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Organic solvent functional group effect on enzyme inactivation by the interfacial mechanism

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Abstract

We have used a bubble column apparatus to study interfacial inactivation of enzymes. The amount of enzyme inactivated was proportional to the area of organic solvent exposed, as is characteristic of the interfacial mechanism. Tests were made with a series of 12 solvents of log *P* close to 4.0, but with different functional groups. With α- and β-chymotrypsin, inactivation was much less severe with amphiphilic molecules like decyl alcohol, than with less polar compounds (heptane as the extreme case). This corresponds to a correlation with aqueous–organic interfacial tension, and presumably reflects a more polar interface as seen by the enzyme adsorbing from the aqueous phase. A 50% mixture of decyl alcohol and heptane behaved similarly to pure decyl alcohol, which would be expected to accumulate at the interface. With pig liver esterase, the correlation was rather weak, however. Accumulated data for interfacial inactivation by alkanes was examined for the above enzymes, and also papain, trypsin, urease and ribonuclease. The differing sensitivities did not show a clear correlation with any enzyme property, although there was some relationship to adiabatic compressibility, thermal denaturation temperature and mean hydrophobicity.

Keywords: Interfacial inactivation; Aqueous–organic interface; Hydrophobicity; Amphiphilic; Solvent choice

1. Introduction

The use of non-conventional media in industrial biocatalysis is well documented [1–5], as are the many advantages that this can bring. One of the most important aspects of using organic solvents as media instead of water is the improved solubility of certain substrates. However, organic solvents can have a deleterious effect on the biocatalyst (e.g., Cantarella et al. [6] and MacNaughton and Daugulis [7]). Inactivation can be caused by dissolved solvent molecules, and/or by contact with the interface between the aqueous and organic phases. In previous studies, we have shown how these mechanisms can be separated and quantified using a bubble column apparatus [8,9]. A similar apparatus has been used by Caussette et al. [10] to study inactivation at the air–water interface.

To understand what determines inactivation, it is important to separate the effects of dis-

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solved solvent and the interface. This is best done by choosing relatively hydrophobic solvents, of very low solubility in water, with which the interfacial mechanism usually dominates. If both mechanisms occur, it is not clear what effect enzyme molecules inactivated by dissolved solvent have on the process of interfacial inactivation. Therefore, merely subtracting the inactivation due to dissolved solvent from the overall rate of inactivation to derive the rate of interfacial inactivation is not completely satisfactory.

It is clear that interfacial inactivation can be strongly dependent on the choice of solvent [6,7,9]. However, it is not clear what property of the organic solvent is responsible for the results obtained. A trend has been found of increased interfacial inactivation with greater hydrophobicity of the organic solvent [6,9,11]. This trend is particularly clear for a series of solvents with the same functional group but varying alkyl chain length [6]. In such correlations, hydrophobicity is generally measured by the log of the partition coefficient of the organic solvent in an octanol/water two-phase system (log P).

In this work, we studied the effect of varying the functional group of a number of organic solvents with a similar level of hydrophobicity. Organic solvents with a log P value as close to 4 as was possible were chosen, as at this level of hydrophobicity the dissolved solvent effect should be minimal.

We chose to investigate the interfacial inactivation of 4 enzymes: α-chymotrypsin (E.C. 3.4.21.1), β-chymotrypsin (E.C. 3.4.21.1), papain (E.C. 3.4.22.2) and pig liver esterase (E.C. 3.1.1.1). The reasons for their choice were as follows.

(1) They have been shown to be interfacially inactivated in previous studies.

(2) They have a convenient assay method.

(3) We wished to consider what bearing structural similarities have on the results: α-chymotrypsin and β-chymotrypsin are very closely related enzymes with a large structural homology. Papain and pig liver esterase have a different fold and no structural homology to the serine proteases.

2. Materials and methods

2.1. Interfacial inactivation

This was quantified using the bubble column apparatus as previously described [8].

2.2. Enzymes

α-Chymotrypsin (E.C. 3.4.21.1) from bovine pancreas (Sigma Type II, C4129 LOT 91H7195, a 3 times crystallised, dialysed and lyophilised preparation) was dissolved in Tris–HCl buffer pH 7.8 at a concentration of 1 mg/ml.

