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Hofmeister Series: The Hydrolytic Activity of *Aspergillus niger* Lipase Depends on Specific Anion Effects

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The specific activity of lipase A (*Aspergillus niger*) toward the hydrolysis of *p*-nitrophenyl acetate (p-NPA) is shown to increase as a result of sodium salt addition according to specific ion effects of the Hofmeister series. This shows explicitly that the Hofmeister effect is due to the different specific interactions between anions and the enzymatic surface.

Introduction

More than a century ago, Hofmeister related the efficiency of precipitation (salting-out) of the protein ovalbumin to specific ion effects due to added sodium salts.[1,2] The precipitation efficiency of the anions, at a fixed ionic strength, was found to decrease in the order:

$$H_2PO_4^- > SO_4^{2-} > F^- > Cl^- > Br^- > NO_3^- > I^- > ClO_4^- > SCN^-$$

The phenomenon embraced by this sequence, referred as a Hofmeister series, was seen later to be ubiquitous. A vast range of "specific ion" effects, of which biochemists are very much aware, show up also in physical, colloid, polymer and surface chemistry.[3] Well-known examples occur with surface tension,[4] ζ and surface potential measurements,[5] as well as for octadecyl monolayers spread on salt solutions,[6] and cationic microemulsions.[7] Until recently, they have remained inexplicable with conventional theories of solution and colloid chemistry that treat water as a continuum.[3] Of the different attempts to account for Hofmeister phenomena, one assigns the matter to changes in bulk water structure, induced by ions. The ion specificity is determined by the ability of ions to form (kosmotropic), or to break (chaotropic) hydrogen bonds in water systems.[8−11] A second model describes specific ion activities in terms of electrodynamic fluctuation (dispersion) forces, which are omitted in the conventional limit DLVO, Onsager, and Debye−Huckel type of electrostatic theories.[12−14] In essence, Hofmeister effects are attributed substantially to bulk effects in the one case, or to surface phenomena in the other. Probably both effects contribute depending on the situation.

In biochemistry, the pH and ionic strength are recognized as important factors that affect protein properties and enzyme activity.[15] Indeed, enzyme conformations, stability and activity are a result of a complex interplay that include local and bulk intermolecular forces subsumed under names such as van der Waals interactions, hydrogen bonds, solvation and polarization effects, and association−dissociation equilibria of charged groups. Buffers also can play a crucial role in enzyme activities. The influence of added salts and buffers in changing protein behavior and enzyme activity has been reported in several investigations.[14−21] Direct or reversed Hofmeister series effects have been often recognized.[18]

The aim of this work is to show explicitly that in at least one case the Hofmeister effect in enzymatic specific activity (SA) measurements is clearly due to the different specific interactions between anions and the enzymatic interface. A standard assay, the hydrolysis of *p*-nitrophenyl acetate (p-NPA) to *p*-nitrophenol (p-NP), was used to assess how the concentrations of sodium salts with different anions affect the activity of the enzyme lipase A (triacylglycerol acyl hydrolase, EC.3.1.1.3) from *Aspergillus niger*.

Experimental Section

Chemicals. Lipase A (from *Aspergillus niger*) was purchased from Amano Enzyme Europe Ltd. The X-ray structure is unknown. For the commercial preparation a pI = 3.7,[22] and a protein content of 14.1%[23] have been recently reported. Sodium dihydrogen orthophosphate 99%, and disodium hydrogen orthophosphate 99% were purchased from Carlo Erba. Sodium perchlorate 99%, sodium bromide anhydrous >99%, and sodium nitrate 99% were from Acros. Sodium chloride 99.5% was from Merck. *p*-Nitrophenylacetate 99% (p-NPA), *p*-nitrophenol > 99.5% (p-NP), and 2-propanol 99.8% were from Fluka.

pH Measurements. The pH measurements of a series of aqueous/buffers salt solutions were performed by using a pH-meter pH-526, WTW, equipped with a pH glass electrode SenTix81 (range of linearity pH 2−10; typical reliable reproducibility of measurements up to electrolyte concentration 1−1.5 M). The water used to prepare buffers and salt solutions was purified through a Millipore system (Simplicity 185; electrical conductivity 0.054 μS/cm). After the addition of each salt, the solution under investigation was stirred for 2 min and allowed

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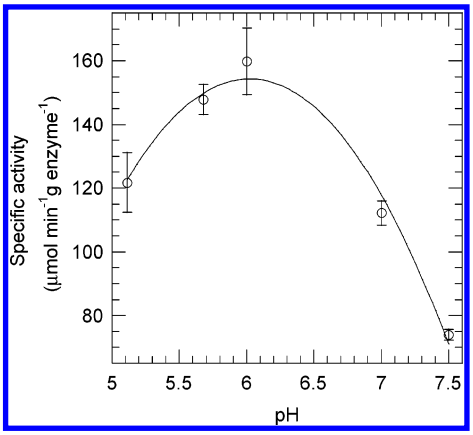

Figure 1. Enzymatic activity of lipase A (*Aspergillus niger*) in phosphate buffer 5 mM solution at different pH values.

