

Role of Organic Solvents on Pa-Hydroxynitrile Lyase Interfacial Activity and Stability

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Abstract: Catalytic activity and adsorption of Pa-hydroxynitrile lyase (Pa-Hnl) was investigated at various organic solvent/water interfaces. We focused on the role of solvent polarity in promoting activity and stability in two-phase systems, specifically for the solvents heptane, dibutyl ether (DBE), diisopropyl ether (DIPE), butylmethyl ether (BME), and methyl *tert*-butyl ether (MTBE). Enzyme activity towards mandelonitrile cleavage was determined in a recycle reactor with a well-defined interfacial area as described by Hickel, et al. 1999. Here the recycle reactor was modified to permit exchange of the aqueous phase. With this modification, irreversibility of enzyme adsorption was determined as a function of the adsorption time at the interface. Irreversibility of enzyme adsorption was also investigated by measuring the surface pressure of a sessile-drop upon washout. We find that Pa-Hnl exhibits the highest stability but the lowest initial catalytic activity at the aqueous/organic solvent interface with the most polar organic solvents. Thus, DIPE and MTBE display no loss in enzyme activity over a period of several hours. However, the more apolar the solvent is the higher the initial Pa-Hnl activity, but the faster the loss of activity. Dynamic tensiometry reveals that Pa-Hnl adsorbs more strongly at the interface of the more apolar solvents. Surprisingly, Pa-Hnl develops some irreversible adsorption after 30 min at the DIPE/water interface, but does not lose catalytic activity. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **74**: 18–28, 2001.

Keywords: hydroxynitrile lyase; interfacial reaction; surface pressure; two-phase system

INTRODUCTION

Hydroxynitrile lyases (Hnls) are used for the production of enantiomerically pure cyanohydrins, which, in turn, provide chiral synthons for a variety of pharmaceuticals. To overcome endemic problems in the use of Hnls in bulk aqueous media, including decreased enzyme stability under the pH conditions necessary to avoid racemic products and low water solubility of many desired substrates and products, two-phase systems have been used for this biocatalytic re-

action (Bauer et al., 1999; Griengl et al., 1998; Hickel et al., 1999; Loos et al., 1995). Although both the stability and the solubility problem can be avoided in two-phase systems, the enzymatic stability of Hnls at the liquid/liquid interface depends strongly on the nature of the organic solvent. For example, the hydroxynitrile lyase from *Prunus amygdalus* (Pa-Hnl, EC 4.1.2.10) has been used successfully in a diisopropyl ether (DIPE)/water two-phase system (Hickel et al., 1999; Kiljunen and Kanerva, 1997a,b) but it loses activity quickly in many other organic solvent/water two-phase systems. Even though Hnls have been employed in several two-phase systems (Bauer et al., 1999; Förster et al., 1996; Hickel et al., 1999; Kiljunen and Kanerva, 1997a,b; Loos et al., 1995; Warmerdam et al., 1996), a fundamental understanding of the molecular mechanisms leading to Hnl adsorption and possible denaturation has not been achieved.

We recently established that Pa-Hnl catalyzes the mandelonitrile cleavage reaction directly at the liquid/liquid interface of the DIPE/water two-phase system and not in the bulk aqueous phase (Hickel et al., 1999). When at the interface, the enzyme follows Michaelis-Menten kinetics with a Michaelis constant K_m of 14.4 mM for mandelonitrile. Pa-Hnl adsorption at the DIPE/water interface appears to follow a Langmuir-like adsorption isotherm, with the caveat that direct measurements of the adsorption behavior were not studied. The surprising result was that Pa-Hnl adsorption, as measured by interfacial substrate cleavage kinetics, occurred for all aqueous enzyme concentrations studied, but the surface pressure changed significantly only at higher bulk enzyme concentrations (above 20 mg L⁻¹). Further, Pa-Hnl activity remained constant over the 2 h time period of the experiment at all enzyme concentrations employed. Apparently, interfacial configurational changes of the adsorbed Pa-Hnl at the DIPE/water interface permit partial unfolding, resulting in an increase in surface pressure, but not sufficient unraveling to alter the molecular structure of the active site. Unfortunately, direct determination of the enzyme structure at the interface cannot be carried out readily due to the difficult practical issues involved with studying deformable fluid interfaces. Therefore, we do not

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have a clear understanding of the enzyme structural changes occurring at the organic solvent/water interface.

In this work, we employ a modified version of the recycle reactor described in Hickel et al. (1999) to determine the interfacial activity of Pa-Hnl. Likewise, we also adopt the cleavage of mandelonitrile to benzaldehyde and HCN as the model enzymatic reaction. Our specific goal is to elucidate the role of solvent polarity in promoting enzyme activity and stability in two-phase systems. We investigated a range of organic solvents in the two-phase system including heptane, dibutyl ether (DBE), diisopropyl ether (DIPE), butylmethyl ether (BME), and methyl *tert*-butyl ether (MTBE). With the new recycle-reactor, enzyme in the bulk aqueous phase and any enzyme reversibly adsorbed at the interface can be washed out of the reactor without disturbing the organic solvent/water interface. Thus, by measuring the enzymatic reaction kinetics before and after washout, the degree of irreversibility of enzyme adsorption was determined as a function of the adsorption time at the interface. The reversibility of interfacial enzyme adsorption upon washout was also investigated by measuring the surface pressure in a sessile-drop apparatus. In dynamic tensiometry, changes in surface pressure during adsorption, washout, and after the washout can be followed. By combining information from kinetic and adsorption washout experiments we construct a mechanistic description of the physicochemical events occurring at the interface during two-phase enzyme catalysis.