β-Chymotrypsin (E.C. 3.4.21.1) from bovine pancreas, (Sigma P4629 LOT 30H8060, an essentially salt free crystallised and lyophilised preparation) was dissolved in 10 mM Tris–HCl buffer pH 7.8 at a concentration of 1 mg/ml.

Papain (E.C. 3.4.22.2) (Sigma P4762 LOT 83117030, a 2× crystallised, lyophilised powder containing approx. 80% protein) was dissolved in H_2O at a concentration of 2 mg/ml.

Pig liver esterase (E.C. 3.1.1.1), (Sigma E2884, suspended in 3.2 M $(NH_4)_2SO_4$ pH 8 solution) was dissolved in H_2O at a concentration of 1 mg/ml, giving a final $(NH_4)_2SO_4$ concentration of 150 mM.

All buffer solutions were prepared using double distilled water. Since Ca^{2+} ions are known to protect the enzymes α and β-chymotrypsin from autolysis $CaCl_2$ at a concentration of 100 mM was added to the buffer solutions of α and β-chymotrypsin.

2.3. Organic solvents

The organic solvents used in this work were cyclooctane (99 + %), 1-octene (98%), 1-chloroheptane (99%), *n*-butylbenzene (99 + %), isoamyl ether (99%), ethylcaprylate (99 + %), 2-undecanone (99%), decylalcohol (98%), 3,7-

dimethyl-3-octanol (99%), undecylenic acid (98%), undecylicaldehyde (97%), undecanenitrile (99%), decylamine (95%) purchased from Aldrich and *n*-heptane (99 + %) purchased from Sigma.

2.4. Enzymatic assays

The substrates *N*-acetyl-L-tyrosine ethyl ester (ATEE), α-*N*-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) were purchased from Sigma. α- and β-chymotrypsin were assayed using the pH-Stat method of Wilcox [12]. Papain was assayed using the pH-stat method of Walsh and Wilcox [13]. Pig liver esterase was assayed using the pH-stat method of Dudman and Zerner [14].

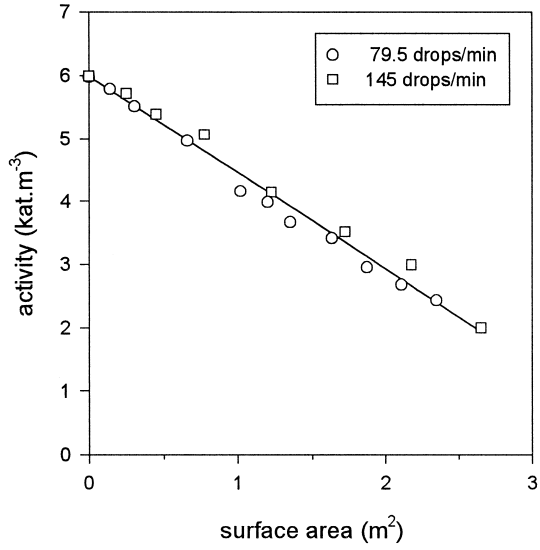

Fig. 2. Inactivation of α-chymotrypsin exposed to heptane as a function of surface area.

3. Results and discussion

Our quantitative method of monitoring interfacial inactivation involves the passing of solvent droplets up through enzyme solution inside the bubble column apparatus [8,9]. In this study, a number of enzymes have been exposed to organic/aqueous interfaces with a similar hydrophobicity, but different functional groups. Solvents with a log *P* value around 4 were chosen so that the interfacial mechanism of inactivation would predominate. This was checked by parallel measurements of inactivation in a solvent-saturated aqueous phase.

