The effects of surface and macromolecular interactions on the kinetics of inactivation of trypsin and α -chymotrypsin

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The autolysis of trypsin and α -chymotrypsin is accelerated in the presence of colloidal silica and glass surfaces. It is proposed that adsorption of the enzymes (favoured by electrostatic factors) results in a conformational change that renders the adsorbed enzyme more susceptible to proteolytic attack. Although the adsorbed enzymes are more susceptible to proteolysis, their activity towards low-molecular-weight substrates is not affected, indicating a relatively minor conformational change on adsorption. The rates of autolysis in solution (i.e. in 'inert' vessels) are second-order for both trypsin and α -chymotrypsin, with rate constants of $13.0 \text{ mol}^{-1} \cdot \text{dm}^3 \cdot \text{s}^{-1}$ for trypsin (in 50 mM-NaCl at pH 8.0 at 25°C) and $10.2 \text{ mol}^{-1} \cdot \text{dm}^3 \cdot \text{s}^{-1}$ for α -chymotrypsin (in 0.1 M-glycine at pH 9.2 at 30°C). In glass vessels or in the presence of small areas of silica surface (as colloidal silica particles), the autolysis of both trypsin and α -chymotrypsin can show first-order kinetics. Under these conditions, saturation of the surface occurs and the fast surface proteolytic reaction controls the overall kinetic order. However, when greater areas of silica surface are present, saturation of the surface does not occur, and, since for a considerable portion of the adsorption isotherm the amount adsorbed is approximately proportional to the concentration in solution, second-order kinetics are again observed. A number of negatively charged macromolecules have been shown similarly to increase the rate of autolysis of trypsin: thus this effect, observed initially with glass and silica surfaces, is of more general occurrence when these enzymes adsorb on or interact with negatively charged surfaces and macromolecules. These observations explain the confusion in the literature with regard to the kinetics of autolysis of α -chymotrypsin, where first-order, second-order and intermediate kinetics have been reported. A further effect of glass surfaces and negatively charged macromolecules is to shift the pH-activity curve of trypsin to higher pH values, as a consequence of the effective decrease in pH in the 'microenvironment' of the enzyme associated with the negatively charged surface or macromolecule.

It has become increasingly recognized that enzyme action in general may be much affected by the presence of various types of surfaces (McLaren & Packer, 1970; Wingard *et al.*, 1976). Many enzymes *in vivo* are localized on membrane surfaces, where the 'micro-environment' of the enzyme differs from that of the neighbouring aqueous phase and where conformational, electrostatic, steric and diffusional restrictions may be imposed. Immobilized or matrix-supported enzymes have been used as models for such systems as well as in applications as recoverable industrial catalysts (Wingard *et al.*, 1976).

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In a preliminary report (Johnson & Whateley, 1972) it was pointed out that, in the autolysis of both trypsin and α -chymotrypsin, surface effects could play a predominant part, not only accelerating the reaction but also controlling the kinetic order of the observed process. The present paper is concerned with a more detailed demonstration of such effects at silica surfaces, particularly for trypsin, although some results for α -chymotrypsin are also reported and these show features very similar to those for trypsin. In order to clarify the mechanisms involved, this work has been extended to a range of macromolecular colloidal systems and the kinetic experiments have been accompanied by adsorption studies.

The first indication of the effect of silica surfaces was observed when studying the interaction of trypsin with polymerizing silicic acid sols, the initial step in an enzyme-immobilization method (Johnson & Whateley, 1971); the rate of autolysis of trypsin was accelerated some 100-fold in the presence of 0.2% silicic acid sol. Since glass surfaces are chemically very similar to developing colloidal silica solutions, it seemed likely that normal glass containers might also have an effect on the rate of autolysis.

To a very large extent, possible surface effects have been ignored in the many studies of autolytic reactions. The absence of 'wall effects' was reported in the α -chymotryptic hydrolysis of acetyl-L-tyrosine hydroxamide, where, though a 20-fold increase in surface area was achieved by the addition of powdered glass or polyethylene beads, both buffer (0.3 M-Tris) and enzyme concentration (190 μ g/ml) were high, tending to mask any such effect (Bernhard & Niemann, 1955). On the other hand, 'wall effects' were noted in pH-stat measurements of the hydrolysis by α -chymotrypsin (0.4 μ g/ml) of acetyl-L-phenylalanine glycolamide ester in 10 mM salt (Applewhite *et al.*, 1958).

Since the classic study of the autolysis of trypsin by Kunitz & Northrop (1934), most workers (e.g. Fraser & Powell, 1950; Nord *et al.*, 1956; Gabel & Kasche, 1972, 1973) have observed second-order kinetics and noted the stabilizing action (against autolysis) of Ca^{2+} ions. However, there have been several observations of first-order (Nord *et al.*, 1956; Yon, 1959) or intermediate-order behaviour for trypsin or its close derivatives.

