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

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A recent Letter by Pinna et al.[1] purports to demonstrate an anionic Hofmeister series affecting the activity of *Aspergillus niger* lipase. A crude commercial lipase preparation (14.1% protein) was used to test the effects of different salts on hydrolysis of the chromogenic substrate *p*-nitrophenyl acetate. The authors report significant lowering of the pH of phosphate buffer solutions by the addition of 0.2−2.0 M sodium chloride, bromide, nitrate, or perchlorate and attempt to deconvolute the effects of pH on enzyme activity from the effects of different salts, concluding that "the Hofmeister effect ... is clearly due to the different specific interactions between anions and the enzymatic interface". However, closer examination of the data suggests that both the experimental results and their interpretation may be questionable.

The authors make no attempt to account for the observed pH changes on addition of salt to phosphate buffer solutions and, indeed, appear ambivalent about the reality of such changes. However, appropriate understanding of the pH changes is critical to the interpretation of the reported enzyme activity data, in order to rule out artifacts in the highly pH-sensitive assay.

Several possible causes may be proposed for the observed pH changes on addition of salt to phosphate buffer: (i) an error in the glass electrode readings, (ii) acid contaminants in the reagents, or (iii) a change in the acid dissociation constant of the buffer and/or other weak acids present in the system, including water itself. The authors have previously published in this Journal a claim of Hofmeister series effects on glass electrode measurements,[2] and although they do not cite this earlier report, they allude in their paper to the possibility that the "measured pH may not be the real bulk pH of the solution". That paper was heavily criticized in a Comment,[3] to which the authors did not respond.

If the glass electrode readings are taken at face value, it is easy to show that changes in the apparent fractional dissociation of the buffer are not linear with the concentration of added sodium salts, ruling out an acid contaminant in the reagents as a source of the pH change. The remaining explanation is then that the acid dissociation constant of the buffer and/or other weak acids in the system is increased at high ionic strength.[3] Ignoring the effects of dilution (salt-containing buffers appear to have been prepared by the addition of solid salts to 5 mM phosphate buffer previously adjusted to pH 6.0 or 7.0), one calculates an apparent change in pK_a' for dihydrogen phosphate from 7.21 to 6.08 on addition of 2.0 M NaBr, in parallel with a pH change from 6.00 to 4.89. (Certainly, no other interpretation would justify the use of phosphate buffer so far outside its conventional buffer range.)

The extent of buffering is important to the interpretation of the enzyme activity data. The release of two acidic products (acetic acid and *p*-nitrophenol) should lead to a pH drop of 0.2 units in the first minute of the enzyme-catalyzed reaction at pH 6.0 in the absence of added salt. Such a pH change will affect both the true enzyme activity and the absorption characteristics of the product (*p*-nitrophenol) used to calculate activity, leading to systematic errors in enzyme activity calculations. The effects will be even more pronounced when added salt is present if, as seems likely, high ionic strength also leads to increases in the acid dissociation constants of acetic acid and *p*-nitrophenol. In particular, greater fractional dissociation of *p*-nitrophenol to the more strongly absorbing *p*-nitrophenolate anion in the presence of added salt would lead to greater absorbance in the absence of greater product formation, hence an apparent, but artifactual, increase in enzyme activity. The likelihood of significant changes in the acid ionization constant of *p*-nitrophenol at high salt is underlined by the fact that the absorbance data presented by the authors, apparently in 5 mM phosphate buffer without added salts, is best fit by a pK_a' for *p*-nitrophenol of 6.95, rather than 7.15 as quoted in the literature and by the authors themselves in the text. An apparent threefold increase in enzyme activity, as reported by the authors, could be accounted for by a further pK_a' shift of 0.6 units at high ionic strength, assuming no other spectral changes than those due to a change in dissociation of the product. A systematic error in glass electrode readings, as alternately proposed by the authors, would give rise to a similar effect. Although the authors report that a "calibration curve for each series of activity measurements was preliminarily determined", it is not clear what constituted a series or how the effects of cosolvent and/or of potentially buffering components in the crude enzyme mixture were addressed.

The proposed Hofmeister series is also difficult to see in the data. At up to 1.0 M salt concentration, results for the four sodium salts tested appear identical within experimental error. Differences between salts are observed only at the sole concentration of 2.0 M, and the significance of the small changes seen (visually amplified by the choice of vertical scale) is difficult to assess. Although some experiments were repeated at pH 6.0, where errors in the molar absorption coefficient for *p*-nitrophenolate due to changes in fractional dissociation may be less likely, only NaBr was tested at this pH, and no comparison with other salts was attempted, making it difficult to assess the claim of a Hofmeister series, as opposed to general effects of ionic strength,. The authors themselves place the salt concentration at which the Hofmeister-like differences occur outside the range of "reliable reproducibility" for their glass electrode, meaning that these data are particularly susceptible to systematic errors. It also appears that no correction was made for the nonenzymatic hydrolysis of *p*-nitrophenyl acetate (which should itself be strongly pH- and salt-dependent).

Even if one assumes that some fraction of the reported activity increase is real rather than artifactual, it is difficult to assess the proposal that the change is due to an interfacial effect (specific anion binding to the enzyme surface) rather than a bulk solution effect (which the authors seem to interpret in terms of pH alone). No data were presented for the effects of salt at constant pH, meaning that it is not possible to assess the shape

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of the response curve to determine its consistency with the authors' proposal of selective anion binding. It would be surprising, though not impossible, to find anion binding sites in an enzyme with an isoelectric point of 3.5. It would be less surprising if high ionic strength affected, for example, the Michaelis constant for binding of *p*-nitrophenyl acetate to the presumably hydrophobic enzyme active site. However, even if convincing salt-specific differences could be demonstrated, the use of a crude lipase preparation for activity assays makes it impossible to definitely assign salt effects on enzyme "specific activity" to any particular component in the mixture.

References and Notes

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