pH Dependence of Subtilisin Dispersed in Organic Solvents

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Abstract: The apparent pH dependence and activity of subtilisin dispersed in organic solvents have been studied. The data demonstrated that the apparent pH dependence displayed by the enzyme in organic media resembled that in water in a broad sense, but it was dependent upon solvent type, water content, and method of water delivery. The effect of organic solvents on the apparent pH dependence of suspended subtilisin powder can be related to the change in polarity of the enzyme's active site. The apparent pK_a of subtilisin was more affected by changing water content than by changing solvents, and it was significantly lower when water was delivered by sodium phosphate salt hydrate ($Na_4P_2O_7 \cdot 10H_2O$) than when 0.05% (v/v) water was added directly. The activity of the enzyme in organic media did not correlate to the classical scales of solvent hydrophobicity.

Introduction

Enzymes not only promote catalytic activity in organic media but also exhibit some remarkable properties which are unusual in aqueous solution, such as molecular memory,[1] greatly enhanced stability,[2] altered substrate and enantiomeric specificities,[3] and the ability to catalyze unusual reaction.[4] "pH memory" is also often referred to as a novel property of enzymes in organic solvents. Zaks and Klibanov[5] have demonstrated that lipase-catalyzed transesterification between tributyrin and heptanol in their mixture was very much dependent on the pH of the last aqueous solution from which the enzyme was lyophilized, with the optimum preparation conditions coincident with the pH optimum for lipase activity in water. Similar behavior was also observed for chymotrypsin in octane,[6] and it is believed that this is a common phenomenon. The most plausible explanation of this effect is that the ionogenic groups of the enzyme acquire an ionization state corresponding to the pH of the aqueous solution from which the enzyme was prepared, and this state is then retained during lyophilization and dispersal in the organic solvent, due to "rigidity" of the protein. This analysis is substantiated by previous reports[7,8] which demonstrate that the resonance frequency of ^{15}N-enriched solid preparations of free imidazole, histidine, or histidine in the active site of α-lytic protease is dependent on the pH from which the amino acid, or the enzyme, was prepared. This pH dependence is, however, little affected when the physical state of the amino acid or the protein is changed from solution state to solid state.

Although there are only a few reported experimental demonstrations of "pH memory", the method of preparing an enzyme from its aqueous pH optimum for use in nonaqueous media has been widely extended to other enzymes and solvents. The generality and method by which organic solvents affect the apparent (or memorized) pH dependence[9] of enzymes in organic media still remain largely unknown. Interestingly, Guinn *et al.*[10] found that, for three pH values selected (2.0, 7.5, and 11.0), alcohol dehydrogenase in water was most active at pH 7.5 while the enzyme in nearly anhydrous heptane showed highest activity when it was lyophilized from a solution of pH 2.0 (the enzyme was virtually inactive in water at this pH). Obviously, an understanding of this phenomenon is important because it is directly related to the optimization of enzyme catalysis in organic media.

The fact that enzymes present variant activities in different organic solvents is another well-known phenomenon in nonaqueous enzymology. Much research has been carried out to investigate the effect of organic solvents on enzyme activity, and the most popular predictive tool for estimating activity has been the use of the logarithm of the partition coefficient of a solvent in an octanol–water (log P),[11] which can reflect the hydrophobicity of the solvent. With addition of the same amount of water in the reaction system, enzymes are usually found to be more active in a solvent with a higher log P, which can be explained by the fact that enzyme activity depends on the amount of water adsorbed to the enzyme rather than on the water content of the whole system.[12] Solvents with different log P values possess distinct capacities for stripping water from the enzyme, resulting in entirely different amounts of water associated with the enzyme and therefore altered enzyme activities. Since discrepancies have been reported while trying to predict enzyme activity with log P, other criteria have also been proposed, such as the Hansen three-dimensional solubility parameter,[13] denaturation capacity,[14] and partition coefficients of substrate and product.[15]

Halling[16] has claimed that in organic media thermodynamic water activity is a particularly important parameter which reflects the water level associated with the enzyme. Indeed, direct solvent effects are best investigated by conducting enzyme kinetics with

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(9) Here we use the word "apparent" because we are studying the effect of the pH, from which the enzyme is prepared, on *subsequent* activity in organic solvents. In the absence of water, pH does not have the same meaning as in aqueous solution, but use of apparent pH and pK_a enables comparisons of "memorized pH dependence" profiles for enzymes in nonaqueous media.
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a constant amount of water bound to the enzyme. Direct addition of salt hydrates provides a convenient means of buffering water activity (a measure of the equilibrium between bound and free water) in the enzymic reaction system.[17] The effectiveness of this method has also been demonstrated by Kuhl *et al.*,[18] Kuhl and Halling,[19] Kvittingen *et al.*,[20] and Yang and Robb.[21]

Subtilisin Carlsberg (a serine protease) was chosen as a model enzyme in this study because it is representative of a large and important family of enzymes whose catalytic mechanism has been fully detailed. In aqueous solution, subtilisin follows the classical acyl-enzyme mechanism. It has been demonstrated that the same mechanism is followed in organic solvents.[22] Subtilisin-catalyzed reactions have been, and continue to be, of much interest, and the kinetics of subtilisin have been extensively studied, not only in aqueous solution but also in organic media.[6,23,24] The pH dependence of subtilisin in aqueous solution has also been investigated, and it has been demonstrated that it can be tailored using protein engineering.[25] Using subtilisin as a model enzyme, we now report the results of a detailed study of the effect of enzyme pH during preparation upon the subsequent activity of the enzyme in a nonaqueous solvent.

Results and Discussion

In order to study the apparent pH dependence of subtilisin in organic media, subtilisin was lyophilized from phosphate buffer (with constant ionic strength of 0.01 M) at 14 different pH's ranging from 5.5 to 8.5. Activity was assessed, in duplicate, in seven organic solvents with different physical properties (for solvent physical parameters as Table II). Two methods were used for introducing water in this study: addition of 1 μL of water to make the water content in the reaction system constant (0.05% v/v) or addition of 0.5 g of $Na_4P_2O_7 \cdot 10H_2O$ to achieve a constant water activity (a_w = 0.52 at 30 °C[26]).