The autolysis of α -chymotrypsin resembles that of trypsin in that the presence of Ca^{2+} ions causes a stabilizing effect (Wu & Laskowski, 1956; Chervenka, 1959, 1962), and kinetics varying from first-order (Bender *et al.*, 1964; Erlanger *et al.*, 1964; Hofstee, 1965) to second-order (Kumar & Hein, 1970; Johnson & Whateley, 1972; Taylor *et al.*, 1973) have been observed.

Although in the present paper the trypsin system is mainly discussed, parallel experiments with α -chymotrypsin were also performed, and these demonstrate very similar general behaviour. To demonstrate the extent to which the effects reported here are of more widespread importance, the influence of a range of colloidal systems (mainly negatively charged macromolecules) on enzyme kinetics was also investigated.

Experimental

Materials

Trypsin (EC 3.4.21.4) (bovine; 3 $\times$ crystallized; salt-free) was obtained from Miles-Seravac, Maidenhead, Berks., U.K. (grade 4, batch 482). It is

known that such trypsin contains α - and β -trypsin as well as inactive material, but, since it has been shown that the kinetic parameters for α - and β -trypsin are very similar (Robinson *et al.*, 1971), no separation was made. α -Chymotrypsin (EC 3.4.21.1) (bovine; 3 $\times$ crystallized; salt-free) was also obtained from Miles-Seravac (grade 2A, batch 385A).

N- α -Benzoyl-L-arginine ethyl ester hydrochloride and acetyl-L-tyrosine ethyl ester were chromatographically homogeneous samples from BDH Chemicals, Poole, Dorset, U.K.

Repelcote (a 2% solution of dimethyldichlorosilane in CCl_4) was from Hopkin and Williams, Chadwell Heath, Essex, U.K.

Buffer salts and EDTA were of AnalaR grade from BDH Chemicals and solutions were prepared with triple-distilled water from a Pyrex glass apparatus.

Benzamidine hydrochloride was obtained from R. N. Emanuel, Wembley, Middlesex, U.K.

An 'oil-free' sample of Syton X30 (previously known as Syton 2X) was kindly supplied by Monsanto Chemicals, Ruabon, Wrexham, Wales, U.K. Syton, a monodisperse sol of non-porous silica spheres, stable to changes in pH, salt and sol concentration, is very similar to Ludox manufactured by Du Pont. The sample contained approx. 25% silica with a weight-average particle diameter of 19 nm (as shown by light-scattering experiments). Dilutions were made up in 50 mM-NaCl or in 50 mM-NaCl/10 mM-Tris/HCl buffer, pH 8.0. Where the presence of Ca^{2+} was required CaCl_2 was added to 2 mM, and where the complete absence of Ca^{2+} was essential the CaCl_2 component was replaced by 2 mM-EDTA.

Poly-L-glutamic acid (sodium salt) (mol.wt. approx. 105 000) was from Sigma Chemical Co., Poole, Dorset, U.K. (grade III). Poly-L-lysine hydrobromide (mol.wt. 70 000 and above) was from Sigma Chemical Co. Chondroitin sulphate (sodium salt) was from Sigma Chemical Co. (grade III). Poly(ethylene oxide) (mol.wt. 300 000) was from BDH Chemicals. Polyvinylpyrrolidone (mol.wt. 360 000) was from Sigma Chemical Co. Polyacrylic acid (mol.wt. 230 000) was from BDH Chemicals. Colloidal magnesium aluminium silicate (Veegum) was from R. T. Vanderbilt Co., New York, NY, U.S.A.

Assay of trypsin esterase activity

The spectrophotometric method of Schwert & Takenaka (1955) was used. This involves following the increase in absorbance at 254 nm when benzoyl-L-arginine ethyl ester is hydrolysed to benzoyl-L-arginine. Benzoyl-L-arginine ethyl ester (1 mM) in 0.1 M-Tris/HCl buffer, pH 8.0, containing 5 mM- CaCl_2 was the assay solution. The specific

activity of the trypsin was found to be 9600 benzoyl-L-arginine ethyl ester units/mg, taking the specific absorption coefficient of trypsin at 280 nm as $1.54 \text{ mg}^{-1} \cdot \text{ml} \cdot \text{cm}^{-1}$ (Robinson *et al.*, 1971). This value of the specific activity was used for determining initial trypsin concentrations from the esterase activity in autolysis experiments. Thus no allowance has been made for inactive trypsin initially present in the trypsin sample.