MATERIALS AND METHODS

Materials

The enzyme used is the hydroxynitrile lyase from almonds (*Prunus amygdalus*; Pa-Hnl, EC 4.1.2.10), obtained from Sigma Chemical Co. (St. Louis, MO). The commercially available solution contains 3.5 mg mL⁻¹ protein with a specific activity of 160 μmol min⁻¹ mg⁻¹ and was stored at 4°C. Benzaldehyde (Certified), NaOH (Certified A.C.S.), and KOH (Certified A.C.S.) were obtained from Fischer Scientific (Pittsburgh, PA); dibutyl ether (DBE, 99%), butylmethyl ether (BME, 99%), methyl *tert*-butyl ether (MTBE, 99%+), diisopropyl ether (DIPE, 99%+), and potassium dihydrogen phosphate (99%+) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Heptane (OmniSolv, Extra Low UV Cut Off) was obtained from EM Science (Gibbstown, NJ, USA). All organic solvents used for experiments were presaturated with 50 mM KH₂PO₄ aqueous buffer (adjusted to the desired pH with a saturated KOH solution).

Mandelonitrile was obtained from DSM-Chemie (Linz, Austria). Because of the cleavage of mandelonitrile by humid air and oxidation of the resultant benzaldehyde to benzoic acid, mandelonitrile was stored at -20°C under a nitrogen atmosphere. The benzaldehyde content in the mandelonitrile is less than 5 wt% initially (determined by UV spectroscopy) and slowly increases over time after opening

the container. To account for the increasing benzaldehyde concentration in the substrate, we measured the background absorption of the substrate in the organic solvent before the enzymatic reaction was started. The initial benzaldehyde concentration is not of critical importance, as only changes of benzaldehyde concentration over time were measured to determine the product formation rate. Benzoic acid, a strong Hnl inhibitor formed from benzaldehyde by oxidation, is absent from the mandelonitrile due to the production method and storage conditions.

Methods

Recycle Reactor Design

The probe reaction for the determination of Hnl activity is the cleavage of mandelonitrile to form benzaldehyde and HCN with product formation followed selectively by UV absorbance in the organic phase. As illustrated in Figure 1, the apparatus designed for these experiments consists of a cylindrical glass recycle reactor, a fiberoptic UV spectrometer, including a flow-through optical cell, and a pump for recycling the organic solvent. The aqueous enzyme solution is placed in the lower section of the reactor. Product formation in the organic phase above is followed by online UV spectroscopy. The organic phase is well mixed by recycling it through the optical cell. Inlet and outlet flow lines are located just above the organic solvent/water interface. The interface is nearly stagnant at the flow rate used (8.3×10^{-3} L min⁻¹). A more complete description of the reactor and experimental setup is given in Hickel et al. (1999).

There are three basic modifications to the setup employed previously. First, the recycling of the organic phase is now accomplished by a L/S® PTFE Tubing Pump (Masterflex® from Cole-Parmer, Vernon Hill, IL, USA), since this pump is more resistant to alkane solvents. Second, the Teflon® stopper that seals the top of the reactor was replaced by a stainless-steel stopper of the same dimensions. Third, to

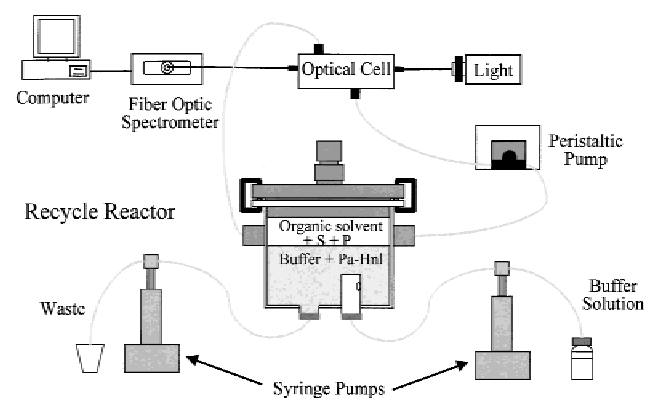


Figure 1. Schematic of the apparatus for the determination of enzyme-adsorption reversibility upon washout. Pa-Hnl catalyses the cleavage of mandelonitrile (substrate = S) to form benzaldehyde (product = P) and HCN at the DIPE/buffer interface.

permit study of enzyme adsorption reversibility we designed a new recycle reactor to allow exchange of the aqueous phase. Figures 1 and 2 demonstrate that inlet and outlet flow lines have now been added to the bottom of the reactor, as shown.

The overall experimental setup for the enzyme reaction kinetics and adsorption studies is illustrated in Figure 1. Two opposed syringe pumps (Model 260D, ISCO, Lincoln, NE, USA), each with a capacity of 200 mL, exchange the aqueous phase in the recycle reactor. Syringe pumps have the advantage of constant and fluctuation-free flow, which is very important to avoid mechanical disturbance of the enzyme at the liquid/liquid interface.

To determine the initial activity of the enzyme at the organic solvent/water interface we followed the procedures described in Hickel et al. (1999). For all experiments, the total aqueous-phase volume was 5.5×10^{-3} L in the reactor without exchange of the aqueous phase and 6.5×10^{-3} L in the reactor for the exchange experiments. The organic-phase volume was 5×10^{-3} L, including that in the reactor and in the recycle tubing. Fifty mM KH_2PO_4 , adjusted to the respective pH by a saturated KOH solution, served as the aqueous buffer. The flow rate for the recycling for the organic phase through the optical cell was 8.3×10^{-3} L min^{-1} .

The rate of chemical cleavage of mandelonitrile in the various two-phase systems without enzyme present depends on the solubility of mandelonitrile and benzaldehyde in the two phases, as well as on the water solubility in the organic phase. Background product formation rates without enzyme present were found to be small and were subtracted from the experimental rate data with enzyme present to allow comparison of the various organic solvents.

