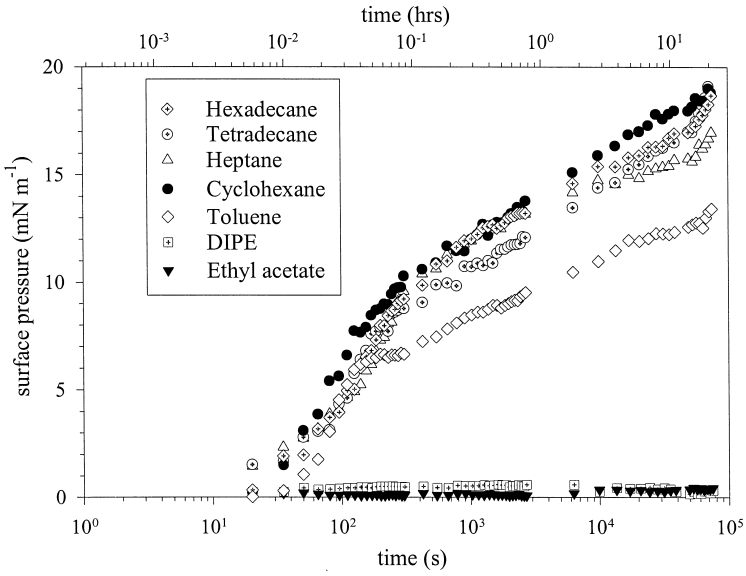

Fig. 1. Dynamic surface pressure of *p*-Hnl enzyme solution with seven different organic solvents. $C_{Hnl} = 10$ g m^{-3}, 50 mM KH_2PO_4 buffer, pH 6.5.

pressure correlate very well with the initial interfacial tensions of the pure solvent/water but, however, do not correlate with log *P* values as they differ for all solvents used. Apparently, clean interfacial tensions are a better indicator of the molecular interactions that affect the enzyme behavior in the adsorbed state. Octanol/water partitioning coefficients are a measure of the ratio of interactions between a dilute solvated molecules in the two homogeneous phases, where as molecules at an interphase are not isolated and totally 'solvated' in either phase. Above a certain hydrophobicity of the organic phase, the interfacial tension remains quite constant suggesting that the state of the water molecules at the interface is the same despite the structure or chain length of the organic solvent molecules.

We also tested for a change in surface pressure when the enzyme was dissolved in a buffer at low pH values and using DIPE as the organic phase. Low-pH buffers have been used for the cyanohydrin synthesis reaction, since the chemical background reaction is suppressed [5]. This leads to a higher enantiomerical purity of the resulting products. Unfortunately, without stabilization, Hnls lose their activity very rapidly under these conditions.

Fig. 2 shows the surface pressure changes at various pH values using DIPE as the organic phase. No changes of surface pressure were determined at pH 6.5 and 5.5, but increases in surface pressure were found for pH values below pH 5.5. There the initial lag-phases are dependent on the pH of the bulk aqueous phase. Upon going to low pH values, the time span of the initial lag-phase decreases. It is known that the *p*-Hnl enzyme activity strongly depends on pH. The enzyme is very stable at pH 6.5 and pH 5.5, but its half life decreases dramatically at lower pH values (the half life is less than 5 min at a concentration of 10 g m^{-3} and pH 3.5). Therefore, the increase in surface pressure can be explained by adsorption of already denatured *p*-Hnl at the interface. The difference in the initial lag-phase for different pH values can be explained by the time dependence of protein denaturation in the bulk aqueous phase. Since the concentration of denatured protein at pH 4.0 and 4.5 is very small for a long period of time

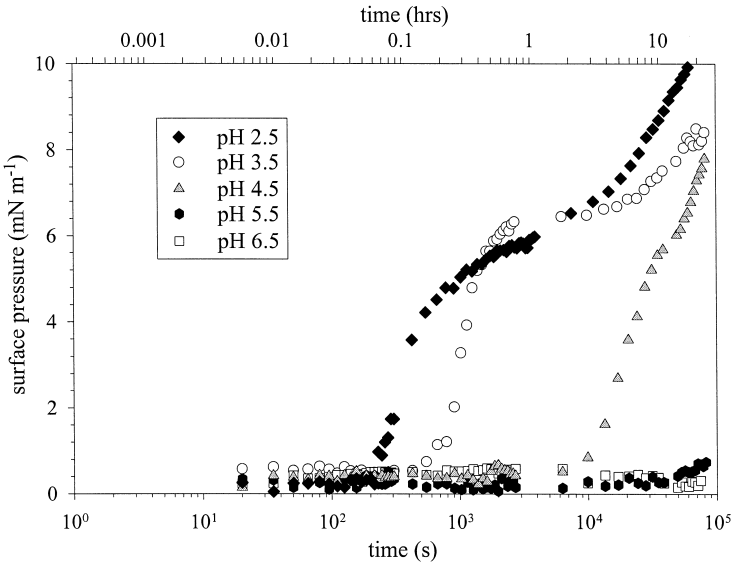

Fig. 2. Dynamic surface pressure of *p*-Hnl enzyme solution at DIPE/aqueous buffer interfaces at pH values between 2.5 and 6.5. $C_{Hnl} = 10$ g m^{-3}, 50 mM KH_2PO_4 buffer.