Assay of α -chymotrypsin esterase activity

The spectrophotometric method used was basically similar to that for trypsin; the hydrolysis of acetyl-L-tyrosine ethyl ester was followed by the decrease in absorbance at 237 nm. The substrate solution used, at 25°C, was acetyl-L-tyrosine ethyl ester (2 mM) in 0.1 M-Tris/HCl buffer, pH 7.0, containing 5 mM- CaCl_2 .

Rates of autolysis

Trypsin. To a suitable volume of buffer (50 mM-NaCl/2 mM-EDTA/10 mM-Tris/HCl buffer, pH 8.0) was added the amount of colloidal silica sol in the same buffer required to achieve the desired silica concentration in 10 ml (e.g. for a final concentration of 0.01% SiO_2 , 0.2 ml of 0.5% SiO_2 was used). After equilibration at $25 \pm 0.1^\circ\text{C}$, stock trypsin solution (10 mg/ml, in the same buffer but adjusted to pH 2–3) was added to give the required final trypsin concentration and a total volume of 10 ml. Immediately after the addition of the trypsin and after mixing by inversion, a sample (20–100 μl) was taken for spectrophotometric assay with benzoyl-L-arginine ethyl ester as indicated above. Further samples were taken for assay at suitable time intervals. A variety of tubes were used for performing the autolysis experiments: glass, polypropylene, cellulose nitrate and silicone-treated glass. Tubes were sealed either with Parafilm or with rubber bungs covered with Parafilm.

α -Chymotrypsin. Essentially similar methods were used as described for the autolysis of trypsin. α -Chymotrypsin autolysis experiments were performed at 30°C so that the results would be directly comparable with those of Kumar & Hein (1970), who found that the rate of autolysis showed a maximum at pH 9.2; this pH was used in the present work. The buffer solution was 1 mM-EDTA/0.1 M-glycine/NaOH buffer, pH 9.2.

Adsorption isotherm

Silica sols (Syton) in NaCl/ CaCl_2 /Tris buffer, pH 8.0, to which was added benzamidine (2 mM), were used. The silica concentration ranged from 0.005 to 0.1%. To 9.9 ml of the silica sol at $25 \pm 0.1^\circ\text{C}$ was added 0.1 ml of trypsin solution (1%) to bring the final trypsin concentration to 4 μM . After gentle inversion three times, the tubes were kept at 25°C for 30 min, samples were taken and

trypsin activity towards benzoyl-L-arginine ethyl ester was determined. (In a series of experiments to determine the equilibration time, similar results had been obtained when the sols were centrifuged after 20, 50 and 120 min.) The tubes were then centrifuged for 90 min at 25 000 rev./min in a Beckman model L instrument with a no. 40 rotor. This corresponded to an average centrifugal force of 41 000 g with a maximum of 56 000 g. The trypsin activity of the supernatant towards benzoyl-L-arginine ethyl ester was then determined. Autolysis was minimized in these experiments by the presence of Ca^{2+} ions and the trypsin inhibitor benzamidine. Other similar experiments were performed with silica sol concentrations of 0.02, 0.05 and 0.1% with a range of trypsin concentrations from 2 to 20 μM .

pH-activity curves

pH-activity curves were measured in a Radiometer TTT1C pH-stat under an N_2 atmosphere at 25°C with the glass vessel and electrodes silicone-treated. Substrate was 50 mM-benzoyl-L-arginine ethyl ester, and 10 mM-NaCl was present. Trypsin concentration was typically 0.4 μM .

Results and discussion

Trypsin autolysis in solution, in silica sols and on surfaces

The inactivation of trypsin in solution under the conditions used in the present work has been shown previously to be due to autolysis (Kunitz & Northrop, 1934; Nord *et al.*, 1956; Gabel & Kasche, 1972, 1973).

Syton colloidal silica sols were used in the present work to avoid the problem of changes in the state of polymerization (due to variations in the pH, salt and sol concentration) that occur with silicic acid solutions. In the presence of 0.2% Syton and 2 mM- CaCl_2 and with an initial trypsin concentration of 4.2 μM , a linear second-order plot was obtained with an apparent second-order rate constant of $37 \text{ mol}^{-1} \cdot \text{dm}^3 \cdot \text{s}^{-1}$. Under similar conditions, but in the absence of colloidal silica, the rate of autolysis was extremely low; after 6 h the activities of the solutions were not significantly different from their initial values. To achieve measurable and reproducible rates for the autolysis reaction in the absence of colloidal silica, experiments were performed in the absence of Ca^{2+} ions. All of these initial experiments were performed in glass (Pyrex) centrifuge tubes (10 cm $\times$ 1.3 cm internal diam.). The second-order-type plots for the autolysis of trypsin (in the absence of colloidal silica) at various initial concentrations of trypsin from 1.5 to 10 μM in 50 mM-NaCl/2 mM-EDTA/10 mM-Tris/HCl buffer, pH 8.0, at 25°C are shown in Fig. 1. The plots are not linear and their shape approaches that for a first-order reaction when plotted in this manner. However, the