All enzyme washout experiments were carried out in the DIPE/water two-phase system. Pa-Hnl was added to the reactor aqueous phase via a syringe inserted through the hole in the stopper. Enzyme was adsorbed for various times at the interface prior to washout (between 15 min and 18 h). Subsequently, the aqueous phase was exchanged by 180 mL of 50 mM K_2PO_4 buffer at pH 5.5 and presaturated with diisopropyl ether (DIPE) to displace remaining enzyme in the bulk aqueous-phase and any enzyme reversibly ad-

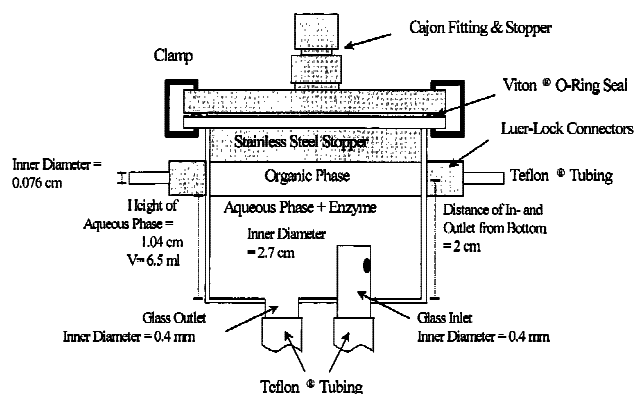


Figure 2. Detailed schematic of the reactor. Organic-phase volume in the reactor, $V_R = 4.2 \times 10^{-3}$ L, aqueous-phase volume, $V_A = 6.5 \times 10^{-3}$ L.

sorbed to the interface. Exchange was carried out by two syringe pumps each operating at a flow rate of 10 mL min^{-1} . To keep the aqueous-phase volume in the reactor constant the pumps were controlled such that one was emptying while the other was refilling, each at the same flow rate. After displacement of the aqueous solution by enzyme-free buffer, mandelonitrile was added to the organic phase with a gas-tight $10\text{-}\mu\text{L}$ syringe and the benzaldehyde concentration change was monitored by UV spectroscopy over time.

Dynamic Interfacial Tension Measurements

The automated pendant-drop apparatus described by Beverung et al. (1998) was used to measure dynamic interfacial tensions. Experimental procedures and detailed description of the equipment are available elsewhere (Beverung, 1996; Beverung et al., 1998; Hickel et al., 1999). Tensiometry results are reported as the reduction in interfacial tension from the initial value. This change is defined as the dynamic surface pressure, $\Pi(t) = \sigma_0 - \sigma(t)$, 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. Measurements of the interfacial tension of the substrate and product at the heptane/water as well as at the DIPE/water interface indicate no perceptible surface activity for either mandelonitrile or benzaldehyde.

To investigate possible reversibility of protein adsorption at the fluid/water interface, an inverted sessile-drop assembly was constructed (Galitzky, 1999), as illustrated in Figure 3. By trapping an organic solvent drop underneath a flattened glass rod immersed in the bulk aqueous solution, enzyme present in the bulk aqueous phase can be washed out without disturbing the organic droplet. Replacement of the aqueous enzyme solution with pure buffer was carried out by a flow system identical to that of the recycle reactor and displayed in Figure 1. The inlet line to the quartz optical cell was placed diagonally opposed from the outlet line, providing gentle flow past the organic solvent drop and also minimizing dead volume in the cell.

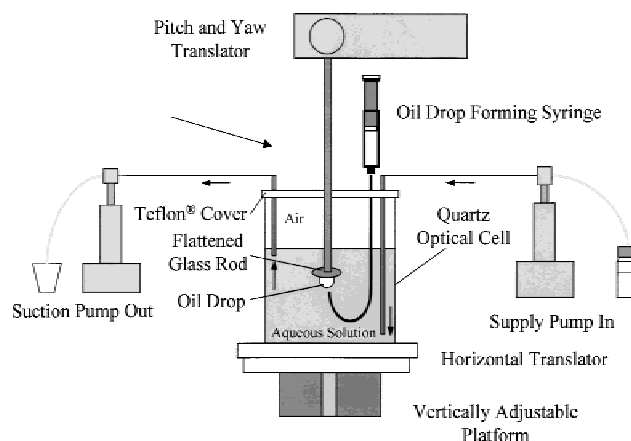


Figure 3. Detailed schematic of inverted sessile drop assembly for the interfacial-tension determination upon washout.

For all experiments the aqueous-phase volume was 20 mL and the enzyme concentration in the aqueous phase was 49.2 mg L⁻¹. The aqueous phase was replaced by 400 mL of 50 mM KH₂PO₄ buffer, pH 5.5, and saturated with DIPE, at a flow rate of 10 mL min⁻¹. Separate mixing experiments demonstrate that complete washout of a nonadsorbing solute occurs within several minutes at this flow rate (Galitzky, 1999).

RESULTS

Influence of Solvent on Enzyme Kinetics and Adsorption

The choice of solvent is limited by constraints on the determination of the enzyme activity and by the reactor design. Solvents are required to be water immiscible, thereby excluding glyme, THF, and dioxane, and to exhibit no UV absorbance at 280 nm, as this interferes with the product concentration determination. Thus, aromatic solvents are not suitable, as well as stabilized ethers, due to the aromatic nature of the stabilizers. A low viscosity of the solvent is needed to minimize mass-transfer limitations in the reactor (Hickel et al., 1999). This criterion excludes long-chain aliphatic solvents such as hexadecane and dodecane. Finally, mandelonitrile and benzaldehyde must readily dissolve in the solvents. This solubility limitation is not met by very apolar solvents, such as hexadecane and cyclohexane.

Based on these restrictions, we studied the following solvents: diisopropyl ether (DIPE), butyl methyl ether (BME), methyl *tert*-butyl ether (MTBE), dibutyl ether (DBE), and heptane. Heptane is the most apolar solvent we used for our experiments, although mandelonitrile does not exhibit much solubility in heptane.

Pertinent physical properties of the solvents are found in Table I. Differences in chemical structures and physical properties among DBE, DIPE, BME, and MTBE are rather small in comparison to heptane. Nevertheless, these differences prove to be significant, especially with respect to enzyme stability at the solvent/water interface. The listed physical properties correlate well to the initial enzyme activity and the enzyme stability, as shown by the half-life of the reaction-rate decline ($t_{1/2}$).

Figure 4 shows the concentration of the product benzaldehyde over time at the DIPE/water, BME/water, and MTBE/water interfaces for a mandelonitrile concentration of 10 mM and a Pa-Hnl concentration of 12.2 mg L⁻¹. Symbols represent the experimental benzaldehyde concentrations; lines represent the first derivative of these data with respect to time, illustrating the product formation rate, or equivalently, the enzyme activity at the interface (Hickel et al., 1999). The product formation rate is highest at the DIPE/water interface (dashed line), followed by the BME/water (solid line) and MTBE/water (dashed-dotted line) interfaces. Concentration data represented here do not include correction for the chemical background reaction. Values of the actual enzymatic interfacial product formation rate corrected for the background reaction are reported in Table I. Note that the product formation rate stays constant over a period of at least 3 h for all three solvents. Thus, as previously reported for DIPE (Hickel et al., 1999), Pa-Hnl does not apparently denature at the aqueous interface with these three solvents within the time period of experiments.