due to a slow pH-induced denaturation, the interfacial tension is not influenced until a sufficient concentration of unfolded *p*-Hnl is reached. Therefore, the initial lag-phase at pH 4.5 is extended (more than 2 h). Nevertheless, it was surprising that the initial lag-phases were also about 6 min and 20 min at pH 2.5 and 3.5, respectively. It is assumed that the entire protein is already denatured at the start of the experiment as a time dependence of the surface pres-

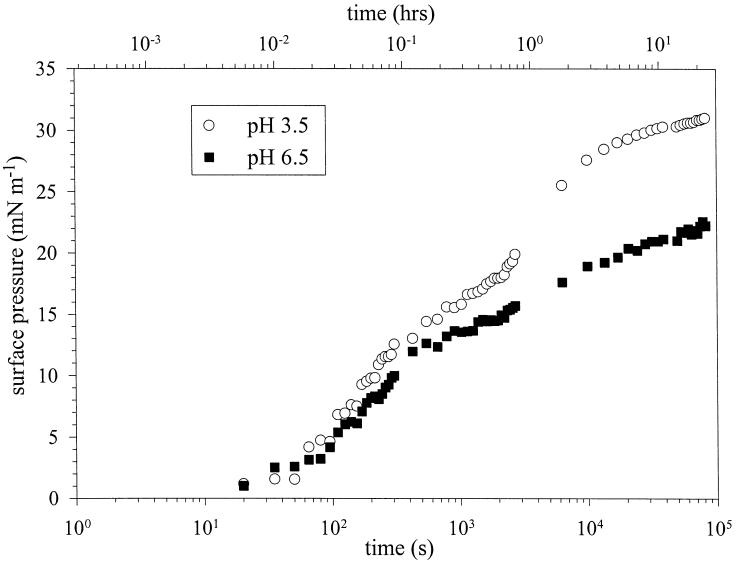

Fig. 3. Dynamic surface pressure of *p*-Hnl enzyme solution at heptane/aqueous buffer interfaces. $C_{Hnl} = 10$ g m^{-3}, 50 mM KH_2PO_4 buffer, pH 6.5 and pH 3.5.

sure change could not be determined when incubating the *p*-Hnl in buffer pH 2.5 and 3.5 for various times prior to the experiment. A slower diffusion of unfolded protein can contribute to some extent to that long phase but seems to be doubtful to explain a lag phase of 20 min. It has been observed with the Hnl from *Hevea brasiliensis* that stable aggregates form at pH 3.5 [4]. Aggregate formation with *p*-Hnl has not been proven but seems very likely. Therefore, one explanation for the longer lag-phase could be that aggregates adsorb and are stable at the DIPE/aqueous buffer interface for a longer time. As most hydrophobic parts of the protein interior are already in contact with hydrophobic parts of other protein molecules, the tendency to unravel at the interface may be minor. This can be especially true in the case of DIPE, a rather polar organic phase. Apparently it is not until some of these aggregates fall apart that the unfolded protein molecules adsorb at the interface to lower the tension.

Fig. 3 shows, in contrast to the results with DIPE, that the initial lag-phase for the protein solution at pH 3.5 and pH 6.5 with heptane as the organic phase are very similar. The influence of the bulk pH denaturation seems to play an unimportant role in the initial lag-phase in this case as heptane is substantially more hydrophobic than DIPE. Nevertheless, the total decrease of interfacial tension is higher at pH 3.5, which suggests that there is an overall influence of the pH-induced denaturation on interfacial tension.

4. Conclusions

Our results demonstrate that polar solvents do not destroy the natural conformation of the *p*-Hnl at the organic/water interface. In contrast, apolar solvents lead to an unfolding of the enzyme after adsorption, as shown by the dynamic decrease in interfacial tension. By changing the pH of the aqueous buffer from pH 6.5 to pH 4.5 (and lower) the surface pressures increase dramatically even with DIPE as the organic phase. There, the bulk inactivation and denaturation of the *p*-Hnl takes place prior to the adsorption at the interface. In contrast, the influence of bulk pH denaturation seems to play an unimportant role in the case of more hydrophobic organic phases, as changes in the initial lag-phase and total increase in surface pressure are minor. Dynamic interfacial tension is a useful tool for establishing the stability of enzymes at fluid/fluid interfaces.

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