1. Salt Hydrates. The ability of $Na_4P_2O_7 \cdot 10H_2O$ to control thermodynamic water activity, and thus increase enzyme activity, is demonstrated in Figure 1. No reaction occurred in a dry state, but the product, *N*-acetyl-L-phenylalanine methyl ester, was produced after direct addition of $Na_4P_2O_7 \cdot 10H_2O$, indicating that this salt hydrate is an efficient activator of subtilisin. A lag period was observed that depended upon how much salt was added, while no lag period was present when water was introduced directly. The data indicate that a short time is necessary for the salt hydrate to release water to the reaction system, and the time is reduced when more salt was added. The lengths of the lag periods, 5 and 12 min for 0.4 and 0.2 g of salt addition, respectively, are comparable to results obtained when $Na_2HPO_4 \cdot 12H_2O$ was introduced to tyrosinase in dry chloroform.[21] When the reaction rate achieves a steady state and is independent of the amount of salt added, a water equilibrium between $Na_4P_2O_7 \cdot 10H_2O$ and its anhydrous form has been established and a constant water activity has been achieved. It should be noted that the degree of conversion during the constant rate period was well within the range necessary to ensure initial rates were being observed.

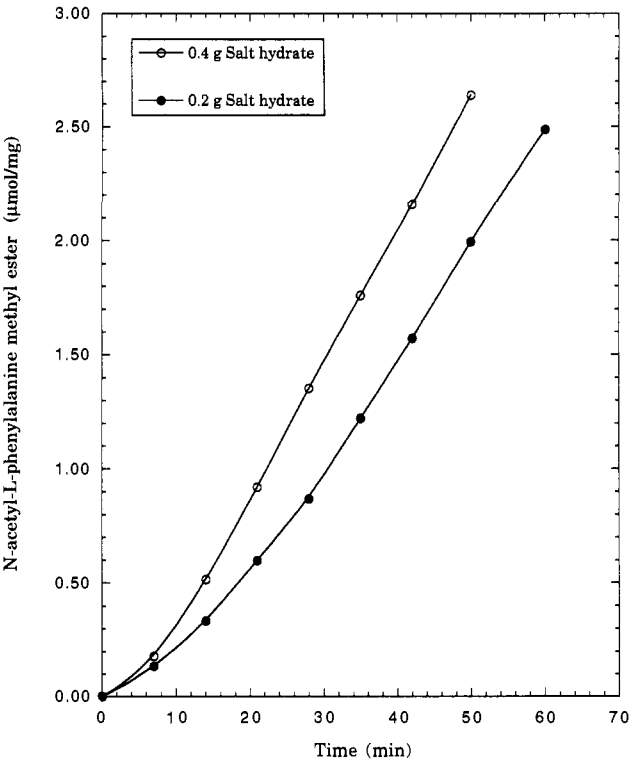

Figure 1. Progress curves of transesterification catalyzed by subtilisin in acetonitrile with the addition of different amounts of $Na_4P_2O_7 \cdot 10H_2O$. A 3-mL substrate solution containing 30 mM *N*-acetyl-L-phenylalanine ethyl ester and 1.2 M methanol in acetonitrile was added to a 4-mL Wheaton vial containing 2 mg of subtilisin (lyophilized from pH 8.42), followed by addition of 0.2 or 0.4 g of $Na_4P_2O_7 \cdot 10H_2O$.

Table I. Subtilisin Activity in Acetonitrile with Addition of Different Salt Hydrates[a]

salt hydrate	water activity at 30 °C	initial rate (nmol/min/mg_{enzyme})
$Na_2HPO_4 \cdot 12H_2O$	0.85	120
$Na_2HPO_4 \cdot 7H_2O$	0.65	77
$Na_4P_2O_7 \cdot 10H_2O$	0.52	59
(1 μL of H_2O)		(1.4)

[a] A substrate solution (2 mL) containing 30 mM *N*-acetyl-L-phenylalanine ethyl ester and 1.2 M methanol in acetonitrile was added to a 4-mL Wheaton vial containing 2 mg of subtilisin lyophilized from pH 8.42, followed by addition of various salt hydrates (0.4 g). The values for water activity are taken from ref 26.

Several salt hydrates with high water activity (a_w = 0.72), such as $Na_2SO_4 \cdot 10H_2O$, $Na_2HPO_4 \cdot 12H_2O$, and $Na_2CO_3 \cdot 10H_2O$, have been demonstrated previously to activate chymotrypsin,[18,19] lipase,[20] and tyrosinase,[21] and in our study, $Na_4P_2O_7 \cdot 10H_2O$ (a_w = 0.52 at 30 °C) has been used successfully as an efficient water donor to subtilisin. This salt hydrate was employed for all subsequent experiments. It should be noted that the use of a salt hydrate to deliver water to an enzyme suspension in organic media has a significant activation effect on subtilisin activity (Table I). Although subtilisin is active in a relatively dry state with addition of only 1 μL of water to 2 mL of dry acetonitrile solution (0.05% v/v), the presence of $Na_4P_2O_7 \cdot 10H_2O$, which gave a water activity of 0.52, activated the enzyme 42-fold, and even higher activity was achieved when $Na_2HPO_4 \cdot 12H_2O$ and $Na_2HPO_4 \cdot 7H_2O$ were employed, suggesting that subtilisin is more active at a higher water activity.