Figure 5 shows the concentration change of the product benzaldehyde over time at the DBE/water interface at the same mandelonitrile and Pa-Hnl concentrations as in Figure 4. The initial product formation rate is 3–10 times higher here than at the other ether/water interfaces described above. A major difference also is that the enzyme loses activity at the DBE/water interface. To describe the loss of enzyme activity quantitatively we ascertain the time when the product formation rate falls to 50% of its initial value ($t_{1/2}$). At the dibutyl ether/water interface, $t_{1/2}$ is 56 min.

Pa-Hnl behavior studied at the interface of the most apolar organic solvent, heptane, can be seen in Figure 6. Here, the initial product formation rate is very high initially (more than 20 times higher than at the DBE/water interface) but rapidly declines. Half of the initial product formation rate is lost within 1 min, which indicates that Pa-Hnl is very unstable when exposed to the heptane/water interface.

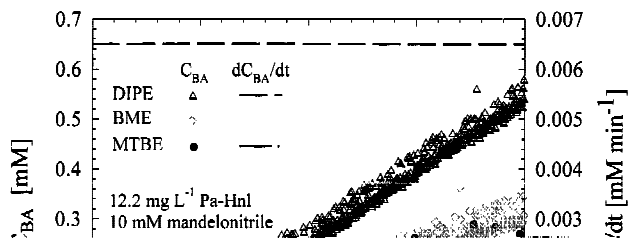
We also determined the dependence of the initial interfacial product formation rate and $t_{1/2}$ on the bulk aqueous Pa-Hnl concentration at the DBE/buffer interface, as illustrated in Figure 7. Similar to DIPE (Hickel et al., 1999), the initial interfacial product formation rate (filled circles) in-

Table I. Comparison of initial interfacial product formation rate and $t_{1/2}$ at different organic solvent/water interfaces with selected physical properties of the respective solvents (50 mM K₂PO₄ buffer, pH 5.5, $C_{\text{Pa-Hnl}} = 12.2 \text{ mg L}^{-1}$, $C_{\text{MN}} = 10.1 \text{ mM}$).

Solvent	Initial interfacial product formation rate [M min ⁻¹]	Time of 50% of initial product formation rate, $t_{1/2}$ [min]	Initial interfacial tension [mN m ⁻¹] ^a	Logarithmic octanol/water partition coefficient ^b log P
Heptane	420	<1	50	4.50
Dibutyl ether (DBE)	15.3	56	28	3.21
Diisopropyl ether (DIPE)	5.0	>300	17.8	1.52
Butyl methyl ether (BME)	1.7	>300	15.8	—
Methyl <i>tert</i> -butyl ether (MTBE)	1.3	>300	10.5	0.94

^aMeasured in our laboratory by pendant drop-tensiometry.

^bCRC Handbook of Chemistry and Physics, 80th ed. p 16–42, 1999–2000. Boca Raton, FL: CRC Press.



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creases linearly with the bulk enzyme concentration until it attains a constant value (about $0.039 \text{ mM min}^{-1}$ at an enzyme concentration of 40 mg L^{-1}). Half-life measurements (open squares), however, reveal that Pa-Hnl is deactivated at the DBE/water interface and does so more quickly when more protein is present in the aqueous phase. At a concentration of 3 mg L^{-1} , $t_{1/2}$ is 112 min; at concentrations above 40 mg L^{-1} , $t_{1/2}$ is constant at a value of about 36 min. The calculated maximum interfacial product formation rate at the DBE/water interface is $0.35 \text{ mmol m}^{-2} \text{ min}^{-1}$ (see Hickel et al., 1999, for the calculation procedure). In comparison to the maximum interfacial product formation rate at the non-denaturing DIPE/water interface (Hickel et al., 1999, Fig. 10), we find that Pa-Hnl activity at the DBE/water interface is initially 2.2 times higher. However, that activity is completely lost within several hours of reaction.

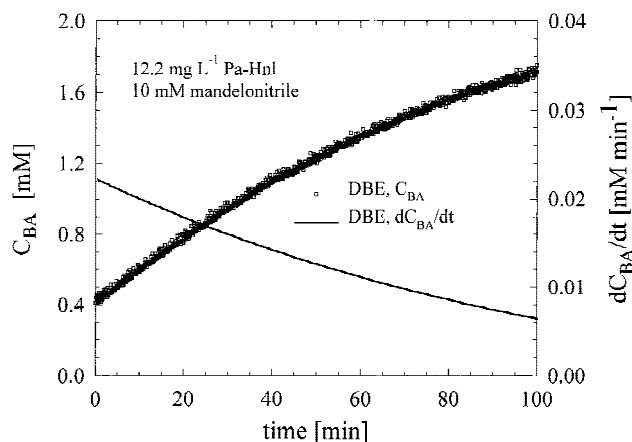


Figure 5. Time dependence of product concentration and product formation rate with DBE as the organic phase in a two-phase system. The initial product formation rate is $15.3 \times 10^{-3} \text{ mM min}^{-1}$ after the subtraction of the background product formation rate ($50 \text{ mM KH}_2\text{PO}_4$ buffer, pH 5.5).

Dynamic surface pressures of Pa-Hnl are illustrated in Figure 8 for three different interfaces: heptane/water, DBE/water, and DIPE/water. At an enzyme concentration of 10 mg L^{-1} , the dynamic surface pressure at the DIPE/water interface does not change from its initial value for over 10 h. Conversely, the surface pressure of Pa-Hnl increases slowly at the DBE/water interface over the same time period. Finally, changes in the surface pressure of Pa-Hnl occur almost immediately after drop formation at the heptane/water interface. No clear equilibrium state is attained with the DBE/water and heptane/water interfaces after 10 h of Pa-Hnl adsorption.