Fig. 1. Plot of the reciprocal of relative activity versus time for the autolysis of trypsin at 25°C in Pyrex glass tubes

The buffer used for the autolysis was 50mM-NaCl/2mM-EDTA/10mM-Tris/HCl buffer, pH 8.0. Initial trypsin concentration: $\square$, $\blacksquare$ and $\square$, 1.6 μM ; $\circ$, 1.9 μM ; $\bullet$, 2.0 μM ; $\blacktriangle$, 4.4 μM ; $\triangle$, 4.5 μM ; $\blacklozenge$, 5.1 μM ; $\diamond$, 5.6 μM ; ∇ , 9.7 μM ; $\blacktriangledown$, 10.0 μM .

curves for the different initial concentrations are not coincident (as required for a first-order reaction), i.e. the overall order of reaction lies between first and second.

In view of the greatly increased rate of trypsin autolysis in the presence of colloidal silica particles, there was the possibility that glass surfaces caused a similar effect. Any such 'wall effect' due to an increased rate of autolysis at the glass surface would be accentuated by stirring. Second-order plots with and without stirring are shown in Fig. 2(a). In both cases the initial trypsin concentration was at or near 2 μM and the solution was stirred with a Perspex rod of rectangular cross-section (approx. 7 mm $\times$ 3 mm) at a rate of 1–3 rev./s.

Reactions in inert vessels

In attempts to find a suitable tube material that did not affect the rate of autolysis of trypsin, similar autolysis experiments were performed with polypropylene, cellulose nitrate and silicone-treated glass tubes. With the polypropylene and cellulose nitrate tubes autolysis runs were also performed in the presence of added fine particles of these same

Fig. 2. (a) Effect of stirring on the autolysis of trypsin in Pyrex glass tubes and (b) effect of stirring and added tube material on the autolysis of trypsin in cellulose nitrate tubes

The buffer and general conditions were as indicated for Fig. 1. (a) Initial trypsin concentration: $\bullet$, 1.9 μM , no stirring; $\blacklozenge$, 2.0 μM , no stirring; $\blacksquare$, 2.0 μM , with stirring. (b) Initial trypsin concentration: $\blacksquare$, 1.9 μM , no stirring; $\blacklozenge$, 1.8 μM , no stirring; $\blacktriangle$, 1.8 μM , with stirring; $\bullet$, 1.8 μM , cellulose nitrate particles added.

materials, to increase significantly the surface area in contact with the solution. With polypropylene there was an increase in the rate of autolysis with added polypropylene surface area and with stirring, similar to the effect observed in glass tubes (Fig. 2a). Both cellulose nitrate and silicone-treated glass tubes were 'inert', the autolysis of trypsin showing a linear

second-order rate plot in the absence of stirring (see Fig. 2*b*), and the effect of stirring was small compared with the effect of stirring in untreated glass tubes (see Fig. 2*a*).

The second-order plots for the autolysis of trypsin at various initial concentrations in cellulose nitrate tubes in NaCl/EDTA/Tris buffer, pH 8.0, at 25°C are shown in Fig. 3. The values of the apparent second-order rate constant, k_{app} , for the three highest initial concentrations of 4.5, 6.4 and 8.0 μM show good agreement, being 12.8, 12.6 and 13.5 $\text{mol}^{-1} \cdot \text{dm}^3 \cdot \text{s}^{-1}$ respectively, with a mean value of 13.0 $\text{mol}^{-1} \cdot \text{dm}^3 \cdot \text{s}^{-1}$. At the lower initial trypsin concentrations, k_{app} shows increased values, i.e. 16 and 21 $\text{mol}^{-1} \cdot \text{dm}^3 \cdot \text{s}^{-1}$ for initial trypsin concentrations of 3.2 and 1.9 μM respectively. This increase in the value of k_{app} at low initial enzyme concentrations (less than 4 μM) may be due either to a change in the mechanism and kinetics of the reaction at low enzyme concentrations (although the second-order rate plots show good linearity) or, more likely, to a small wall effect on cellulose nitrate surfaces that only becomes apparent at low enzyme concentrations. The small effect of stirring, evident in Fig. 2*b*), supports the latter view.