(17) This method has been discussed by Halling (ref 26), who suggests that a salt hydrate pair can establish an equilibrium with water, providing a constant vapor pressure at a fixed temperature. If a more hydrated form of a salt is added to a dry system, some of the hydrate will liberate its water to the system, meanwhile producing its anhydrous or lower hydrate form, thus establishing an equilibrium, e.g. $Na_2HPO_4 \cdot 12H_2O \rightleftharpoons Na_2HPO_4 \cdot 7H_2O + 5H_2O$; likewise, the anhydrous salt or the lower hydrate can adsorb water from a relatively wet system, generating its partner hydrate. As long as each of the hydrate forms involved in equilibrium is present in the system, no matter what the ratio is, a constant water activity can be achieved at a fixed temperature.

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Table II. Maximal Reaction Rates Obtained from Experiments in Various Organic Solvents[a]

solvent	log P[b]	dielectric constant[c]	1 μL of water V_{max}[d]	1 μL of water pH_{opt}[d]	$Na_4P_2O_7 \cdot 10H_2O$ V_{max}[d]	$Na_4P_2O_7 \cdot 10H_2O$ pH[d]
hexane	3.5	1.89	20.5	8.20		
toluene	2.5	2.38	48.5	8.20	61.8	8.20
chloroform	2.0	4.81	0.89	8.42	4.40	8.20
tetrahydrofuran	0.28	7.32	2.00	7.54	21.3	8.11
acetone	−0.23	20.7	27.3	8.20	104.2	8.20
acetonitrile	−0.36	36.2	1.72	8.20	80.7	8.20
dioxane	−0.47	2.21	0.60	7.78	8.64	7.78

[a] Experimental detail is provided in the text. [b] The log P values were taken from ref 11. [c] Dielectric constants were taken from *CRC Handbook of Chemistry and Physics*, 60th ed.; Weast, R. C., Ed.; CRC Press: Boca Raton, FL, 1979. [d] V_{max} values are the initial rates obtained at optimal pH in each solvent (nmol/min/mg of enzyme).

2. Enzyme Activity. Activities of subtilisin in various organic solvents were determined as functions of pH, and the maximal reaction rates obtained at pH optima are listed Table II. When 1 μL of water was added, the enzyme activity varied essentialy in the order of toluene > acetone > hexane > tetrahydrofuran > acetonitrile > chloroform > dioxane, but this order is to some extent pH dependent. Addition of 1 μL of water was necessary to ensure good activity in the more hydrophilic solvents. The activity of subtilisin, at all pH's, in these solvents did not follow the "log *P* rule",[11] and interestingly, the enzyme showed a high activity in acetone, which possesses a negative log *P* (similar results appeared previously[6]). Chloroform, with a relatively high log *P* value, promoted a rather poor enzyme activity. It could be argued that failure to fit the log *P* rule was due to utilization of a different water content in all the reaction systems. When the water content present in the dried solvents (<0.01% v/v) and the lyophilized enzyme (10–15% w/w) was considered, the 2-mL reaction mixture with addition of 1 μL of water might contain a total of 1.2–1.5 μL of water, of which different proportions are bound to the protein. However, when the water content of the enzyme in the reaction system was controlled by using the salt hydrate, enzyme activity still did not follow the log *P* rule (for all pH's, acetone > acetonitrile > toluene > tetrahydrofuran > dioxane > chloroform). The high activity in acetone and low activity in chloroform were observed again, the enzyme remained poorly active in tetrahydrofuran and dioxane, and surprisingly, acetonitrile promoted a very high activity in the presence of salt hydrate. Enzyme activity in hexane with addition of salt hydrate was not obtained because the reaction rate was too fast to follow.[27] The two sets of data suggest that, in addition to stripping water from the enzyme, solvents also have a direct and specific effect on enzyme activity. Therefore as pointed out by Schneider,[13] a single-parameter approach such as log *P* is presumably limited in its ability to reflect the spectrum of possible enzyme–solvent interactions. Other factors, such as Hansen parameters,[13] denaturation capacity of the solvents,[14] and partitioning of substrates and products between bulk organic phase and the aqueous layer around the enzyme,[15] are currently being assessed for their utility in predicting the experimental data.

Another important conclusion that can be drawn from this data set concerns the use of water activity control in investigating the effect of solvents on enzymes. All water activity control can do is to ensure that a catalyst preparation has a constant water content in different solvents.[29] It cannot prevent the direct effect of solvents on enzymes, and thus caution must still be exercised when correlating solvent physical properties to enzyme activity at constant water activity. Naturally, this is even more true for comparisons of activity in different solvents without ensuring that the water content of the enzyme is constant. Take for example the data for chloroform in the presence of salt hydrate. The amount of water on the enzyme should be the same as for other solvents, but the enzyme activity is very low. Thus, chloroform must have a direct, and deleterious, interaction with the protein.

The rates of the enzymic reactions when water activity was controlled at 0.52 by addition of $Na_4P_2O_7 \cdot 10H_2O$ were all higher than those when 1 μL of water was added, simply because of the higher enzyme hydration level with salt hydrates. The reaction rates in acetonitrile using subtilisin lyophilized from pH 8.11 with addition of 1, 3, and 5 μL of water and 0.5 g of $Na_4P_2O_7 \cdot 10H_2O$ were 1.72, 3.38, 19.0, and 76.2 nmol/min/mg of enzyme, respectively. The data confirm the conclusion drawn from Table I that the activity of subtilisin in organic solvents depends on the water content in the system, with higher reaction rates being obtained when more water was associated with the enzyme.