Irreversibility of Enzyme Adsorption at the DIPE/Water Interface

Pa-Hnl does not lose activity at the DIPE, BME, or MTBE/water interfaces over many hours of exposure (see Fig. 4

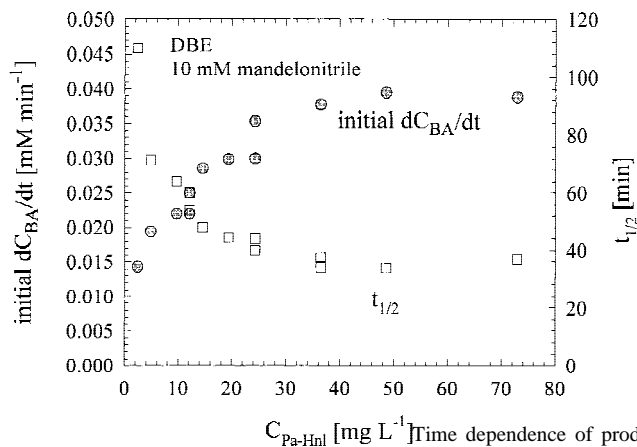


Figure 7. Dependence of the initial product formation rate and half-life of product formation rate on aqueous phase bulk Pa-Hnl concentration (10 mM mandelonitrile, $50 \text{ mM KH}_2\text{PO}_4$ buffer, pH 5.5) for the DBE/water interface.

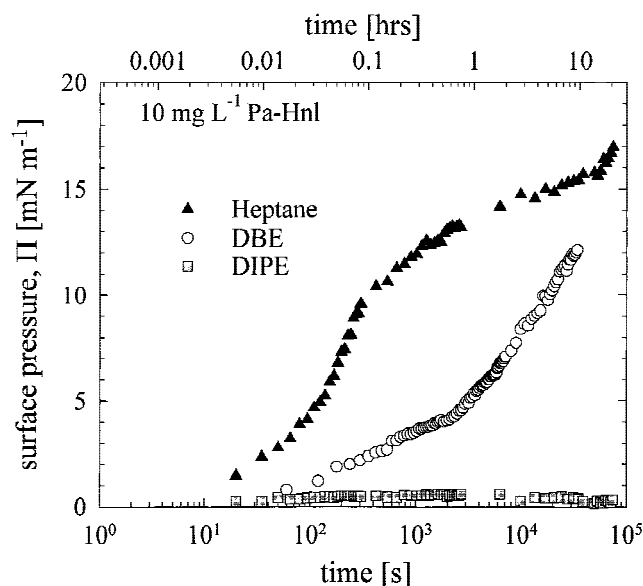


Figure 8. Dynamic surface pressure changes of three different organic solvent/water two-phase systems upon Pa-Hnl adsorption: heptane, DBE, and DIPE (50 mM KH_2PO_4 buffer, pH 5.5).

and Hickel et al., 1999). This indicates that the enzyme does not change conformation of the active site upon adsorption at these interfaces. On the other hand, dynamic surface pressures do significantly increase, depending on the enzyme concentration in the bulk aqueous phase (Hickel et al., 1999). It is likely that proteins do not substantially alter the interfacial tensions without at least partial unfolding (Anderson et al., 2000; Beverung, 1996; Hickel et al., 1998; Tripp et al., 1995). Therefore, significant decreases in interfacial tension are taken here as an indication of enzyme conformational change upon adsorption at the fluid/fluid interface. If such adsorption-caused structural changes are insufficient to decrease the activity of Pa-Hnl at certain interfaces, the question arises whether they are sufficient to initiate irreversible Pa-Hnl adsorption. Therefore, we investigated the possible irreversibility of enzyme adsorption at the DIPE/water interface by two independent techniques: enzyme washout studies in the recycle reactor and in the sessile-drop tensiometer.

Possible change of product formation rate at the interface after washout of enzyme in the aqueous phase was examined in the recycle reactor shown in Figures 1 and 2. After Pa-Hnl was adsorbed for various times at the DIPE/water interface, the aqueous phase was replaced by pure buffer, and the subsequent product formation rate was remeasured as reported in Figure 9. The two different symbols in this figure (open squares and filled circles) represent data obtained with two different Pa-Hnl lots. Also, since the initial enzyme activity changes slightly for every enzyme batch the data have been normalized to allow direct comparison.

From Figure 9 we find that no product is formed after washout when Pa-Hnl is permitted to adsorb for only a short time (<15 min) at the DIPE/water interface. The longer the

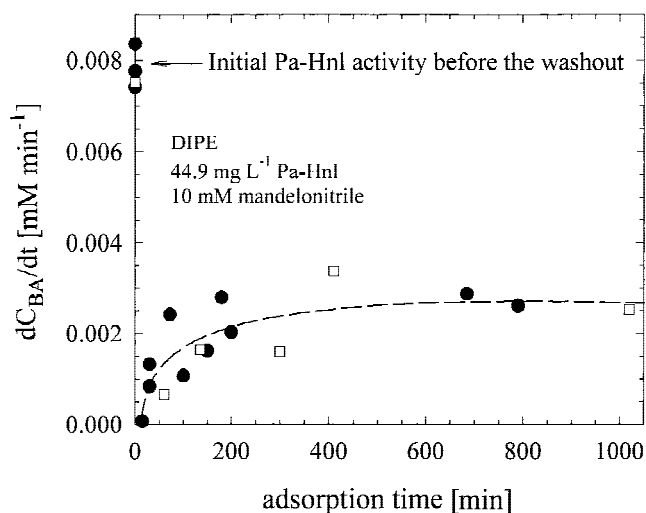


Figure 9. Dependence of the product formation rate on the adsorption time of Pa-Hnl at the DIPE/water interface measured after washout of the aqueous phase. The two different symbols represent data obtained with two Pa-Hnl lots. The initial enzyme activity changes for every enzyme batch and, in order to compare them, data have been normalized. The dashed line is a guide to the eye (50 mM KH_2PO_4 buffer, pH 5.5).