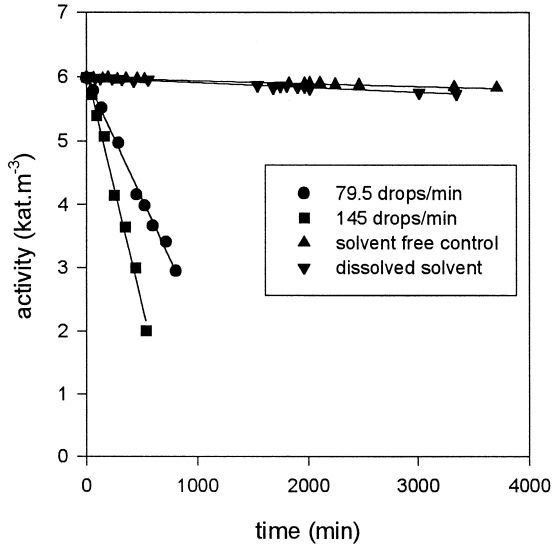

Fig. 1. Inactivation of α-chymotrypsin exposed to heptane as a function of elapsed time.

In most cases, the rate of dissolved solvent inactivation was less than 0.5×10^{-3} h^{-1} and little faster than a purely aqueous control. A typical example is shown in Fig. 1 for the inactivation of α-chymotrypsin exposed to heptane. This figure demonstrates that the inactivation is not a simple function of time.

However, Fig. 2 demonstrates that a relationship exists between interfacial inactivation and surface area of organic solvent that the enzyme experiences. The gradient of this line is a measure of the rate of interfacial inactivation. In the majority of cases studied similar behaviour was observed, showing that interfacial inactivation is the dominant effect. The few exceptions to this general trend will be dealt with where appropriate. All rates of inactivation have been determined in at least duplicate experiments, and the values agreed within 10%.

We will first consider the enzymes individually, before trying to draw any general conclusions for the set of results as a whole.

3.1. α-Chymotrypsin

The result of the successful inactivation experiments for α-chymotrypsin are shown in Table 1. It is obvious that the solvents do not inactivate the enzyme to the same extent. Therefore, a property other than solvent hydrophobicity must be important in controlling the level of enzyme inactivation.

Generally, the solvents with more polar functional groups have less of an inactivating effect on the enzyme. Solvents with a polar functional group, combined with a longer hydrophobic chain, will have an amphiphilic character. Such solvents will have a lower surface tension. The surface tension measured at an aqueous/organic interface has been shown in some instances to correlate well with the level of interfacial inactivation an enzyme experiences at that particular interface [7,20–22]. Therefore, we chose to correlate the interfacial tension of the solvent used to inactivate the enzyme with the resultant rate of interfacial inactivation (Fig. 3).

If we look at the trend established in Fig. 3, it is clear that the surface tension of the aqueous/organic interface has a bearing on the level of interfacial inactivation of this enzyme. This is probably related, at least in part, to the driving force for adsorption. Literature studies have shown that adsorption and spreading of proteins at the aqueous/hydrocarbon interface reduces the surface tension by about 20 mN m^{-1} [23,24]. With organic solvents having more polar functional groups, the interfacial tension is lower than this even for the clean interface. Hence, the reduction in interfacial tension by the protein, which is a measure of the driving force for adsorption, must be lower with such interfaces.

Another way of looking at the degree of adsorption and resulting inactivation is by considering the way an enzyme views the different interfaces it experiences. The interfaces with the highest interfacial tension are those that are hydrocarbon in nature. To the enzyme, these interfaces are exclusively hydrophobic; therefore, there should be a strong hydrophobic effect, which is a major driving force in protein adsorption. In the case of interfaces with a lower interfacial tension like decyl alcohol and

Table 1
Rate of inactivation for α-chymotrypsin

Solvent	log *P*	Interfacial tension (mN m^{-1})	Rate of inactivation by:	
			Dissolved solvent (10^{-3} h^{-1})	Interface (μkat m^{-2})
10-Undecylenic acid	4.1	10.1[a]	1.4	10.8
1-Octene	4.1	50[b]	< 0.5	5.1
1-Chloroheptane	3.9	37[b]	6.7	5.0
2-Undecanone	4.0	18[b]	1.8	4.6
Heptane	4.0	50.2[c]	< 0.5	4.3
Butyl benzene	4.1	39.6[c]	8.9	3.8
Cyclooctane	4.1	46[b]	< 0.5	3.7
Isoamyl ether	4.0	28[b]	2.0	3.4
3,7-Dimethyl-3-octanol	3.9	16[b]	< 0.5	2.5
Ethyl caprylate	3.8	25.5[a]	< 0.5	2.4
Undecylnitrile	4.0	18[b]	< 0.5	1.3
Decyl alcohol	3.9	8.3[d]	< 0.5	0.85

[a] Measured interfacial tension taken from Girifalco and Good [15].
[b] Estimated interfacial tension using method of Freitas et al. [16], using the log of Henry's law constant [17] and the surface tension of the pure organic compound [18].
[c] Measured interfacial tension taken from Demond and Lindner [19].
[d] Measured interfacial tension taken from Freitas et al. [16].