3. Precision and Accuracy. Determinations of pH dependencies for enzymes solubilized in aqueous solution have been performed with varying degrees of detail. In organic solvents the only precedents are ref 5 (five points between pH 4 and 10), ref 6 (nine points between pH 2 and 10), ref 7 (six points between pH 5.5 and 8.4, ref 8 (three points between pH 5.1 and 9.3), and ref 10 (three points between pH2 and 11). In order to elucidate whether the presence of an organic solvent affects the pH dependence of enzyme catalysis in the solid state, we have adopted a somewhat more detailed approach. Selecting a minimum of 14 separate pH points in the pH range 5.5–8.5, we measured the activity of suspended enzyme powders, in duplicate, in seven organic solvents, all at two different water contents. Each experiment measured the initial rate of the enzyme, which under the conditons used ([S] $\ll K_m$, and K_m has been determined for this enzyme and substrate in the solvents tested)[22] is equivalent to the determination of V_{max}/K_m. Each initial rate was determined gas chromatographically by injection of the reaction mixture at 6–10 time points. The slope of the progress curve (initial rate) was subject to less than 2% error in each case. Taking all the duplicate points and averaging the error obtained for each pair of determinations gives the experimental error for initial rate determination: ±6.0%. Gas chromatography is the standard method for analysis of nonaqueous transesterification reaction rates. Thus, the independent points for activity at different pH's are both accurate and precise to within ±10% (we have added 4% error for pH measurements). Figure 2 shows typical data for our determinations of activity at different apparent pH's. Since a plot of all the experimental data would contain approximately 190 points and seven lines, it is not presented.

The experimental data, which are not subject to significantly more experimental error than would be experienced with a pH-stat device in aqueous solution, must now be analyzed in a way which will enable comparison of data in different solvents. In aqueous solution, the pH dependence of subtilisin follows the ionization of the catalytically important base (His 64) at this active site. Between pH 5.5 and 8.5, the enzyme activity is dependent on the microscopic pK_a of His 64.[30] It is important to assess whether the aqueous solution parameters pH and pK_a retain meaning for enzymes suspended in organic media and whether the memorized "pH dependence" of enzyme activity is affected by changing the environment in which the enzyme is placed. Determination of the apparent pK_a provides a means of comparing the pH dependence of an enzyme in different organic solvents and aqueous solution, although in organic environments the meaning of pK_a of an enzyme is obviously different from that in water.

The apparent pK_a values in organic solvents and aqueous solution were obtained by fitting the initial rates of subtilisin-catalyzed reactions as a function of pH to theoretical ionization

(27) The conversion reached 13% after only 10 min, while each injection took at least 7 min.

(28) Ryu, K.; Dordick, J. S. *Biochemistry* **1992**, *31*, 2588–2598.
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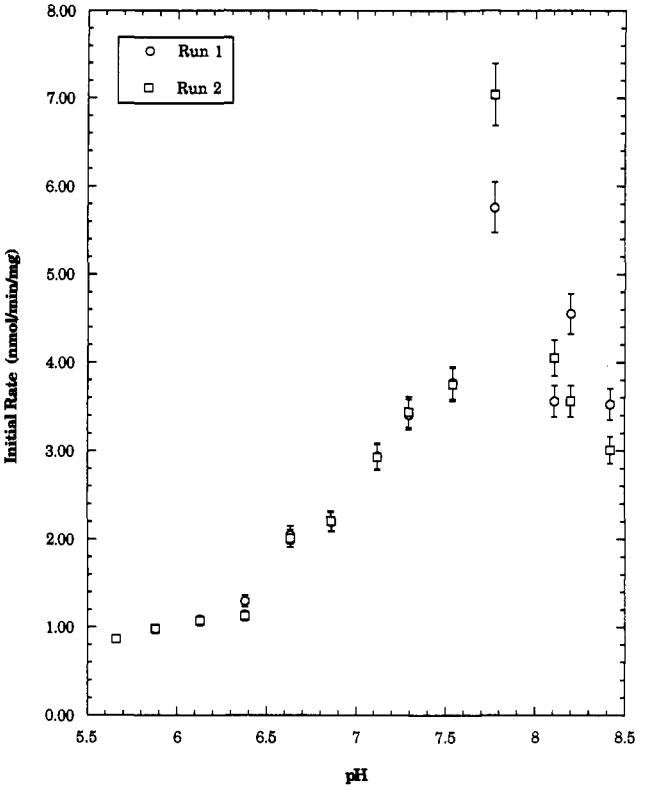

Figure 2. Example of the pH dependence of the initial rate of subtilisin-catalyzed transesterification in dioxane with 0.5 g of $Na_4P_2O_7 \cdot 10H_2O$. The reactions were followed as described in the Experimental Section, and each point was duplicated as shown.

curve(s) by nonlinear regression (Kaleidograph).[31] It must be stressed once again that, under our experimental conditions, the initial rates obtained either in organic solvents or in aqueous solution are all proportional to k_{cat}/K_m. The pH dependence of k_{cat}/K_m for enzyme catalysis represents the pH dependence of the free enzyme and substrate. The errors derived from fitting the data to the ionization equation, which are listed in Table III, describe how well the experimental data (subject to, on average, ±10% experimental error) fit the theoretical ionization curves. Given the reported accuracy in determination, significant deviation from theoretical single ionization kinetics can only be explained by a solvent effect on the enzyme. Such deviations are predictable given the work of Arnold[32] and others who have argued convincingly that enzyme activity in organic solvents can be increased by protein engineering of charged groups. The very basis of this approach is that proteins with different charged states will have discrete stabilities and activities in nonaqueous media. Preparation of subtilisin from varying pH's must, of course, yield different charges on the enzyme. These charges will not only be at His 64, and thus, unlike water, there will be both direct and indirect effects of pH on enzyme activity in organic solvents. The indirect effects on activity in organic solvents will be displayed by loss of adherence to classical single-ionization kinetics.

In our data analyses we have introduced double-ionization kinetics to improve the fit of the data at high pH. Using this standard approach, we can determine the apparent pK_a and the optimal pH. Since the pH range was limited to between pH 5.5 and 8.5, the error for optimal pH determination is significant (~±0.2 pH units). For pK_a determinations, however, the fit of the data is somewhat better (~±0.1 pH units). If the pH range is limited to between pH 5.5 and 8.0 and a single-ionization equation is used to fit the data, the pK_a is found to be the same (±0.02) as that derived from the complete data set with double-ionization kinetics. We note that performing more experiments would not improve this fit, since the error in the initial rate determinations is only ±10%, at worst. For the discussion that follows, it is important to remember that while the errors in apparent pK_a are significant (±0.1 pH units) they are not unusual for pK_a determinations, and the effect of solvent on apparent pK_a is nearly 10 times greater (±1 pH unit).