TABLE 1: Molar Extinction Coefficients (ϵ) and Wavelength (λ) of P-NP at Different pH Values

pH	λ (nm)	ϵ (L cm^{-1} mol^{-1})
5.11	360	3100
5.68	360	3180
6.00	400	1740
7.00	400	8990
7.50	400	13270

to rest for 1 min before the pH value was recorded. All readings were made at 25 °C.

Lipase Assay. Lipase-catalyzed hydrolysis of p-NPA to p-NP was used to determine the enzymatic activity. A typical experiment was performed by using 200 μL of a 50 mM p-NPA solution in 2-propanol that was mixed with 1.6 mL of phosphate buffer (5 mM, pH = 5−7.5) with or without salt. The reaction was started by adding 200 μL of crude lipase solution, 7.5 mg/mL. The formation of p-NP was determined by measuring the absorbance at 360−400 nm depending on pH value (Table 1), using a UV−visible spectrophotometer Cary 50. Because the pK_a of p-NP is 7.15, the neutral or the ionized species is dominant, depending on the pH value. These species have different molar extinction coefficients (Table 1). Therefore a calibration curve for each series of activity measurements was preliminarily determined.

Spontaneous hydrolysis may also occur. Its extent was found to fluctuate in the range 2−10% (both with and without added salt) of enzymatic activity and was not considered in the presentation of data in Figure 2. One unit of hydrolytic activity is defined as the amount of enzyme that releases 1 μmol of p-NP per minute.

Results and Discussion

The enzymatic activity of lipase A (*Aspergillus niger*) was first measured as a function of pH. To do this, the ratio [$H_2PO_4^-$]/[HPO_4^{2-}] was changed to prepare different sodium phosphate buffer solutions in the range pH 5.0−7.5 ([$H_2PO_4^-$] + [HPO_4^{2-}] = 5 mM), and without any added salts. The results are shown in Figure 1. Lipase A shows its maximum activity at pH = 6. The points in Figure 1 were fitted by a second-order polynomial to enable the inference of specific activity at a given salt concentration (see below).

In a parallel systematic investigation on Hofmeister series effects due to the addition of salts having different anions and cations to different buffer solutions (work in progress), it was found that sodium salts added to sodium phosphate buffer 5 mM at initial pH = 7 decrease the pH, as measured by a conventional glass electrode significantly. A reverse Hofmeister series characterizes the decreasing measured pH. This is shown in Table 2. The effectiveness of the salts in decreasing pH is ClO_4^- > NO_3^- > Br^- ≥ Cl^- if data obtained for 1 M salt concentration are considered. Nitrate follows after chloride at 2 M salt concentrations.

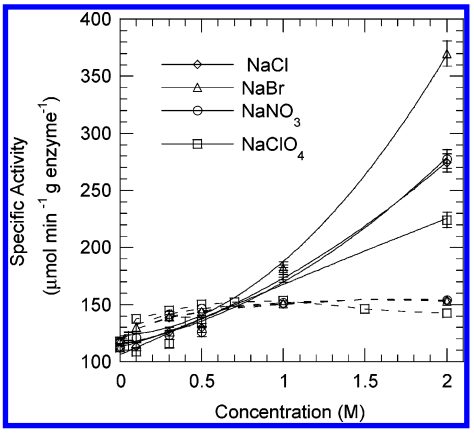

Figure 2. Anions effect on the hydrolytic activity of lipase A in phosphate buffer (5 mM, pH = 7) solution with increasing concentration of sodium salts. Experimental activities (solid lines), calculated activities (dashed lines).

TABLE 2: pH of Phosphate Buffer (5 mM, Initial pH = 7) Solution with Increasing Concentration of Sodium Salts

Salt concn (M)	NaCl	NaBr	$NaNO_3$	$NaClO_4$
0.2	6.75	6.72	6.65	6.63
0.5	6.56	6.54	6.47	6.35
1.0	6.35	6.33	6.29	6.06
1.5	6.01	5.97	6.02	5.55
2.0	5.88	5.84	5.90	5.47

[a] Standard deviations are ±0.02 pH units.