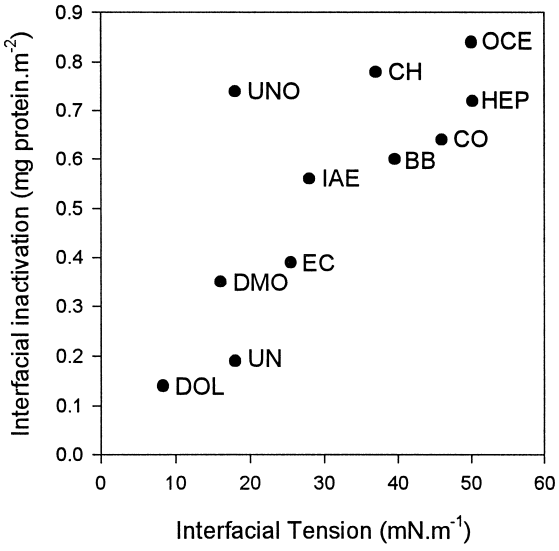

Fig. 3. Correlation between level of interfacial inactivation of α-chymotrypsin and interfacial tension. Solvents are identifed as: DOL — decyl alcohol; UN — undecylnitrile; DMO — dimethyloctanol; EC — ethyl caprylate; IAE — iosamyl ether; BB — butyl benzene; CO — cyclooctane; HEP — heptane; UNO —undecanone; CH — chloroheptane; OCE — octene.

undecylnitrile, many polar groups will be displayed at the interface with the aqueous phase. This should lessen the hydrophobic effect in adsorption processes, and hence the driving force for protein adsorption. In addition, adsorbed protein molecules may be less likely to unfold, because this is also driven by the possibility of contact between the hydrophobic core and the interface.

A particular comparison worth noting is between decyl alcohol and 3,7-dimethyl-3-octanol. Since these contain exactly the same groups in the log *P* system, they have identical calculated hydrophobicities. However, the terminal hydroxyl and linear alkyl chain of decyl alcohol gives it a significantly stronger amphiphilic character. This leads to an expected lower interfacial tension, and a lower tendency to cause interfacial inactivation (Table 1).

Even when they are only one component in a mixed organic phase, amphiphilic molecules will tend to accumulate at the interface, and hence dominate its properties. Hence, we tested the inactivation of α-chymotrypsin by a mixture of decyl alcohol and heptane (50% v/v), finding 0.23 μkat inactivated/m^2 of interface. This is much closer to the value for pure decyl alcohol than for pure heptane, as expected.

The use of such mixtures indicates a valuable method of protecting against interfacial inactivation while maintaining desired properties in the bulk of the organic phase. The extreme case would be to use a small addition of a strongly surface active component. Feliu et al. [22] found that the addition of a surfactant protected enzymes from inactivation in a system where the interfacial mechanism was probably important.

One solvent, undecylenic acid, is clearly an exception (Table 1), and is excluded from Fig. 3. In this case the inactivation is a great deal higher than one would expect from the interfacial tension and log *P* of undecylenic acid. It may be that the carboxyl groups clustering at the interface have a special inactivating effect, perhaps by an acid–base mechanism.

It was not possible to obtain useful measurements of interfacial inactivation with two other solvents. Decylamine was found spontaneously to form a fine emulsion when it was introduced into the aqueous phase. With undecylaldehyde the experiment started to proceed as normal but after a few minutes the solvent stopped passing up through the bubble column. This was due to blockage of the Teflon tubing near the delivery nozzle with an insoluble precipitate which was probably a result of a reaction taking place between the solvent and water.