4. pH Dependence. Since all nonionizing substrates should give the same pK_a,[30] succinyl-L-alanyl-L-alanyl-L-propyl-L-phenylalanyl-*p*-nitroanilide was chosen as a substrate for convenience because the hydrolysis reaction catalyzed by the enzyme can be followed spectrophotometrically. As can be seen from Figure 3 (top), the data for experiments at three fixed ionic strengths (0.01 M, 0.5 M, and 1 M) fitted single-ionization curves well, giving pK_a values of 6.52 ± 0.04, 7.07 ± 0.03, and 7.07 ± 0.03, respectively. It is reasonable that there is an increase in both pK_a and reaction rate with an increase in ionic strength of the buffer solution from 0.01 to 0.5 M, since ionization of the imidazole ring of His 64, which is nonprotonated when the enzyme is active, should be depressed by high ionic strength. Once the ionic strength is high enough (e.g. >0.5 M), further increases would not be expected to affect pK_a. In water, as reported previously for subtilisin BPN',[25] there is a significant buffer effect on the activity of subtilisin Carlsberg. Figure 3 (bottom) shows that in Tris-HCl the optimum pH of the enzyme in water is between 8.0 and 8.2.

The apparent pK_a values and pH optima obtained in organic solvents are listed in Table III, and examples of fitting the experimental data to ionization curves are shown in Figure 4. Inspection of Table III reveals that the apparent pK_a values were between 6.7 and 7.6 when 1 μL of water was added, and between 6.3 and 7.0 when salt hydrate was used. The pH optima obtained by fitting the equation in both situations varied from 7.5 to >8.5. Immediately apparent is that, while the apparent pH dependence of the enzyme in organic media does lie in the same order of magnitude as in water, the data do not fit the standard single-ionization curve well, giving higher errors when compared with the aqueous data. This seems to indicate that the activity of subtilisin suspended in organic media is not simply controlled by the ionization of His 64. Rather, the solvent and the solvent water content may have a significant effect on the apparent pH dependence of subtilisin. Indeed, the apparent pK_a varied between 6.3 and 7.6 (an order of magnitude change in K_a), and optimum pH varied from 7.5 to >8.5. Take, for example, the data for chloroform and tetrahydrofuran with 1 μL of water: for enzyme prepared from pH 8.4 the enzyme was almost four times as active in chloroform as that prepared from pH 7.5, but in tetrahydrofuran the optimal pH of preparation was below pH 7.5.

The activity data discussed above demonstrate that chloroform has a direct interaction with the enzyme. Excluding the data for chloroform and tetrahydrofuran, the apparent pK_a values obtained in organic solvents when 1 μL of water was added were between 7.1 and 7.4, corresponding to the results obtained in aqueous solution at high ionic strength. The pH optima observed in the experiments (see Table II) also fell in the same range of pH optima observed in water. The ionic strength within the water associated with the enzyme powder, as calculated from the water content and known salt content, was approximately 4 M. It has been reported previously[8] that when α-lytic protease with histidine

(31) The fitting equations are as follows. For aqueous solution

$$V = \frac{10^{(pH-pK_a)}A}{10^{(pH-pK_a)} + 1}$$

For organic solvents

$$V = \frac{A}{1 + (10^{-pH}/10^{-pK_1}) + (10^{-pK_2}/10^{-pH})}$$

(32) Arnold, F. H. *Trends Biotechnol.* **1990**, *8*, 244–249.