Let us assume that the measured pH is the real pH of the bulk solution and not a reflection competition between ion pair and hydronium adsorption at the surface of the glass electrode. Once the effect of the pH of the buffer on the activity, and the effect of salt addition on the pH of the buffer, have been determined the effect of salt addition on the enzymatic activity could be studied.

The p-NPA assay was performed in a 5 mM phosphate buffer at pH = 7. Measurements were carried out at 25 °C reading the absorbance at 400 nm. Figure 2 shows the increase of the specific activity (solid lines) of lipase A as a result of the addition of NaBr, NaCl, $NaNO_3$ and $NaClO_4$ salts up to 2 M along with the activities calculated (dashed lines) on the basis of data in Table 1 and of the polynomial used to fit the curve in Figure 1.

Salt addition enhances enzymatic activity significantly, particularly when salt concentration exceeds 1 M. The increase of specific activity is much larger than that expected for a decrease of pH from 7 to about 6, as predicted by the dashed lines in Figure 2.

The effect is remarkable and is different with respect to the influence of the various salts on the pH measurements. Thus enhanced activities are in the order: Br^- > Cl^- ≈ NO_3^- > ClO_4^-. These experiments on the enzymatic activity of lipase A toward p-NPA hydrolysis, in 5 mM phosphate buffer at initial pH = 7, clearly indicate a specific role of the Br^- anion.

To ascertain the real effect of the pH decrease and of the salt addition, activity assays were repeated using again a 5 mM phosphate buffer solution, but at initial pH = 6, and in the presence of NaBr. Table 3 shows the specific enzymatic activities determined without and with 1 and 2 M added NaBr.

TABLE 3: pH and Hydrolytic Activity of Lipase A in Phosphate Buffer (5 mM, Initial pH = 6) and NaBr (0, 1, 2 M) Solutions

[NaBr]	pH	Specific Activity (μmol min^{-1} g^{-1})
0.0	6.00	160 ± 5[a]
1.0	5.17	244 ± 4
2.0	4.89	324 ± 7

[a] Standard deviation.

Strikingly, enzymatic activity increased significantly, even though the addition of NaBr induced a further significant decrease of (measured) pH below the optimum value of 6 (Figure 1). These results emphasize the crucial role of Br^- in increasing the enzymatic activity. The comparison between activities at [NaBr] 2 M in Figure 2 (where pH = 5.84 as given in Table 2) and those in Table 3 (pH = 4.89) indicates a clear effect of pH on the enzymatic activity. However, this pH effect seems to be less important than the global specific effect produced by the Br^- anion. A sort of reinforced synergism is found in the case of data in Figure 2 (SA = 370 μmol min^{-1} g^{-1}) where the optimal pH is obtained by adding NaBr to a phosphate buffer solution at initial pH = 7. Addition of NaBr to the optimal buffer solution at pH = 6, the case of Table 3 (SA = 324 μmol min^{-1} g^{-1}), brings about still a synergistic effect but induces a smaller increase of the specific enzymatic activity because a concurrent and significant decrease of pH occurs.

Conclusions

From this work some important conclusions on an understanding of the real nature of the Hofmeister effect emerge. If the major effect of salt addition was due to the salt induced water structure modification (bulk phenomena), the corresponding pH change should have produced the increase of enzymatic activity predicted by theoretical curves (dashed lines in Figure 2). But this is not so. The present results can be justified only in terms of a specific interaction (adsorption) of Br^- anions at the enzyme surface. To these interactions, dispersion forces are likely to contribute.[12] The final outcome is that the enzyme is driven thereby to a conformation very active for the catalytic process. Hence, the main conclusion is that, in this particular case, enzymatic activity is intimately related to anion specific surface phenomena. As mentioned above, it should be recalled that surface adsorption phenomena will occur at the glass electrode surface also. Consequently, the measured pH may not be the real bulk pH of the solution. One may not even be sure of the pH reading, but the superactivity induced by adding specifically NaBr 2 M in phosphate buffer at pH = 7 (which is not the optimal pH without added salts) seems to be a quite remarkable result. In summary it turns out that it is more convenient to obtain the apparent optimal pH conditions by adding salts rather than by using an "ad hoc" buffer solution only. The phenomenon may well be quite general, in which case it provides a clue to specific ion regulation mechanisms and superactivities of enzymes.

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Supporting Information Available: A table of the enzymatic activity data displayed in Figure 2 is available free of charge via the Internet at http://pubs.acs.org.

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