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The effect of glass and silica surfaces on trypsin and α-chymotrypsin kinetics

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SUMMARY

The pH–activity curve for the trypsin (EC 3.4.4.4) hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) in glass vessels is shifted to higher pH values at low enzyme concentrations (1 $\mu g \cdot cm^{-3}$) and low ionic strength (10 mM) due to adsorption of the trypsin onto the negatively charged glass surfaces.

For a similar reason, though the autolysis of α-chymotrypsin (EC 3.4.4.5) shows second-order kinetics in 'true' solution, faster rates of autolysis are observed in glass vessels or in the presence of low concentrations of colloidal silica (0.004%); under these conditions the kinetics are first order. This much increased rate of autolysis of adsorbed α-chymotrypsin probably explains the confusion in the literature regarding the kinetics of autolysis of α-chymotrypsin where both first- and second-order kinetics have been reported.

The kinetics of autolysis of trypsin (EC 3.4.4.4) and α-chymotrypsin (EC 3.4.4.5) and of trypsin hydrolysis of the low molecular weight substrate benzoyl-L-arginine ethyl ester (BAEE) are all affected by the presence of glass surfaces of such areas as may normally be present in standard experimental conditions, *e.g.* autolysis experiments in glassware and BAEE hydrolysis followed in a pH stat. 'Wall' effects have been considered previously[1] in connection with the α-chymotrypsin-catalysed hydrolysis of acetyl-L-tyrosine hydroxamide, but their absence is probably due to the high buffer (0.3 M Tris) and enzyme (about 190 $\mu g \cdot cm^{-3}$) concentration. On the other hand[2], 'wall'

Abbreviation: BAEE, benzoyl-L-arginine ethyl ester.

effect problems arose in pH-stat measurements of the hydrolysis by α-chymotrypsin (0.4 $\mu g \cdot cm^{-3}$) of acetyl-L-phenylalanine glycolamide ester in 10 mM salt.

Fig. 1 shows the shift to higher pH values of the pH–activity curve for the trypsin hydrolysis of BAEE ($2 \cdot 10^{-3}$ M) in the pH-stat on reducing the ionic strength to 10 mM (trypsin concentration 1 $\mu g \cdot cm^{-3}$). That this shift in the pH–activity curve at low ionic strength is due to adsorption of trypsin onto the glass surfaces (and not due to an intrinsic change in the trypsin pH profile due to changes in ionic strength) is shown by

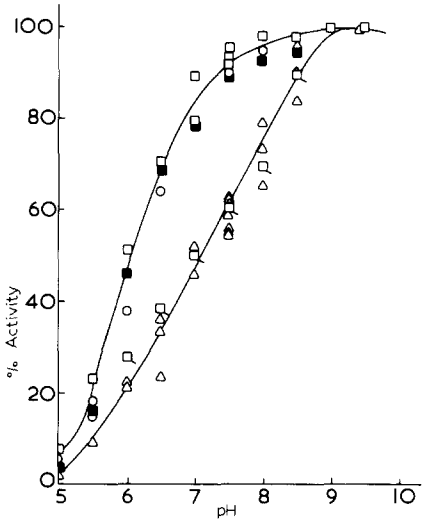

Fig. 1. Apparent effect of ionic strength on the pH–activity curve of trypsin-catalysed hydrolysis of BAEE at 25°C. □, I 0.2 (0.05 M KCl; 0.05 M $CaCl_2$); ■, I 0.05 (0.05 M NaCl); △, I 0.01 (0.01 M NaCl); ○, I 0.01 (0.01 M NaCl) + lysozyme (25 $\mu g \cdot cm^{-3}$); ▽, I 0.01 (0.01 M NaCl) + bovine serum albumin (250 $\mu g \cdot cm^{-3}$).

blocking the adsorption of trypsin by the addition of lysozyme (EC 3.2.1.17) (25 $\mu g \cdot cm^{-3}$) to the assay mixture prior to the addition of trypsin. In the presence of lysozyme the pH–activity curve at low ionic strength is restored to the same shape and position as at higher ionic strengths. Lysozyme was used because its isoelectric point (11.4) is higher than that of trypsin (10.8), so that its net positive charge at the pH values used will be higher than that of trypsin and it is thus likely to be more firmly adsorbed on the negatively charged glass surface. Such a lysozyme film was not removed by washing the cell and electrodes well with water, allowing them to stand overnight in water and then washing again and drying with paper tissue, for the pH–activity curve in 10 mM salt was then the same as that in the higher ionic strength solutions. However, after washing the cell and electrodes in 1 M NaCl and detergent, the 'shifted' pH–activity curve in 10 mM salt could be reproduced. Bovine serum albumin at a concentration of 250 $\mu g \cdot cm^{-3}$ did not alter the pH–activity curve from its 'shifted' low ionic strength position.