Although the level of dissolved solvent inactivation for α-chymotrypsin was generally little more than for the aqueous control, it was significant with some solvents (Table 1). Butyl benzene and chloroheptane gave the fastest rates of dissolved solvent inactivation. Interestingly solvents with these functional groups have been shown to give anomalous results in other studies [6,7,9]. However, two of these studies did not attempt to separate dissolved solvent inactivation from interfacial effects and therefore the results are not easy to interpret. Because the

solvents we have tested all have similar hydrophobicity, the higher rates of inactivation cannot simply be assigned to greater solubility in the aqueous phase.

3.2. β-Chymotrypsin

β-Chymotrypsin is similar in size, structure and specificity to α-chymotrypsin [25,26]. Like α-chymotrypsin, it is interfacially inactivated when exposed to a tridecane interface [28]. With this enzyme we tested only a selected range of solvents, based on the findings with α-chymotrypsin. The rates of inactivation obtained are shown in Table 2. In common with α-chymotrypsin there is a positive correlation with the interfacial tension of the aqueous/organic interfaces involved (Fig. 4).

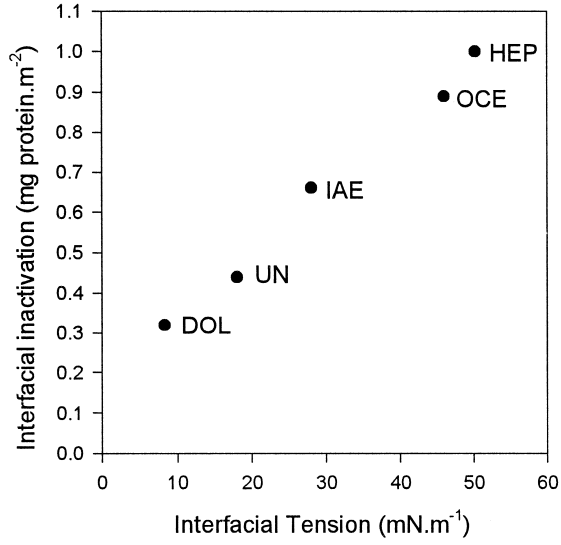

Fig. 4. Correlation between level of enzyme inactivation and interfacial tension for β-chymotrypsin. Solvents are identifed as: DOL — decyl alcohol; UN — undecylnitrile; IAE — iosamyl ether; OCE — octene; HEP — heptane.

3.3. Papain

A recent study by Feliu et al. [22] suggested that this enzyme is interfacially inactivated by most organic solvents, despite the general view that it is highly stable. However, in our experiments, we could not detect interfacial inactivation (< 0.2 μkat m^{-2}) with any of the solvents tested (heptane, 1-chloroheptane, 2-undecanone or decyl alcohol). Heptane was the most hydrophobic solvent studied by Feliu et al. [22], and the least inactivating. It could be that this enzyme is generally stable with more hydrophobic solvents, as often found where the dissolved solvent mechanism predominates. Unfortunately, Feliu et al. [22] did not distinguish between interfacial and dissolved solvent inactivation and therefore the published results cannot be assigned to either inactivation mechanism. They did report inactivation of papain by agitation of the aqueous phase in the absence of any organic solvent. They attributed this to shear effects, although inactivation at the air–water interface may be more likely. In our apparatus there is no air–water interface, and shear is very low compared with a stirred tank reactor.

Table 2
Rate of inactivation of β-chymotrypsin

Solvent	Interfacial tension (mN m^{-1})	Interfacial inactivation (μkat m^{-2})
Heptane	50.2	6.5
1-Octene	46	5.8
Isoamyl ether	28	4.0
Undecylnitrile	18.0	2.7
Decyl alcohol	8.3	2.2

The rate of inactivation by dissolved solvent effect was less than 0.5×10^{-3} h^{-1} in all these cases.