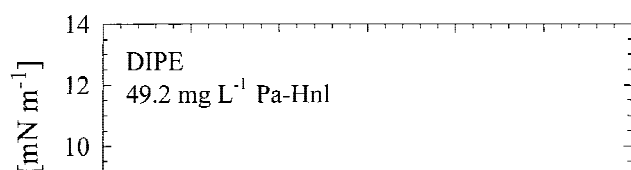
enzyme adsorbs at the interface, however, the higher is the remaining product formation rate. After several hours of enzyme adsorption, the product formation rate after washout reaches a constant value of about 30% of the initial value and does not change with further increases in time of enzyme adsorption. The results in Figure 9 suggest that Pa-Hnl readily desorbs initially from the DIPE/water interface but that at later time some irreversible adsorption develops.

Protein desorption from the interface should lead to a decrease in surface pressure. Therefore, to ascertain further whether Pa-Hnl adsorption changes from reversible to irreversible over time, we measured the change in surface pressure before, during, and after exchanging the aqueous phase as a function of the adsorption time of the protein at the interface. Figure 10 shows on a linear time scale the change in surface pressure of a 49.2 mg L^{-1} Pa-Hnl solution upon adsorption at the DIPE interface over a period of 300 min. The surface pressure first increases after a lag of about 20 min. After about 100 min, the rate of increase in surface pressure slows down but no steady value is reached over 5 h.

Washout experiments were then carried out under the same conditions as those in Figure 10. Figure 11 shows the results for dynamic surface pressure upon Pa-Hnl adsorption for 60 min followed by washout of Pa-Hnl in the bulk aqueous phase for 65 min. Next, Figure 12 exhibits a similar graph only now adsorption occurs for 120 min followed by 53 min of washout. Dynamic surface pressures early on in Figure 11 and 12 vary somewhat from those observed in Figure 10, even though all conditions were identical. This is due to differences in the enzyme solutions employed. Tem-

perature differences and age of the enzyme solution also slightly influence the initial lag phase of enzyme adsorption, but they do not influence the overall change in surface pressure.

After the initial increase in surface pressure during the first hour of adsorption, the surface pressures in Figure 11 slightly increase during washout. After washout ends 115 min, the surface pressure continues to rise slowly. The increase in surface pressure after 300 min is only a third of the increase for the experiments conducted in the absence of washout seen in Figure 10. After 120 min of enzyme adsorption in Figure 12, washout of the aqueous phase does not influence the dynamics of surface pressure. The data in Figure 12 illustrate that the surface pressures follow the same trends as those observed in Figure 10 without washout.



Replacement of Native Enzyme by Denatured Enzyme

We have shown previously that native enzyme cannot displace denatured enzyme from the DIPE/water interface (Hickel et al., 1999). Hence, bulk denatured enzyme adsorbs more strongly at the DIPE/water interface than does the native enzyme. Thus, we investigated by reaction kinetics whether bulk denatured enzyme can replace adsorbed native enzyme at the DIPE/water interface. Figure 13 shows the product concentration over time for a Pa-Hnl concentration of 26.5 mg L⁻¹. After 24 min of reaction, the same concentration of heat-denatured Pa-Hnl (40 min at 90°C) was

added to the aqueous buffer. As seen in Figure 13, there is no decrease in product formation rate after addition of denatured enzyme, which proves that for this adsorption time native Pa-Hnl is not replaced at the interface by denatured enzyme. As the irreversibility of enzyme adsorption strongly depends on the exposure time of the enzyme to the interface, this result only applies for adsorption times greater than 24 min and may be different for very short adsorption times.

Influence of Aqueous pH on Pa-Hnl Kinetics at the Interface

The pH of the aqueous buffer is critical for successful application of Pa-Hnl in industrial processes. Low pH values (below pH 4) suppress the chemical cleavage and formation of racemic cyanohydrins in the aqueous phase, but, unfortunately, low pH denatures Hnls at the same time (Bauer et al., 1998; Hickel et al., 1997). Thus, two-phase systems are better suited for industrial applications. Since the substrate and product are primarily soluble in the organic phase, the background reaction can be strongly suppressed even at pH values higher than 4.0. The influence of the pH on Pa-Hnl adsorbed at the solvent/water interface has not yet been investigated.

Figure 14 records the transient product concentration in the DIPE phase over time for four different aqueous-phase pH values. Lines in this graph represent the mean value of the data points, and the standard deviations are illustrated by the reported error bars. Above pH 5.0, the enzyme is very stable at the DIPE/water interface (Hickel et al., 1999). At pH 4.0, Pa-Hnl slowly loses interfacial activity, as indicated by the slight decrease in the slope of the corresponding curve in Figure 14. Below pH 4.0, the enzyme deactivates more quickly the lower is the pH of the aqueous phase.

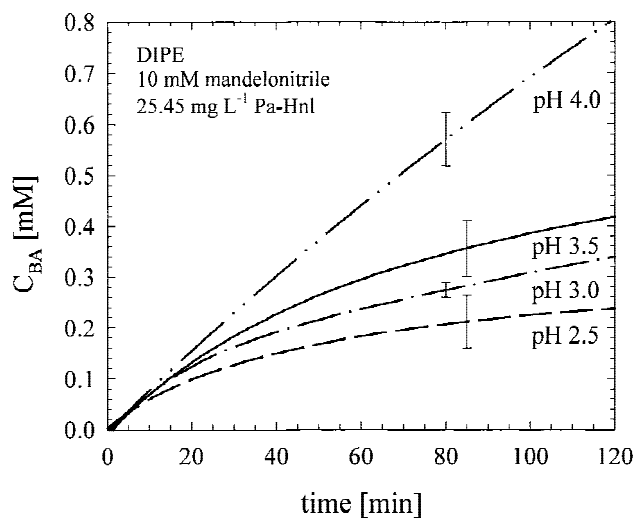


Figure 14. Dependence of the product formation rate measured at the DIPE/water interface on the aqueous phase pH. Lines in this graph represent the mean value of the data points. The SD is shown by the error bars (50 mM KH₂PO₄ buffer).