It is difficult to compare these results with other results given in the literature because of the wide variation in conditions of pH, temperature, buffer

and enzyme concentrations used, as well as type of reaction vessel surface. The only results at pH 8 and 25°C are those of Buck *et al.* (1962), who found a value of 8.3 $\text{mol}^{-1} \cdot \text{dm}^3 \cdot \text{s}^{-1}$ in 0.1 M-borate buffer, and those of Gabel & Kasche (1972, 1973), from whose Arrhenius plots a value of 2.5 $\text{mol}^{-1} \cdot \text{dm}^3 \cdot \text{s}^{-1}$ (at pH 8.1 and in 10 mM-borate buffer) can be estimated. In 0.1 M-NaCl/2 mM-EDTA/10 mM-borate buffer, pH 8.0, at 25°C we have found $k_{app} = 1.95 \text{ mol}^{-1} \cdot \text{dm}^3 \cdot \text{s}^{-1}$, whereas under similar conditions with the substitution of 10 mM-Tris buffer for the 10 mM-borate buffer $k_{app} = 13.5 \text{ mol}^{-1} \cdot \text{dm}^3 \cdot \text{s}^{-1}$. Thus it appears that borate acts as an inhibitor to the autolytic reaction; boric acid has been shown to act as an inhibitor of both the serine hydrolases chymotrypsin and *Streptomyces griseus* proteinase 3 (Bauer & Pettersson, 1974).

Possessing now a relatively inert surface, it was possible for us to introduce controlled areas of silica surface. In the presence of 0.001% Syton colloidal silica, in cellulose nitrate tubes, and in the absence of Ca^{2+} , the rate of autolysis of trypsin was increased, and in the second-order-type rate plots the results for all the initial enzyme concentrations fell approximately on the same curve (Fig. 4), indicating

Fig. 3. Autolysis of trypsin in cellulose nitrate tubes Buffer and general conditions were as indicated for Fig. 1. Initial trypsin concentration: ■ and □, 1.9 μM ; ● and ○, 3.2 μM ; ▲, 4.5 μM ; ◆, 6.4 μM ; ▼, 8.0 μM . Lines were fitted by least-squares regression.

Fig. 4. Effect of Syton colloidal silica (1 mg/100 ml) on the autolysis of trypsin in cellulose nitrate tubes Buffer and general conditions were as indicated for Fig. 1. Initial trypsin concentration: ■, 2.2 μM , silica absent; ●, 5.5 μM , silica absent; ▲, 11.0 μM , silica absent; □, 2.2 μM , silica present; ◆, 5.5 μM , silica present; ▼, 11.0 μM , silica present. Straight lines were fitted by least-squares regression.

first-order kinetics. The shape of the curve was confirmed as first-order by the linear plot of $\log(\% \text{ activity})$ versus time, as shown in Fig. 5. Results obtained in the absence of colloidal silica are included in Fig. 4 for comparison. The amount of colloidal silica present in 10 ml of reaction mixture was $100\mu\text{g}$ (10 ml of 0.001% SiO_2), which corresponds to a silica surface area of about 120cm^2 for silica spheres of diameter 19 nm (determined by light-scattering). This silica surface area was small in relation to the area that the trypsin present could cover as a monolayer (see below: approx. $0.25\text{m}^2/\text{mg}$); at the lowest initial enzyme concentration ($2.2\mu\text{M}$) only about 10% of the trypsin could be adsorbed to form a monolayer. Since there was an increased rate of autolysis of trypsin in the presence of a relatively small area of silica surface as well as a change in the observed kinetics from second-order to first-order, it follows that the surface reaction must be relatively fast.

When much higher concentrations of colloidal silica are used, Ca^{2+} has to be present (usually 2 mM- CaCl_2) to lower the rates of autolysis to conveniently measurable values. In the absence of Ca^{2+} (i.e. in the presence of 2 mM-EDTA) a value of k_{app} of $310\text{mol}^{-1}\cdot\text{dm}^3\cdot\text{s}^{-1}$ was found in the presence of 0.1% colloidal silica, the initial trypsin

Fig. 5. Data of Fig. 4 plotted in first-order form. Symbols are as defined in Fig. 4. Lines were fitted by least-squares regression.

concentration being $3.2\mu\text{M}$. The presence of 2 mM- CaCl_2 decreased this value to $43\text{mol}^{-1}\cdot\text{dm}^3\cdot\text{s}^{-1}$. As mentioned above, in the presence of 2 mM- CaCl_2 the rate of autolysis of trypsin in solution with no added colloidal silica was negligible. Fig. 6 shows some

Fig. 6. Effect of colloidal silica concentration on the autolysis of trypsin at 25°C in cellulose nitrate tubes. The buffer used for the autolysis was 50 mM- $\text{NaCl}/2\text{ mM-}\text{CaCl}_2/10\text{ mM-Tris}/\text{HCl}$ buffer, pH 8.0. Initial trypsin concentration: ∇ , $4.6\mu\text{M}$, silica concentration 10 mg/100 ml; $\bullet$, $4.5\mu\text{M}$, silica concentration 20 mg/100 ml; $\blacktriangle$, $4.2\mu\text{M}$, silica concentration 0.1 g/100 ml; $\blacksquare$, $4.1\mu\text{M}$, silica concentration 0.5 g/100 ml; $\blacklozenge$, $4.0\mu\text{M}$, silica concentration 2.0 g/100 ml. Lines were fitted by least-squares regression.

