Stoichiometric complexation of bovine trypsin with potassium poly(vinyl alcohol sulphate) and enzymatic activity of the complex

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The complexation of bovine trypsin with potassium poly(vinyl alcohol sulphate) (KPVS) was investigated at different pHs by means of colloid titration. The number of moles of the basic (amino, imidazolyl, and guanidyl) groups in 1 g of trypsin, which were bound to the sulphate groups in KPVS by salt linkages, was evaluated from the titration data. From a comparison of the results obtained with the number of basic groups counted from the amino acid sequence already reported, it was found that the complexation follows a stoichiometric relationship in the range pH <3 where all the basic groups in trypsin are protonated. The enzymatic activity of the resulting stoichiometric complex was examined by using $N\alpha$-benzoyl-DL-arginine-*p*-nitroanilide and casein as substrates. The complexed enzyme showed appreciable retention of activity, not only towards the low molecular weight substrate but also towards the polymeric substrate. Therefore, it became apparent that the stoichiometric complexation of trypsin with KPVS does not have a large influence on conditions for the active site of the enzyme, because the salt linkages for maintaining the structure of the complex are very loose.

(Keywords: complexation; bovine trypsin; potassium poly(vinyl alcohol sulphate); colloid titration; enzymatic activity)

INTRODUCTION

Colloid titration is a useful method for obtaining information about the stoichiometry of polyelectrolyte complex formation through salt linkages between the oppositely charged groups of polyions[1,2]. Previously, we applied this titration technique to study the salt linkage formation of flexible synthetic polyelectrolytes with several non-flexible substances having irregularly distributed charged groups: proteins[3–5], ion exchangers[6] and charged colloid particles[7,8]. Evidence has accrued which indicates that the fixed charges of a flexible polyion stoichiometrically form salt linkages or ion pairs, with the opposite charges being irregularly located in the global protein molecule or on the colloid surface. This could suggest that electrical neutralization between the oppositely charged groups follows a stoichiometric relationship even if they lie quite far apart[8].

For protein molecules, it is important to learn how such salt linkage formation affects their three-dimensional conformation and also the configuration of a biologically active site. Enzymes could serve to elucidate this problem because of possible investigations on their catalytic activities towards different substrates after complexing with polyions. Bovine trypsin was used here in view of the available information on the amino acid sequence[9–11], the three-dimensional conformation[12,13], the structure of the active site[14,15], enzymological properties[11] and changes in the enzyme activity before and after binding to a suitable supporting matrix[16,17]. Potassium poly(vinyl alcohol sulphate) (KPVS) was used for the complexation with trypsin since its polyelectrolyte properties have been investigated in detail[18]. The stoichiometry of the salt linkage formation between the sulphate groups in KPVS and the basic groups in trypsin was studied as a function of pH by colloid titration. The resulting complex was examined by potentiometric titration, by infra-red spectroscopy and by measuring its enzyme activities towards low and high molecular weight substrates.

EXPERIMENTAL

Materials

Trypsin (from bovine pancreas, Sigma type XIII) was used after the purity was confirmed by amino acid analysis and electrophoresis in polyacrylamide gel. The active site titration with *p*-nitrophenol-*p'*-guanidinobenzoate showed that 1 mg of trypsin yields $3.91 \pm 0.17 \times 10^{-5}$ mmol of liberated *p*-nitrophenol (this value corresponds to $91 \pm 4\%$ of the theoretical value calculated on Walsh's data[11] concerning the amino acid sequence of bovine trypsin). Casein and $N\alpha$-benzoyl-DL-arginine-*p*-nitroanilide (BANA) were purchased from Sigma Chemical Co. (USA). KPVS was the same sample as used previously[2–5,18]: number average degree of polymerization, 1.5×10^3; equivalent weight, 166; degree of esterification, 0.924. All other reagents were of analytical grade and obtained from commercial sources. Carbonate-free distilled water was used in all the experiments.