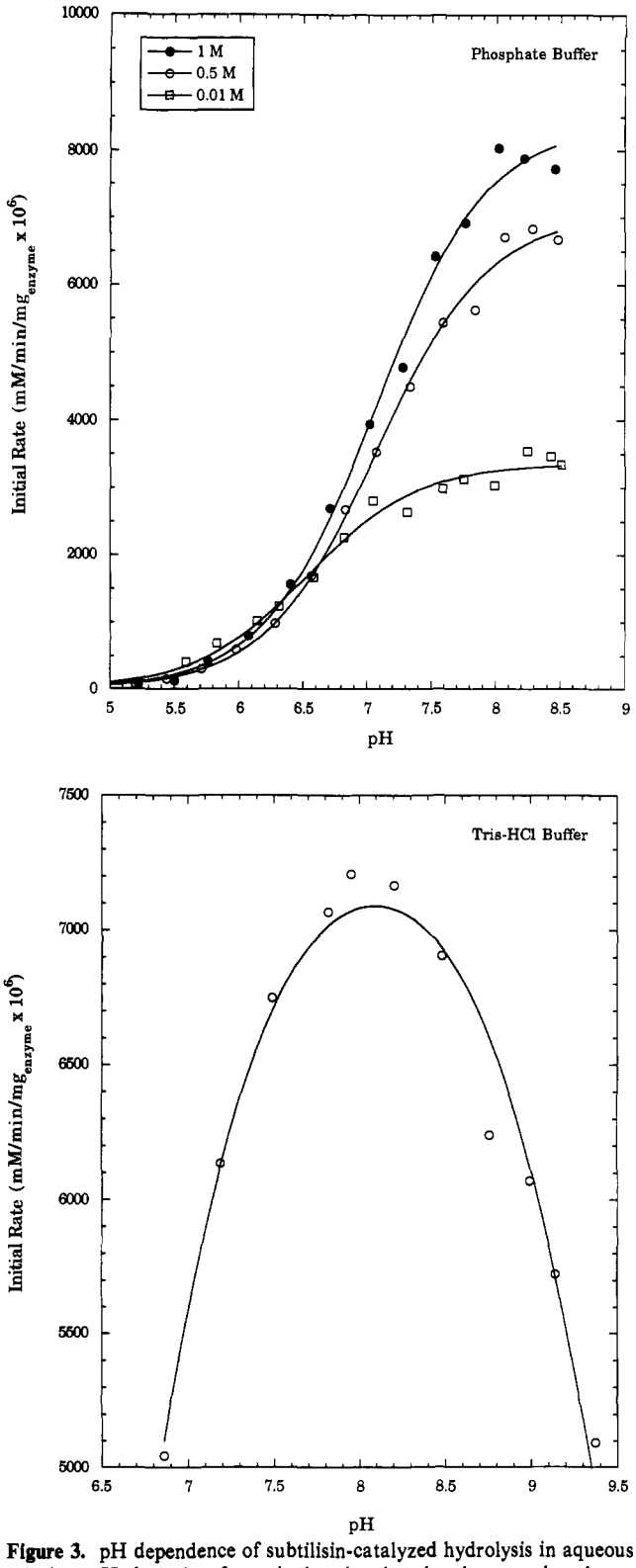

Figure 3. pH dependence of subtilisin-catalyzed hydrolysis in aqueous solution. Hydrolysis of succinyl-L-alanyl-L-alanyl-L-propyl-L-phenylalanyl-*p*-nitroanilide in phosphate buffer with different ionic strengths (top) and in 0.01 M Tris-HCl buffer (bottom) were followed as described in the Experimental Section. The data obtained in phosphate buffer were fitted to the theoretical ionization curve by nonlinear regression (Kaleidograph).[30]

enriched in ^{15}N in the imidazole nitrogens was lyophilized from aqueous solutions with pH values ranging from 4.9 to 9.3, the high-resolution ^{15}N NMR spectra revealed that the behavior of the ^{15}N resonances as function of pH in the solid samples closely parallels that observed in the corresponding solution-state study,

Table III. Apparent pK_a Values and pH Optima Obtained by Fitting Ionization Curves[a]

	1 μL of water		$Na_4P_2O_7 \cdot 10H_2O$	
solvent	pK_a	optimal pH	pK_a	optimal pH
hexane	7.22 ± 0.23	7.94		
toluene	7.16 ± 0.05	8.16	6.62 ± 0.10	>8.50
chloroform	7.64 ± 0.34	>8.50	6.33 ± 0.10	>8.50
THF	6.70 ± 0.15	7.49	6.52 ± 0.10	>8.50
acetone	7.06 ± 0.12	>8.50	6.55 ± 0.16	7.64
acetonitrile	7.40 ± 0.11	8.01	6.88 ± 0.11	7.80
dioxane	7.18 ± 0.22	7.76	7.03 ± 0.19	7.77

[a] Experimental detail is provided in the text, as is the method for determination of apparent pK_a's. THF is tetrahydrofuran. An optimal pH of >8.50 is given when the activity of the enzyme has not decreased significantly by pH 8.5.

indicating that the active site catalytic triad structure of Asp-His-Ser is maintained in the lyophilized enzyme powder. The pK_a of the imidazole ring was assumed to be 7.0 ± 1.0. Similar pH dependencies for lyophilized powders of ^{15}N-enriched histidine and imidazole have also been reported.[7] These reports demonstrate that the physical state of the enzyme has little or no effect on the ionization state of the enzyme's active site, and pH and pK_a (within 1 pH unit) retained meaning for the powders in the sense that the conjugate acid/conjugate base ratio found in a solution, as controlled by pH and pK_a, was also found in the powder prepared from that solution. Our results appear to substantiate this point of view and indicate that the apparent pH dependence of subtilisin in organic media which do not interact directly with the enzyme is similar to that in water.

However, when sodium phosphate salt hydrate was used to fix the water activity of the system, the apparent pK_a values (6.3–7.0) were almost 0.5 pH units lower (three times the measured experimental error) than those obtained in the absence of salt hydrate. The lower pK_a values are clearly not due to a reduction in ionic strength because, with the concentration of phosphate buffer during lyophilization and addition of excess amount of salt hydrate, the ionic strength in both sets of experiments can be computed to be 4 M or higher. Bone and Pethig[33] have found that the dielectric parameter of a protein increased greatly upon an increase in its hydration, and they presumed that it is due to water's role as a plasticizer, which increases the flexibility, and hence the polarizability, of the protein structure. Furthermore, Affleck *et al.*[34] have demonstrated that addition of up to 1% (v/v) water to tetrahydrofuran leads to a substantial increase in the transesterification activity of subtilisin Carlsberg that correlates with an increase in the active site polarity. The enhancement in the polarity of the medium should also favor the ionization of a nonprotonated base. This could be used to account for the low apparent pK_a values obtained in our study with addition of the salt hydrate. When 1 μL of water was added to a dry system (0.05% v/v water), the active site of the enzyme would still be expected to be rather rigid, and therefore due to the lack of water clusters which could migrate from one polar residue to another, the degree of protonation of the charged groups should be "frozen" according to their pK_a in the last buffer. Addition of $Na_4P_2O_7 \cdot 10H_2O$ introduces significantly more water to the system (~0.4% v/v water in the solvent phase when acetonitrile was used as a solvent). This additional water could enhance local polarity in the enzyme's active site, therefore favoring the ionization of the neutral imidazole ring of the His 64 in the active site. This hypothesis *is* supported by an experiment *in* which the addition of 3 μL of water to the substrate solution in acetonitrile results in the reduction of the apparent pK_a to 6.73, again lower than 7.40 when only 1 μL of water was added.

(33) Bone, S.; Pethig, R. *J. Mol. Biol.* **1984**, *181*, 323–326.
(34) Affleck, R.; Xu, Z.-F.; Suzawa, V.; Focht, K.; Clark, D. S.; Dordick, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 1100–1104.