DISCUSSION

Pa-Hnl Adsorption, Activity, and Stability in Two-Phase Systems

Pa-Hnl adsorption and deactivation stability at liquid/liquid interfaces depends strongly on the physical properties of the organic solvent. The initial product formation rate, which directly corresponds to the interfacial enzyme activity, increases with the nonpolar nature of the solvent. Table I provides values of the nascent organic solvent/water interfacial tensions and the logarithm of the octanol/water partition coefficient (log P) for the solvents employed. Simply put, the higher the solvent/water interfacial tension and the log P value is, the higher is the initial interfacial Pa-Hnl activity. Conversely, the enzyme stability at the solvent/water interface decreases with decreasing polarity of the organic solvents. Accordingly, Pa-Hnl is stable over many hours at DIPE, BME, and MTBE/water interfaces, but quickly loses activity at those interfaces with initial interfacial tensions above about 20 mN m⁻¹.

A definitive explanation of these results is not possible, as many factors influence the enzyme-activity determination. A major problem for comparing the results of different two-phase systems is the difference in the partition coefficients of mandelonitrile between the organic and aqueous phases, which leads to varying substrate concentrations in the organic phases in every two-phase system. Since we do not measure kinetics in a concentration range where there is total substrate saturation of the enzyme (due to experimental limitations, the enzyme concentration is around the Michaelis K_m value measured for the DIPE/water system; Hickel et al., 1999), a change in the organic-phase substrate concentration by dissolution into the aqueous phase can influence the measured product formation rate. One might consider this partitioning as the reason for the determination of varying interfacial activity in the different two-phase systems. That this reasoning is not valid is argued as follows.

The partition coefficient of mandelonitrile between the organic phase and water is highest for the DIPE/water system, followed by MTBE/water, DBE/water, and heptane/water (also see Bauer et al., 1999). Upon comparing this trend in partition coefficients with the initial interfacial product formation rates of the respective systems shown in Table I, no correlation can be found. Rather, Pa-Hnl shows the highest initial activity in the heptane/water system, followed by DBE/water, even though the substrate concentrations in the organic phase are lowest there. Therefore, the difference in substrate solubility and concentration in the various organic phases alone does not explain the high initial enzyme activity with the very apolar solvent/water interfaces.

We propose that Pa-Hnl adsorbs more strongly at the more apolar interfaces. Further, strong adsorption leads to denaturation of the enzyme by partial unfolding, which is shown by a loss in activity over time measured by interfacial product formation rate (see Figs. 5–7). In addition, the

dependence of the initial interfacial product formation rate on the Pa-Hnl concentration in the aqueous bulk phase for two different two-phase systems, DIPE/water (Hickel et al., 1999) and DBE/water (Fig. 7), provides information on adsorption strength of Pa-Hnl at these two interfaces. In both cases, the initial interfacial product formation rate increases with bulk concentration until a constant value is reached. These two systems differ not only in the maximum initial interfacial product formation rates but also in the enzyme concentrations in the aqueous phase when this constant value is reached. For the DIPE/water interface, the maximum interfacial product formation rate is $0.16 \text{ mmol m}^{-2} \text{ min}^{-1}$ at a high bulk Pa-Hnl concentration (above 200 mg L^{-1} , see Fig. 10 of Hickel et al., 1999). However, at the DBE/water interface, the maximum initial interfacial product formation rate is $0.35 \text{ mmol m}^{-2} \text{ min}^{-1}$ above a bulk Pa-Hnl concentration of 40 mg L^{-1} (calculated by dividing the maximal initial product formation rate seen in Figure 7 by the known interfacial area). Thus, the DBE/water interface is saturated with protein at a lower bulk Pa-Hnl concentration, indicating stronger adsorption. Stronger adsorption also explains the difference in initial interfacial product formation rates at the same aqueous bulk phase Pa-Hnl concentration for the DBE/water and DIPE/water systems by a factor of three. Increased adsorption leads to a higher interfacial enzyme concentration at the same aqueous bulk phase concentration and, therefore, to an increased initial product formation rate. Nevertheless, the interfacial enzyme concentration likely does not exceed a maximal interfacial coverage. For the heptane/water system, the initial product formation rate increases by a factor of 80 in comparison to the DIPE/water system. Stronger enzyme adsorption may not be the only explanation, as the factor of 80 demands a surface coverage far exceeding estimates of the maximum (Hickel et al., 1999). A possible explanation for the extremely high initial interfacial product formation rate at the heptane/water interface is an activation due to conformational changes in the active site, which eventually lead to enzyme denaturation in the long run. As has been shown for lipases, small conformational changes in one part of the enzyme can lead to activation of the enzyme at the interface (Faber, 1995; van Tilbeurgh et al., 1993). Since the X-ray structure of Pa-Hnl is not known, we have no available structural information for the validity of this assumption.

A second significant result from Figure 7 is the dependence of the interfacial enzyme stability (i.e., $t_{1/2}$) on the enzyme concentration in the bulk aqueous phase. In the case of DIPE, Pa-Hnl is stable for all concentrations over the entire time period of the experiment, as shown in Figure 4 and by Hickel et al. (1999). At the DBE/water interface, however, the enzyme loses activity and does so more rapidly the higher is the enzyme concentration in the bulk aqueous phase.

Another striking indication for stronger Pa-Hnl adsorption to the more apolar solvent/water interfaces can be found in Figure 8. The more apolar the solvent, the greater is the increase in dynamic surface pressure, which is in

agreement with faster unfolding of Pa-Hnl at apolar interfaces. The surface pressure changes almost immediately at the heptane/water interface, indicating small diffusion or kinetic barriers to adsorption and unraveling (Anderson et al., 2000). In contrast, the surface pressure remains the same at the DIPE/water interface for the identical bulk aqueous phase Pa-Hnl concentration of 10 mg L^{-1} . Although Pa-Hnl activity does not change over a period of hours at any concentration at the DIPE/water interface, the dynamic surface pressure does increase over time when the bulk enzyme concentration is higher than of 25 mg L^{-1} (Hickel et al., 1999). Apparently, changes in surface pressure are an indication of enzyme adsorption and partial unfolding at the solvent/water interface, but they do not directly diagnose enzyme denaturation.