Preparation of complexed trypsin

The trypsin–KPVS complex was prepared at pH 2.7 ± 0.2 because the complexation at this pH followed a stoichiometric relationship. The samples used for all the measurements were obtained by repetition of the colloid titration procedure (see the next section), using enzyme solution (167 ml containing 25 mg trypsin) and KPVS solution (0.002 49 N, as expressed in amounts of

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sulphate groups). The addition of the KPVS solution into the gently stirred enzyme solution was automatically stopped when the end point of the titration was reached. The resulting complex was then separated by centrifugation, sufficiently washed with distilled water and, if necessary, dried at 50°C under reduced pressure. No dissolution of trypsin was observed even when a wetting complex was vigorously stirred for 24 h in the same buffer solutions as used in the activity measurements. The sulphur content of the complex was 3.69%, as established by elemental analysis. This value was in fair agreement with that (3.73%) calculated by assuming a stoichiometric complexation.

Colloid titration

The titration was done at 25±0.1°C in a nitrogen atmosphere, using a Hirama automatic recording titrator. Trypsin (1–6 mg) was dissolved in 40 ml of distilled water and then the pH of the solution was adjusted with 0.1–1 N HCl or NaOH. This preparation was titrated with 0.002 49 N KPVS solution previously adjusted to the pH of the preparation. The end point of the titration was indicated by measuring turbidity at 420 nm (References 1–5).

Potentiometric titration

The dissociation of acidic groups remaining in the stoichiometric complex was examined by potentiometric titration. The complex (27.75 mg containing 25 mg of trypsin) was dispersed into 80 ml of distilled water and titrated with 0.0190 N NaOH at 25±0.1°C in a nitrogen atmosphere, using a Beckman digital pH meter (model 4500) and a microburette with a precision of ±0.005 ml.

I.r. spectroscopy

Infra-red (i.r.) spectra of native and complexed trypsin were investigated to obtain information about the formation of the salt linkages between the basic groups in the enzyme and the sulphate groups in KPVS. The complexed enzyme was washed with a NaOH solution (pH 10.8), lyophilized and then dried at 50°C for 1 d under reduced pressure. The uncomplexed native enzyme as a control sample was obtained by lyophilizing an aqueous enzyme solution adjusted to pH 10.8 with NaOH. The spectral measurements for both samples in a KBr disk were made with a Hitachi 2500-50 i.r. spectrophotometer.

Enzymatic studies

The activities of native and complexed trypsin were assayed at different pHs, using both BANA as a low molecular weight substrate and Hammarsten casein as a high molecular weight substrate. Various buffer solutions at an ionic strength of 0.1 were used: pH <5, acetate; pH 5–8, phosphate; pH >8, carbonate. Regardless of the native or the complexed enzyme, the net protein concentrations in the assay systems were fixed: 0.04 mg ml^{-1} for the assay with BANA and 0.033 mg ml^{-1} for the assay with casein. The hydrolytic reaction of BANA was initiated by quick mixing of the substrate solution (10 ml), ranging in concentration from 0.55 to 2.66 mM, with 30 ml of the enzyme solution or the complexed enzyme suspension, which had previously been thermostated at 30°C. The formation of *p*-nitroaniline was followed at 410 nm with a Hitachi model 220-20 spectrophotometer. When 4% (w/v) casein was used, a mixture of the substrate solution (10 ml) with the enzyme solution or the complex suspension (30 ml each) was incubated at 30°C to make the digestion reaction proceed. The reaction was stopped at suitable time intervals (5–30 min) by adding 5 ml of 70% (w/v) trichloroacetic acid, and the protein remaining in the supernatant solution was spectrophotometrically measured at 280 nm.

RESULTS AND DISCUSSION

Complexation

The complexation was studied by means of colloid titration of trypsin with KPVS under various conditions of pH and protein concentration. The end point of the titration was determined by monitoring a turbidity change[1–5] due to the formation of a water-insoluble complex (*Figure 1*). No residual trypsin was detected in the supernatant solution after the titration by the usual dye-binding protein assay method using Coomassie brilliant blue G-250. In addition, as shown in *Figure 2*, the titrant volume at the end point is exactly proportional to the protein weight of the sample solutions at different pHs. It was thus found that the complex consisting of KPVS and trypsin is formed quantitatively.