Fig. 7. Plot of apparent second-order rate constant, k_{app} , versus $\log(\% \text{ SiO}_2)$ for the autolysis of trypsin in solution in the presence of Syton colloidal silica.

Buffer and general conditions were as indicated for Fig. 6.

second-order-type plots for the autolysis of trypsin (initial concentrations in the range $4.0\text{--}4.6\mu\text{M}$) in the presence of concentrations of Syton from 0.01 to 2.0%. In all these cases linear second-order plots were found, and the values of k_{app} are plotted versus the silica concentration in Fig. 7. There is a clear maximum in the rate of autolysis at a concentration of silica near 0.1% with a value of k_{app} of $43\text{ mol}^{-1}\cdot\text{dm}^3\cdot\text{s}^{-1}$. To help in the interpretation of these results, the isotherm for the adsorption of trypsin on Syton colloidal silica was determined under similar conditions.

Adsorption experiments and the interpretation of kinetic results

The uptake of trypsin by the silica surfaces was monitored by the loss of enzymic activity in the supernatant liquid after centrifuging. To prevent autolysis during these experiments it was confirmed that 2 mM-benzamidine (a competitive inhibitor with $K_i = 1.66 \times 10^{-5}\text{ M}$) was effective (Mares-Guia, 1968), though it did not affect the rate of hydrolysis of benzoyl-L-arginine ethyl ester by trypsin in the spectrophotometric assay method. The concentration of benzamidine in the assay cell was typically $70\mu\text{M}$ (i.e. 4 times K_i), whereas the concentration of the substrate, benzoyl-L-arginine ethyl ester, was 1 mM, which is approx. 230 times its K_m value of $4.3 \times 10^{-6}\text{ M}$ (Baines *et al.*, 1964). It was also shown that the presence of low concentrations of colloidal silica did not affect the spectrophotometric assay. As shown in Fig. 8, the adsorption isotherm approximated to the Langmuir type, with a limiting adsorption of 0.48 mg of trypsin adsorbed/mg of SiO_2 or a coverage of $0.25\text{ m}^2/\text{mg}$. The close packing of spheres of diameter 3 nm would correspond to $0.28\text{ m}^2/\text{mg}$.

These adsorption experiments also served to resolve certain questions of mechanism. The presence of the inhibitor benzamidine at the active site of trypsin is most unlikely to affect the adsorption properties of the enzyme. Its susceptibility to surface denaturation is also unlikely to be affected greatly. If surface denaturation of adsorbed trypsin were responsible for the loss in activity, then the presence of the inhibitor should not decrease this rate of activity loss so greatly. In the presence of the inhibitor there was no loss in activity of trypsin in the presence of colloidal silica; thus surface denaturation is unlikely to be responsible for the loss in activity. This conclusion was confirmed by experiments at pH 4.0; at this pH the activity of trypsin is negligible, and thus autolysis will not occur. However, the adsorption of trypsin on silica is likely to be not very dissimilar at this pH value from that at pH 8, as both pH values lie between the isoelectric points of silica and trypsin. Both with and without colloidal silica the loss in activity at pH 4.0

Fig. 8. Isotherm for the adsorption of trypsin on Syton colloidal silica in 50 mM-NaCl/2 mM- CaCl_2 /10 mM-Tris/HCl buffer, pH 8.0, containing 2 mM-benzamidine at 25°C

was not significant after 5 h. It seems unlikely therefore that surface denaturation of trypsin is involved in these experiments.

To explain these observations, it is postulated that trypsin adsorbed on silica or glass surfaces is very much more susceptible to proteolytic attack than is trypsin in solution. This increased susceptibility is probably due to a change in conformation of the enzyme on adsorption, leading to a 'deformed' or 'loosened' conformation. An analogous but opposite effect on trypsin is caused by Ca^{2+} : in the absence of Ca^{2+} trypsin adopts a 'loose' conformation that is much more susceptible to proteolytic attack than is the 'tight' conformation adopted in the presence of Ca^{2+} (Gabel & Kasche, 1973). The presence of Ca^{2+} has only a slight effect on the activity of trypsin towards the low-molecular-weight substrates but has a large effect on the susceptibility of trypsin to autolysis. Similarly, the adsorption of trypsin on a silica surface has no detectable effect on the activity of trypsin towards the low-molecular-weight substrate benzoyl-L-arginine ethyl ester, but greatly increases its susceptibility to proteolytic attack; this occurs both in the presence and in the absence of Ca^{2+} . Thus the conformational change caused by trypsin adsorption on silica can be only a small 'deformation' or 'loosening' of the tertiary conformation. This effect is analogous to the co-operative unfolding transition found by Gabel &

Kasche (1972) at 40–45°C in β -trypsin, where activity is also retained.