3.4. Pig liver esterase

Pig liver esterase has been shown to be interfacially inactivated [27]. Table 3 shows the rates of inactivation we found with the bubble column apparatus. It is clear that the level of inactivation varies considerably between solvents. In particular, the rate of inactivation with undecanone is unusually large. The rate corresponds to 3.7 mg of enzyme protein per m^2 of interface, which is probably more than can be

Table 3
Rate of inactivation of pig liver esterase

Solvent	Interfacial tension ($mN\ m^{-1}$)	Interfacial inactivation ($\mu kat\ m^{-2}$)	Dissolved solvent ($10^{-3}\ h^{-1}$)
2-Undecanone	18	23.4	14
1-Chloroheptane	37	4.4	2.8
3-7-Dimethyl-3-octanol	16	4.0	< 0.5
Heptane	50.2	3.3	< 0.5
Decyl alcohol	8.3	2.5	< 0.5

accommodated in an adsorbed monolayer. This solvent also had a large dissolved solvent effect, and it may be that this cannot be corrected for properly to obtain the true interfacial rate. There may be some specific effect of this solvent, such as action at the active site like a substrate analogue.

Even considering just the other solvents, it is, however, clear that with pig liver esterase there is no correlation between inactivation rate and interfacial tension, unlike the chymotrypsins.

3.5. Comparison of different enzymes

We are now in a position to consider the relative susceptibility of different enzymes to interfacial inactivation. This will be based on not just the data above, but also that obtained by the same methods for inactivation of: α-chymotrypsin, β-chymotrypsin and trypsin by tridecane at different pH values [28], urease, α-chymotrypsin and ribonuclease by various solvents [9]. The comparison will not include lipase, because this enzyme is expected to be a special case because of its evolutionary selection for action at interfaces.

The first problem in comparing enzyme sensitivities is the influence of the type of solvent. As we have seen, this can have major effects on the rate of inactivation of a given enzyme. However, the various aliphatic hydrocarbon solvents have been found to give rather similar rates of inactivation. Hence, we can compare different enzymes using data for any alkane solvent. Table 4 shows the rates of inactivation for the 3 aliphatic hydrocarbon solvents we have studied, together with some relevant enzyme parameters.

To allow a fair comparison of the enzymes, the rates of inactivation in Table 4 have been converted to a mass of protein basis, using the measured specific activities. On this basis, the highest inactivation rates are a 1.0 mg of protein per m^2 of interface. This is consistent with the normal range of monolayer densities of up to 2.5 mg m^2 found in protein adsorption studies [29,30]. As can be seen, where more than one of the alkanes have been studied with a given enzyme, the rates of inactivation are similar.

When considering the interfacial behaviour of proteins, it is common to place them on a scale from ‘hard’ to ‘soft’ [39–42]. Hard proteins are rigid with a good internal structural stability and do not undergo structural change upon adsorption. Soft proteins have a more flexible structure that can undergo structural rearrangement upon adsorption. This structural rearrangement can lead to inactivation of adsorbing enzyme molecules. Therefore a prerequisite for interfacial inactivation may be that a protein should be soft. In this context, the usual measure of protein flexibility is adiabatic compressibility ($\bar{\beta}s$) [43]. For example, it has been shown to correlate with the level of structural change induced when enzymes are adsorbed onto solid surfaces, as monitored by circular dichroism (CD) [40,41]. There was also some indication of a relationship with interfacial inactivation in biphasic systems [8,9].

Table 4
Properties of different enzymes and their interfacial inactivation

Enzyme	M.W. ($\times 10^{-3}$)	T_m[a] (°C)	Hydrophobicity[b]	Compressibility ($\bar{\beta}s$) (10^{-12} cm^2 dyn^{-1})	Interfacial inactivation (mg m^{-2}) by:		
					Hexane	Heptane	Tridecane
α-Chymotrypsin	25.2	43.9[c]	0.131	4.15[d]	1.0[e]	0.8[f]	1.0[e], 0.9[g]
β-Chymotrypsin	22.5	41.8[h]	0.169	7.02[i]		1.0[f]	0.9[g]
Trypsin	23.4	46[j]	0.122	0.92[d]			0.5[g]
Urease	546	40–60[e]	0.06	−9.3[i]	1.0[e]		0.9[e]
Pig liver esterase	165	–	0.165	6.53[i]		0.6[f]	
Papain	23.4	66[k]	0.023	−4.60[i]		< 0.2[f]	
Ribonuclease	13.7	62[l]	−0.13	1.12[d]	< 0.2[e]		< 0.15[e]

[a] T_m = denaturation temperature.
[b] Calculated as a mean of all amino acid values obtained from the polarity scale of Eisenberg et al. [31].
[c] Lozano et al. [32].
[d] Gekko and Hasegawa [33].
[e] Ghatorae et al. [9].
[f] Converted from data above.
[g] Values for pH 7.8 from Ross et al. [28].
[h] Delaage et al. [34].
[i] Predicted value using the equation of Gromiha [35].
[j] Lazdunski and Delaage [36].
[k] Stockell and Smith [37].
[l] Von Hippel and Wong [38].