The fact that this shift in the pH--activity curve is only observed at low ionic strength is in agreement with the view that the adsorption of trypsin onto glass and silica surfaces is primarily due to an electrostatic attraction at the pH values used. The pH shift may be interpreted as being due to a lowering of the pH at the surface of the negatively charged glass, where the adsorbed enzyme is acting, by the electrostatic attraction of hydrogen ions, *i.e.* the 'surface' pH is lower than the 'bulk solution' pH. Similar effects have been observed with adsorbed[3] and immobilized enzymes[4, 5].

Surface effects may also seriously affect observed reaction kinetics. Thus the kinetics of the autolysis of α-chymotrypsin have been investigated by several workers, most of whom have observed first-order kinetics[6-9] for this reaction, but second-order kinetics were observed recently[10]. The results presented here confirm that the true 'solution' kinetics (*i.e.* in the absence of 'wall effects') of α-chymotrypsin autolysis are second order (at 30°C, pH 9.2) and also show that when α-chymotrypsin is significantly adsorbed onto glass or silica surfaces there is a much increased autolysis rate with apparent overall first-order kinetics.

In Fig. 2a, the second-order kinetic plots of $(\%\ \text{Activity})^{-1}$ *vs.* time for the autolysis of α-chymotrypsin at various concentrations in cellulose nitrate tubes show

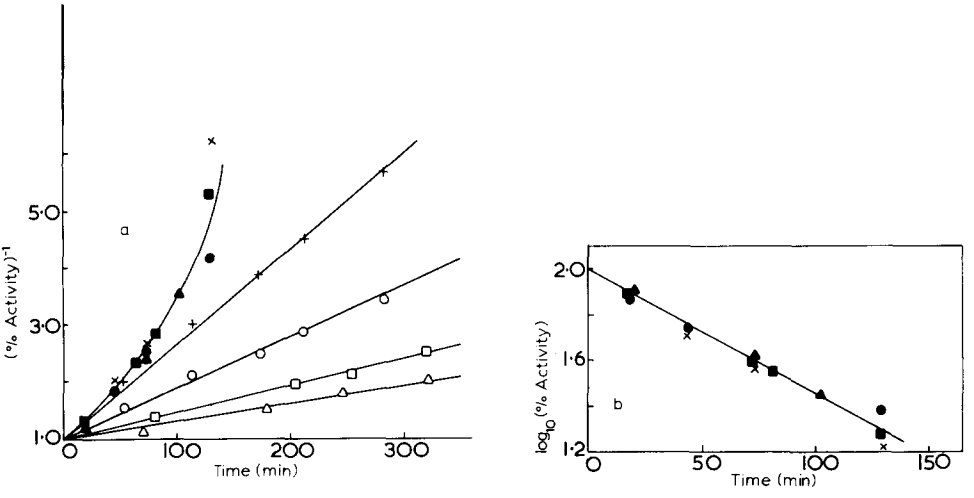

Fig. 2. Effect of colloidal silica particles on the autolysis of α-chymotrypsin at 30°C, pH 9.2, 0.1 M glycine. (a) Second-order plot. (b) First-order plot.