Irreversibility of Pa-Hnl Adsorption at the DIPE/Water Interface

For a period of several hours the transient product concentrations in the organic phase increase linearly in Figure 4, indicating a constant reaction rate of Pa-Hnl at the interface. It appears that the active site does not undergo conformational changes leading to a loss in activity over this time period. One might conclude, therefore, that Pa-Hnl is reversibly adsorbed in its native conformation at the DIPE/water interface.

However, our kinetic washout experiments show that Pa-Hnl adsorption at the DIPE/water interface changes slowly from reversible to irreversible. Even after 20 min of adsorption, Pa-Hnl cannot be completely removed from the interface (see Fig. 9). The amount of irreversibly adsorbed protein increases slowly in time until it reaches a constant value after about 2 h, as determined by both interfacial activity (Fig. 9) and surface pressure (Figs. 11, 12). The dynamic surface pressures in Figures 10 and 12 are identical, showing that within 2 h of exposure the amount of tension-lowering enzyme at the interface is irreversibly bound. From Figure 9, an interesting result is that the maximum amount of irreversibly adsorbed protein after about 2 h is apparently only about 30% of the amount before washout. There are two possible explanations for these findings. First, a reversibly adsorbed second layer of Pa-Hnl influences the activity measurements but does not affect the tension lowering. Second, only around 30% of the Pa-Hnl at the interface shifts from reversible to irreversible adsorbed protein over time, and reversibly adsorbed enzyme negligibly influences tension. In both cases, however, enzyme conformation changes must occur; otherwise, complete reversibility should be observed. Nevertheless, the conformational changes of Pa-Hnl at the DIPE/water interface do not alter the active site, since the overall enzyme activity does not decrease, at least, for up to several hours.

As mentioned above, it appears from the surface pressure experiments that irreversibly adsorbed protein dominates the changes in the dynamic surface pressure. There is no decrease in surface pressure during or after the washout of

the enzyme solution, independent of the time that Pa-Hnl adsorption has occurred. Clearly, there are also long-time processes occurring caused by the irreversibly adsorbed Pa-Hnl influencing the surface pressure, but not strongly influencing the interfacial Pa-Hnl activity at the DIPE/water interface.

The exchange experiment in Figure 13 provides another confirmation of the irreversibility of Pa-Hnl adsorption at the DIPE/water interface. Denatured enzyme very likely adsorbs more strongly at the interface than native enzyme (Hickel et al., 1999). Nevertheless, denatured Pa-Hnl does not displace native Pa-Hnl from the DIPE/water interface after 20 min of initial adsorption. Thus, given enough adsorption time the interaction between the initially native enzyme and the interface is too strong to be overcome by the bulk denatured enzyme. Even after 20 min of exposure to the DIPE/water interface, enough conformational changes of the Pa-Hnl occur to prevent exchange with bulk denatured enzyme.

Influence of Aqueous pH on Pa-Hnl Kinetics at the Interface

Aqueous pH influences the interfacial Pa-Hnl activity qualitatively in the same way as it does for Pa-Hnl activity in the aqueous phase. The lower the pH, the faster the enzyme loses activity. There is no enhanced pH stability for Pa-Hnl in the DIPE/water two-phase system in comparison to the aqueous-phase system. Aqueous-phase pH is critical for the enzyme stability both in bulk water and at the solvent/water interface.

CONCLUSIONS

There are three major outcomes of the experiments described in this work and also in Hickel et al., (1999). First, Pa-Hnl adsorbs at organic solvent/water interfaces and catalyzes the interfacial cleavage of mandelonitrile to benzaldehyde. This outcome is shown by the dependence of product formation rate on interfacial area, by measuring dynamic surface pressure at the DIPE/water and DBE/water interfaces, by the suppression of the aqueous-phase background reaction in two-phase systems, and by the irreversibility of the interfacial enzyme adsorption upon washout. Second, Pa-Hnl eventually adsorbs irreversibly, even at solvent/water interfaces where it does not lose activity over the several-hour time course of the kinetic experiments. All enzyme adsorption washout experiments lead to this conclusion. Third, the more apolar the solvent, the stronger is the Pa-Hnl adsorption at the interface and the higher is the initial activity, but at the same time, the faster is the loss of catalytic activity.

Upon combining results for both Pa-Hnl adsorption by dynamic tension and activity measurements, two extreme situations can be distinguished, depending on the polarity of the solvent. Pa-Hnl adsorbs quickly and highly irreversibly at solvent/water interfaces of very apolar solvents, such as

heptane. Here, the total loss of enzyme activity within seconds to minutes documents a very fast denaturation of the enzyme. Pa-Hnl adsorbs and unfolds nearly immediately at highly apolar solvent interfaces. At the relatively polar solvent interfaces, such as DIPE and MTBE, the initial activity is much smaller but remains constant over several hours. Pa-Hnl adsorption is weaker at those solvent/water interfaces and apparently does not immediately lead to conformational change of the active site. Nevertheless, small changes in the enzyme structure, maybe only in a part of the enzyme remote from the active site, do occur as enzyme adsorption slowly becomes irreversible. At solvent/water interfaces with a polarity between heptane and DIPE the results can be explained by a combination of the two extreme cases.

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NOMENCLATURE

BME	butyl methyl ether
C_{BA} [mM]	benzaldehyde concentration
C_{BA0} [mM]	initial benzaldehyde concentration
C_{MN} [mM]	mandelonitrile concentration
C_{Pa-Hnl} [mM]	Pa-Hnl concentration in the aqueous bulk phase
DBE	dibutyl ether
DIPE	diisopropyl ether
Hnl	hydroxynitrile lyase
MTBE	methyl <i>tert</i> -butyl ether
Pa-Hnl	hydroxynitrile lyase from <i>Prunus amygdalus</i> (almond)
P	product, benzaldehyde
S	substrate, mandelonitrile
$t_{1/2}$	time of 50% of the initial product formation rate
V_A [L]	aqueous-phase volume in the reactor
V_R [L]	DIPE-phase volume in the reactor
$\Pi(t)$ [mN m ⁻¹]	surface pressure at time t
σ_0 [mN m ⁻¹]	initial interfacial tension of the pure liquids
$\sigma(t)$ [mN m ⁻¹]	dynamic interfacial tension at time t

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