When the autolysis of trypsin was investigated in glass tubes, curved second-order-type plots were obtained (Fig. 1); the glass surface area in contact with the solution was about 30 cm². In the presence of 120 cm² of silica surface and similar trypsin concentrations, much more rapid first-order kinetics were observed (Figs. 4 and 5). At such concentrations of adsorbate, the adsorption of trypsin on silica approaches the constant surface coverage region of the Langmuir isotherm. Thus the substrate concentration (adsorbed trypsin) remains constant and the rate of the surface reaction becomes proportional to the bulk enzyme concentration, i.e. a first-order reaction. Since the overall kinetics are also first-order, it is clear that the bulk second-order process is much slower than the surface reaction under these conditions. At the lower values of silica or glass surface area, e.g. 30 cm² in glass tubes, the first-order reaction contributes much less and the second-order solution is now appreciable. The observed kinetics thus fall between first-order and second-order. On the other hand, at much higher surface areas (as at the SiO₂ concentrations of Fig. 6) the amount of trypsin adsorbed varies with trypsin concentration and first-order kinetics are not to be expected (see below). Rather, since at low surface coverages of the Langmuir isotherm the amount adsorbed is approximately proportional to concentration in solution, we may expect second-order kinetics, as is shown.

The mechanism for the autolysis of trypsin in the presence of silica or glass surfaces may be summarized as shown in Scheme 1.

The digestion of trypsin in solution is negligibly slow in the presence of Ca²⁺ (under the conditions

of pH, temperature and enzyme concentration considered here). In the absence of Ca²⁺ the rate of autolysis in solution is no longer negligibly slow but the rate at the surface is much faster.

The occurrence of a maximum in the plot of k_{app} versus SiO₂ concentration still requires explanation. From the adsorption experiments at similar concentrations of trypsin and silica, the relative amounts of trypsin adsorbed and in solution could be determined. Table 1 gives such values and also indicates the percentage of surface covered. The mechanism proposed predicts that, as the rate of autolysis is proportional to $[T][T \text{---} \text{SiO}_2]$, i.e. the product of the concentration of trypsin in solution and the concentration of adsorbed trypsin (autolysis in solution may be neglected in the presence of Ca²⁺), then, for a given amount of trypsin, the rate should be maximal for 50% trypsin in solution and 50% adsorbed trypsin. The results in Table 1 show

Table 1. Autolysis of trypsin in the presence of colloidal silica

Conditions were as described for Fig. 6. The initial trypsin concentration was approx. 4 μ M.

Concn. of colloidal silica (%)	Rate constant k_{app} (mol ⁻¹ · dm ³ · s ⁻¹)	Trypsin in solution (%)	Trypsin adsorbed (%)	% of SiO ₂ surface covered
0.01	12	62	38	76
0.02	30	40	60	60
0.05	39	22	78	31
0.1	43	17	83	17
0.2	35	9.9	90.1	9
0.5	20	5.6	94.4	4
1.0	14	2.2	97.8	2
2.0	10	1.3	98.7	1

Scheme 1. Autolysis of trypsin (T) in the presence of silica or glass surfaces

that the maximum rate of autolysis is attained with approx. 80% trypsin adsorbed and 20% in solution. However, the simple theory does not take into account many factors, e.g. the effect of surface coverage on the rate of reaction (at high surface coverages, approaching saturation or monolayer coverage, adsorbed trypsin might be somewhat less susceptible to proteolytic attack owing to shielding by adjacent molecules). Light-scattering (total intensity and photon correlation) and viscosity measurements have shown that trypsin can cause flocculation of Syton colloidal silica sols: this could distort the simple model proposed.

Similar effects in other systems

(a) *Other proteolytic enzymes: α -chymotrypsin autolysis in silica sols and on surfaces.* Conditions of pH and temperature (1 mM-EDTA/0.1 M-glycine/NaOH buffer, pH 9.2, at 30°C) were chosen to allow meaningful comparison with the results of the work of Kumar & Hein (1970). The results on the effect of silica sols and glass surfaces on the autolysis of α -chymotrypsin have been presented in a preliminary communication (Johnson & Whateley, 1972) and are very similar to those found for trypsin autolysis. In solution, in inert tubes (cellulose nitrate or silicone-treated glass) good second-order linear rate plots were obtained with values of k_{app} of 10.8, 9.8 and 9.6 mol⁻¹·dm³·s⁻¹ (mean value 10.2) for initial α -chymotrypsin concentrations of 6.4, 15.4 and 32 μ M. In the absence of EDTA Kumar & Hein (1970) obtained a value of 3.9 mol⁻¹·dm³·s⁻¹, under otherwise similar conditions. The effect of a small amount of Syton colloidal silica was similar to the effect on trypsin autolysis, i.e. more rapid first-order kinetics were observed.