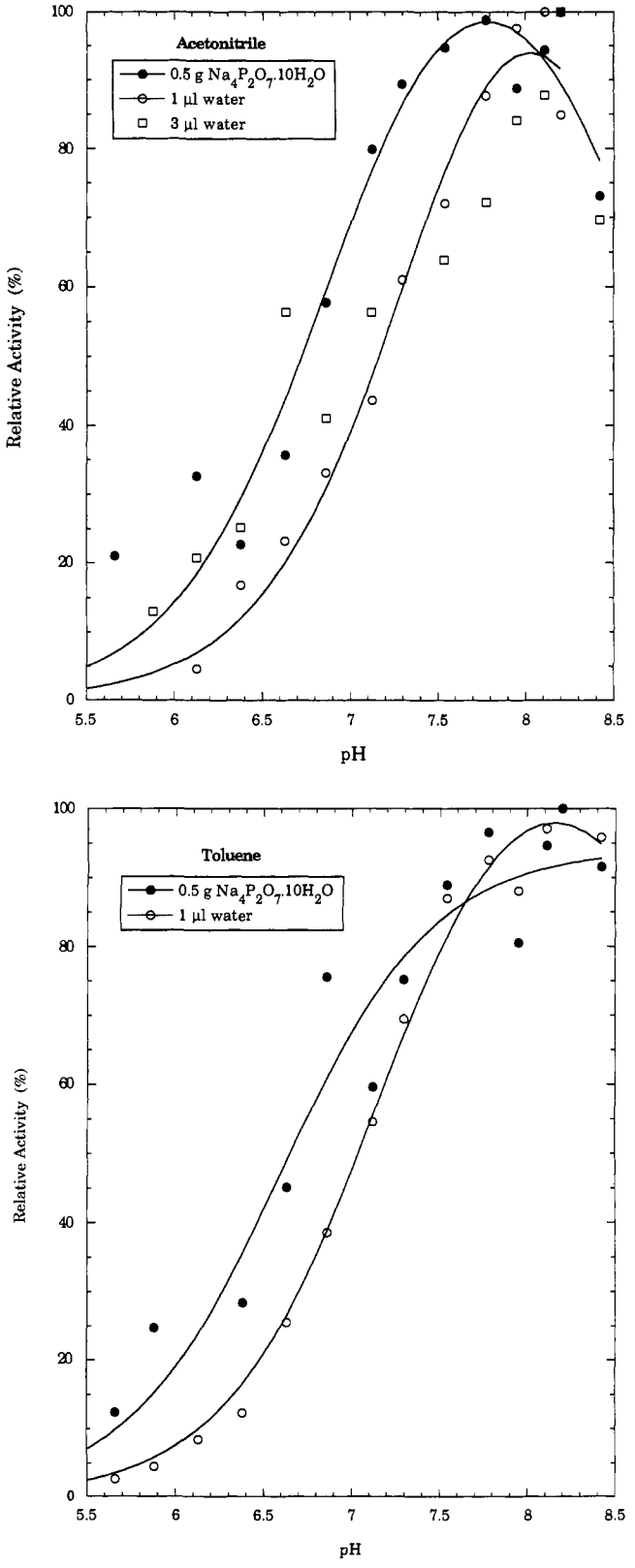

Figure 4. Effect of pH on relative reaction rates in acetonitrile (top) and toluene (bottom). Data points represent the average of duplicate determinations.

The above discussion demonstrates that the water content of the enzyme has a significant effect on the apparent pH dependence in solvents. When the two sets of results were compared by plotting the reaction rates in all the solvents used against pH, an interesting phenomenon was observed. Almost all the apparent pH dependence curves were left shifted when salt hydrate was added

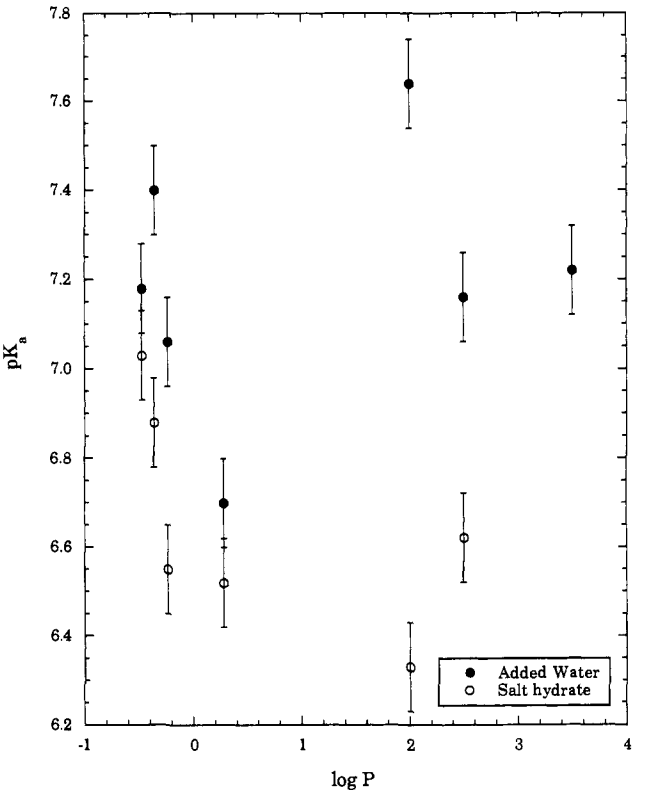

Figure 5. Effect of log P of organic solvents on the apparent pK_a for subtilisin-catalyzed transesterification of *N*-acetyl-L-phenylalanine ethyl ester.

compared to the results obtained with addition of 1 μL of water. The enzyme was nearly inactive at low pH (pH < 6.0) when 1 μL of water was added, whereas with addition of salt hydrate the reaction rates remained comparatively high, even at that low pH level. When 3 μL of water were added to the reaction mixture in acetonitrile, which afforded an intermediate water amount between those provided by addition of 1 μL of water and 0.5 g of $Na_4P_2O_7 \cdot 10H_2O$, an intermediate situation was observed as expected (Figure 4 (top)). Thus, increasing water content in the reaction system, either by direct addition of water or by introduction of salt hydrate, can not only lead to a reduction in the enzyme's apparent pK_a but also help maintain enzyme activity at rather low pH.