	Concentration (mg·cm⁻³)			*Concentration (mg·cm⁻³)*	
	Chymotrypsin	*SiO_2*		*Chymotrypsin*	*SiO_2*
○	0.1	0	▲	0.1	0.04
□	0.2	0	■	0.2	0.04
△	0.5	0	●	0.5	0.04
+	1.0	0	×	1.0	0.04

good linearity, with slopes proportional to initial concentration as expected (except at the lowest concentration of 0.1 mg·cm^{-3}, where the 'wall effect' must still be significant even in cellulose nitrate, the most inert material found for studying the autolysis reaction). The presence of 200 μg of colloidal silica spheres, (Syton, Monsanto Co.) approx. 20 nm diameter, (equivalent to total of about 240 cm^2 silica surface area) results in increased rates of autolysis with all the enzyme concentrations falling on the same curve, indicating first-order kinetics. The shape of the curve is confirmed as first order by the linear plot of log (% Activity) *vs* time shown in Fig. 2b. The silica surface area is small in relation to the α-chymotrypsin present, *i.e.* at the 0.2 mg·cm^{-3} concentration only about 10% of enzyme is adsorbed to form a monolayer. This demonstrates the much increased reaction rates at the surface as compared with those in solution.

When the autolysis is carried out in glass tubes with gentle stirring (conditions approaching those pertaining in the pH-stat, which has been used[8, 9] to follow the autolysis reaction) the results, shown in Fig. 3a, show that the rates for initial concentration of 0.1 and 0.2 mg·cm^{-3} are increased relative to those observed in cellulose nitrate tubes. Further, they deviate from linearity in the second-order plots and fall on the same curve, indicating apparent first-order kinetics. The linear log (% Activity) *vs* time plot in Fig. 3b

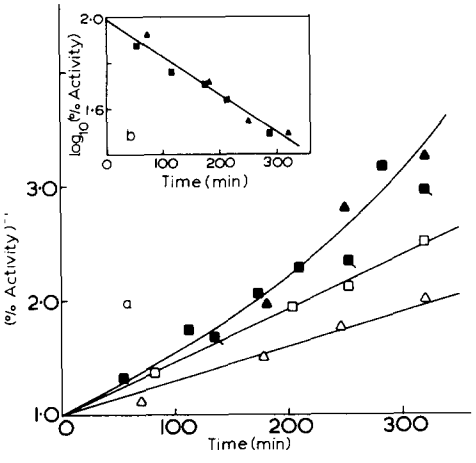

Fig. 3. Effect of different surfaces and of stirring on observed kinetics for autolysis of α-chymotrypsin at 30°C, pH 9.2, in 0.1 M glycine. (a) Second-order plot. (b) First-order plot.

	Chymotrypsin concn (mg·cm^{-3})	*Vessel material*	*Conditions*
△	0.1	Cellulose nitrate	Unstirred
□	0.2	Cellulose nitrate	Unstirred
▲	0.1	Glass	Stirred
■	0.2	Glass	Stirred
◥	0.2	Glass	Unstirred

confirms this. In glass tubes, but without stirring, the observed rates are non-linear and fall between those observed in cellulose nitrate and those in glass with stirring. Similar results have been obtained with the trypsin autolysis reaction.

Thus, it is concluded that the true 'solution' autolysis of α-chymotrypsin follows second-order kinetics, but that it is possible to observe apparent first-order kinetics in the presence of glass and silica surfaces. Stirring (and no doubt low ionic strength) increases the significance of the surface reaction and hence the tendency to show first-order kinetics. A further practical consequence of the results presented here is that care should be taken to avoid conditions in assay methods for trypsin and other basic enzymes [chymotrypsin (EC 3.4.4.5), papain (EC 3.4.4.10), lysozyme] such that adsorption of a significant fraction of the enzyme on the glassware occurs. Thus, particular care is required at low ionic strengths (< 0.02) and low enzyme concentrations ($< 5\ \mu g \cdot cm^{-3}$). In the case of typical pH-stat assays, the glass surface area in contact with 10 cm^3 of solution is of the order of 30 cm^2 and for trypsin, a concentration of 1 $\mu g \cdot cm^{-3}$ is just sufficient to cover this area completely with an adsorbed monolayer. Unless the enzyme concentration is greater than 5 $\mu g \cdot cm^{-3}$, the surface reaction may contribute significantly. For trypsin assay methods involving the hydrolysis of BAEE in a pH-stat, pH values of 7.6[11], 7.8[12,13] and 8.0[14] have been used: from Fig. 1 it can be seen that at pH 7.6, if adsorption on the glassware has largely occurred, the measured activity is only 60–65% the maximum activity rather than the 90–95% activity observed under conditions where adsorption is not significant.

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