With untreated glass the autolysis was faster, the second-order plots deviated from linearity and, with stirring, the rates were further increased. The plots fell on the same curve for initial enzyme concentrations of 3.2 and 6.4 μ M, indicating apparent first-order kinetics. These conditions of stirring in glass are similar to those of the pH-stat experiments of Hofstee (1965) and Ingles & Knowles (1966), where first-order kinetics were found.

(b) *Presence of macromolecules.* To determine whether the effects found with negatively charged glass and silica surfaces were of more general occurrence, the effects of a number of macromolecular materials (in particular, negatively charged macromolecules) on the kinetics of autolysis of trypsin were investigated.

Under standard conditions (50 mM-NaCl/2 mM-EDTA/10 mM-Tris/HCl buffer, pH 8.0, at 25°C, initial trypsin concentrations approx. 8 μ M) and in the absence of any added material, the apparent second-order rate constant k_{app} for autolysis was 13.5 mol⁻¹·dm³·s⁻¹.

Poly-L-glutamic acid (mol.wt. approx. 105 000) at a concentration of 2 mg/ml increased K_{app} to 19.5 mol⁻¹·dm³·s⁻¹. At the lower salt concentration of 5 mM-NaCl, k_{app} was increased to 105 mol⁻¹·dm³·s⁻¹. The importance of electrostatic interactions is confirmed by these results.

With poly-L-glutamic acid and with all the systems studied, it was shown that denaturation was not the cause of the increased rate of activity loss (by showing that the rate of activity loss was not increased by the addition of the macromolecular material when the competitive inhibitor of trypsin, benzamidine, was present). It was also shown in all cases that the presence of the added material did not affect the spectrophotometric assay of trypsin with benzoyl-L-arginine ethyl ester.

Chondroitin sulphate, at a concentration of 1 mg/ml, had no effect when the salt concentration was 0.1 M, but with 10 mM-NaCl k_{app} was increased to 74 mol⁻¹·dm³·s⁻¹.

Polyacrylic acid (mol.wt. 230 000) at a concentration of 1 mg/ml in 10 mM-NaCl similarly increased k_{app} to 34 mol⁻¹·dm³·s⁻¹.

The finding that a colloidal suspension of magnesium aluminium silicate (1 mg/ml) increased k_{app} to 50 mol⁻¹·dm³·s⁻¹ (in 10 mM-NaCl) confirms the view that colloidal silica is not the only colloidal suspension to increase the rate of autolysis.

The two uncharged macromolecules poly(ethylene oxide) (mol.wt. 300 000) and polyvinylpyrrolidone (mol.wt. 360 000) had no effect on the rate of autolysis of trypsin. Similarly the positively charged macromolecule poly-L-lysine (mol.wt. 70 000 and above) had no significant effect on the rate of autolysis.

Thus the increased rate of autolysis of trypsin and α -chymotrypsin occurs not only on adsorption or interaction with silica and glass surfaces, but also on interaction with negatively charged macromolecules, and probably on interaction with negatively charged particles and surfaces in general.

pH-activity-curve shifts due to interaction with macromolecules

A further effect of surfaces on enzyme kinetics, preliminary results of which have been reported previously (Johnson & Whateley, 1972), is the shift in the pH-activity curve when the enzyme becomes adsorbed on a glass surface. This pH-activity-curve shift, well known for immobilized enzymes, was shown to occur under conditions used for trypsin and α -chymotrypsin assays in the pH-stat.

Measurements of the pH-activity curve for trypsin in the presence of macromolecules has shown that this pH-activity-curve shift is a general phenomenon for trypsin in the presence of negatively charged macromolecules. Chondroitin sulphate, polyacrylic acid and poly-L-glutamic acid, all in

10mM-NaCl at 100 μ g/ml, cause pH-activity-curve shifts to higher pH values of 0.6, 0.5 and 0.8 respectively (measured at the 50%-activity level). The electrodes and vessels of the pH-stat were silicone-treated to prevent adsorption of trypsin on these components. Douzou & Balny (1977) have reported a similar pH-activity-curve shift for trypsin in the presence of DNA, also in 10mM-NaCl. The pH-activity curve for lysozyme was also found to be shifted on interaction with the polyanionic bacterial cell wall (Maurel & Douzou, 1976).

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