Although the apparent pK_a values obtained in our experiments were less affected by changing solvent then by changing water content, an interesting effect of organic solvents on the apparent pK_a values can still be observed when plotting the pK_a results versus log P values (Figure 5). A V-shaped relation was observed with the results obtained by addition of either water or salt hydrate. This can still be explained by changes in the local polarity at the enzyme's active site. Penetration of an organic solvent would lower the local polarity at the enzyme's active site[28] and therefore stabilize the nonprotonated imidazole ring of His 64 by increasing its apparent pK_a. This could also be the reason why the apparent pK_a values obtained in organic solvents with addition of 1 μL of water were slightly higher than those obtained in aqueous buffer at a high ionic strength. Local polarity will, however, be increased by introduction of water into the enzyme's active site,[34] which in turn would enhance its apparent pK_a. As solvent log P changes, the balance between water and solvent distribution in and around the active site of an enzyme would also be expected to change. These two factors balance each other, offering one explanation of a V-shaped relationship between the apparent pK_a of the enzyme and the value of log P for the solvent.

The above discussion has attempted to explain the effect of solvents on the memorized pH dependence via direct effects of

solvents on active site polarity. While this can account for the general trends observed, it does not account for the loss of adherence of kinetic data in organic solvents to classical single-ionization curves. The fit of the data to ionization curves has been discussed above. Current studies are in progress to elucidate and separate, in more detail, the role of direct and indirect effects on apparent pH dependence of enzymes in organic solvents.

Conclusion

In a broad sense, the apparent pH dependence of subtilisin suspended in organic media is similar to that in water. However, its variation upon changing solvents, water content, and method of water delivery affords another example of the unusual properties acquired by enzymes in nonaqueous environments. Our results demonstrate that the pH dependence of enzyme catalysis can be tailored not only by protein engineering but also to some degree by solvent engineering. Indeed, both techniques when used with subtilisin can change the enzyme's apparent pK_a by greater than 1 pH unit. Solvents presumably affect the activity and the apparent pH dependence of the enzyme by changing its hydration level and the local polarity at its active site, thus disturbing the delicate balance of the charges interacting with the enzyme's active site. Additionally, the apparent pK_a is more affected by changing water content than by changing solvents. Increasing water content not only can greatly enhance the enzyme activity but also can reduce its apparent pK_a by nearly one unit and help maintain relatively high enzyme activity at rather low pH level.

Experimental Section

Materials. Subtilisin Carlsberg (11 units/mg), *N*-acetyl-L-phenylalanine ethyl ester, succinyl-L-alanyl-L-alanyl-L-propyl-L-phenylalanyl-*p*-nitroanilide, and all salt hydrates used were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents used were of the HPLC grade from Aldrich Chemicals (St. Louis) and were dried over molecular sieves prior to use.

Enzyme Preparation. Subtilisin (0.1 g) was dissolved in 25 mL of phosphate buffer of 0.01 M ionic strength with 14 pH values (5.5–8.5) and snap frozen in liquid nitrogen, then freeze dried for 48 h on a Labconco lyophilizer (Model 4451F). The lyophilized enzyme powder was stored in a tightly sealed Falcon tube at −20 °C. The water content of the powder, determined by the modified Karl Fischer titration in a Fisher Coulometric apparatus, was in the range of 10–15% (w/w), depending on the pH. The protein content was the same for all the enzyme powders, according to the measurements of absorbance of the resolubilized protein at 280 nm.

Enzyme-Catalyzed Transesterification Reactions in Organic Solvents. Transesterification reactions between *N*-acetyl-L-phenylalanine ethyl ester and methanol were undertaken in various organic solvents with the addition of either water or salt hydrates. The product *N*-acetyl-L-phenylalanine methyl ester, was detected gas chromatographically as follows. Substrate solution (2 or 3 mL) containing 30 mM *N*-acetyl-L-phenylalanine ethyl ester and 1.2 M methanol in a given solvent was added to a 4-mL Wheaton vial containing 2 mg of lyophilized enzyme powder, followed by addition of 1 μL of distilled water or 0.5 g of $Na_4P_2O_7 \cdot 10H_2O$, respectively, to start the reaction. The reaction mixture was placed in a New Brunswick G-24 incubator/shaker at 30 °C with agitation of 300 rpm. The speed of agitation was shown to be above that necessary to overcome external diffusional limitations. Samples (0.5 μL) were taken at intervals (typically ~10 min) and injected to a Hewlett-Packard 5890 Series II gas chromatograph with an FID detector after flow through an HP-1 cross-linked methyl silicone gum capillary column (30-m × 0.53-mm × 0.88-μm film thickness). An initial temperature of 100 °C was maintained for 1.5 min, the temperature was then raised at 25 °C/min to 200 °C, and after 1 min, it was raised again at 40 °C/min to 280 °C and maintained for 0.5 min. Under these conditions, the retention times for *N*-acetyl-L-phenylalanine methyl ester and ethyl ester were 5.25 and 5.50 min, respectively. Reactions were followed for up to 5% of total conversion (the initial rate period). We note here that under these conditions the initial rate is actually proportional to k_{cat}/K_m since the K_m for the enzyme in these solvents is greater than the substrate concentration used.[24]

Enzymic Reactions in Aqueous Solution. pH dependence of subtilisin in aqueous solution was determined by following the hydrolysis of succinyl-L-alanyl-L-alanyl-L-propyl-L-phenylalanyl-*p*-nitroanilide in phosphate buffer (at a fixed ionic strength of 0.01, 0.5, or 1 M, pH range 5.5–8.5, for determination of pK_a values) or in 0.01 M Tris-HCl buffer (pH range 6.5–9.5, for estimation of pH optimum). The enzyme solution (25 μL, 20 μg/mL) was added to a cuvette containing 0.02 mM substrate (2 mL) in a given buffer to initiate the reaction, and the increase in absorbance at 412 nm on the release of the product *p*-nitroaniline was monitored to calculate the initial reaction rate, which is also proportional to k_{cat}/K_m.[25]

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