Inspection of Table 4 shows that the two enzymes most resistant to interfacial inactivation, papain and ribonuclease, are definitely 'hard' proteins with low values of adiabatic compressibility. However, there is no general correlation. Trypsin has a low measured compressibility and urease has a very low predicted compressibility, but are sensitive to interfacial inactivation.

One reason for the imperfect correlation may be the mistaken view that compressibility is a fixed value for a given protein. It has recently been shown that an enzyme's $\bar{\beta}s$ varies with pH and salt concentration [44]. We have shown that interfacial inactivation rates are also strongly affected by pH [28]. Ideally, both measurements should be made under identical conditions, but in general the $\bar{\beta}s$ data required are not available. Most of the literature measurements of $\bar{\beta}s$ are reported as made in 'pure water', but it is not clear whether this means that the pH would equal the pI, or would be controlled by residual impurities.

Ribonuclease and papain, the two enzymes most resistant to interfacial inactivation, also have the highest thermal denaturation temperatures. However, once again the relationship is not yet clearly established. Thermal denaturation temperature is another parameter that is known to reflect in part the 'hardness' of the structure.

The total hydrophobicity of an enzyme is another parameter often used to try and explain adsorption phenomena. Protein hydrophobicity is thought to be important because one of the most important driving forces of protein adsorption is the hydrophobic interaction. A hydrophobic region of an enzyme attaches itself onto a hydrophobic surface replacing many water molecules. This is thermodynamically very favourable. and if possible an enzyme will try to spread out to maximise this effect, leading to inactivation of the enzyme due to conformational changes. Therefore, the total hydrophobicity of an enzyme may be an indicator of the likelihood of adsorption and interfacial inactivation. However, Table 4 shows that there is at best a weak correlation between protein hydrophobicity and interfacial inactivation rates. The most stable enzymes papain and ribo-

nuclease do however stand out again as having the lowest hydrophobicity.

A more intuitive parameter to relate to adsorption would be the surface hydrophobicity of an enzyme, which can be measured using Hydrophobic Interaction Chromatography [45]. However, surface hydrophobicity values will not take account of conformational changes at the interface, leading to the exposure of buried hydrophobic residues.

We should return to the effect of the conditions under which measurements are made. For example, we have shown that pH has substantial effects on interfacial inactivation [28], and the same may be true for other factors. This can lead to very different conclusions about relative stabilities, e.g., α-chymotrypsin is inactivated at only one third of the rate of β-chymotrypsin at pH 5.0, but more than 2 times faster at pH 9.0. Many enzyme properties are also greatly affected by pH, for example. This was noted above for compressibility, and is also true for thermal denaturation. It seems unrealistic to hope for a good correlation with a single value of a protein parameter determined under different conditions.

A comment is appropriate on the different behaviour of the chymotrypsins and pig liver esterase with respect to different solvents. The protein parameters are rather similar for all three enzymes, and so are their inactivation rates with alkanes (Table 4). However, as noted above, only the chymotrypsins show a clear correlation with interfacial tension. Studies on further enzymes will be required before we can tell whether a relationship with interfacial tension is typical. The similar fold of the two chymotrypsins may account for their similar behaviour.

4. Conclusions

Among organic solvents of similar hydrophobicity, the type of functional group present has a large bearing on the level of interfacial inactivation. The aqueous–organic interfacial tension was found to correlate well with the level of inactivation for α and β-chymotrypsin, but not for pig liver esterase. Interfacial inactivation by a mixture of decyl alcohol and heptane occurs at a rate close to that of the more polar solvent alone. The sensitivity to interfacial inactivation does not show a simple correlation with any enzyme property, though adiabatic compressibility, thermal denaturation temperature and mean hydrophobicity appear to be important factors.

Acknowledgements

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