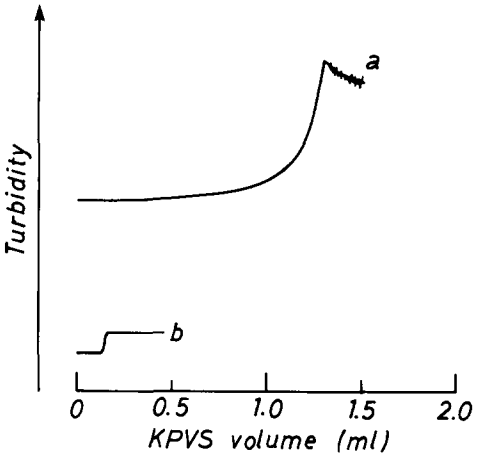

Figure 1 Typical turbidimetric titration curves of trypsin with 0.002 49 N KPVS titrant at (a) pH 2.5 and (b) 8.8. The sample solution (40 ml) containing 4.0 mg trypsin was subjected to the titration. The titration curve was not represented by absolute turbidity because the change in turbidity was measured with an automatic recording titrator

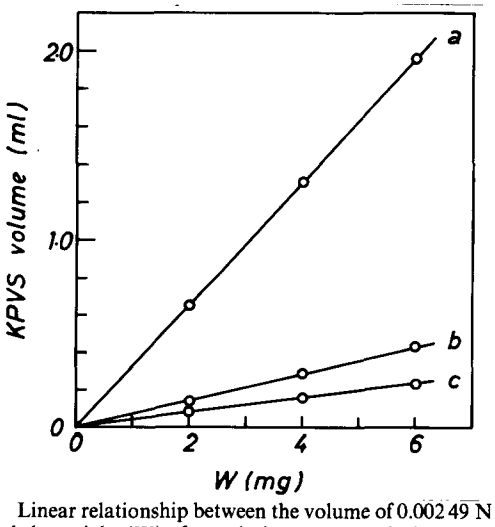

Figure 2 Linear relationship between the volume of 0.002 49 N KPVS titrant and the weight (*W*) of trypsin in aqueous solutions at different pHs: (a) 2.5; (b) 7.5; (c) 8.8

The resulting complex was satisfactorily washed with an aqueous NaOH solution adjusted to pH 10.8, dried *in vacuo*, and subjected to i.r. spectroscopy. Washing with the alkaline solution was done to remove the protons from the protonated basic groups in trypsin, which were free of salt linkages with the KPVS anions. A control sample used in the i.r. measurement was prepared by lyophilizing an alkaline solution (pH 10.8) of trypsin. *Figure 3* compares the spectra of the complexed and native enzymes. The absorption (shoulder) at 2700–2400 cm^{-1}, assigned to amine salts (mainly $-NH_3^+$), was observed in the complexed but not in the native enzyme. This reveals that the complexation takes place through the formation of the salt linkages between the protonated basic groups in trypsin and the sulphate ions in KPVS.

Stoichiometry of the complexation

The number of moles (M_s) of sulphate anions in KPVS bound by salt linkages to the protonated basic groups in 1 g of trypsin was estimated from the titrant volumes at various pHs, and the colloid titration curve was constructed by plotting M_s against pH (see *Figure 4*). An increase in M_s is seen with decreasing pH, followed by a constant value (0.818 ± 0.019 mmol g^{-1}) below pH 3. Since the sulphate groups in KPVS keep a completely dissociated state at least up to pH 2 (see References 2–5, 18), the increase in M_s can be related to the protonation of the basic groups in trypsin with decreasing pH. Also, the constant M_s value at pH < 3 is regarded as an intrinsic value for a complete protonation of all the basic groups.

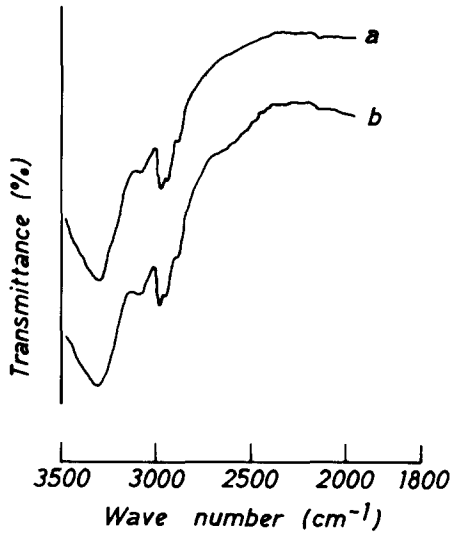

Figure 3 I.r. spectra of (a) trypsin and (b) complexed trypsin in KBr disk. The preparation methods of both samples are described in the text

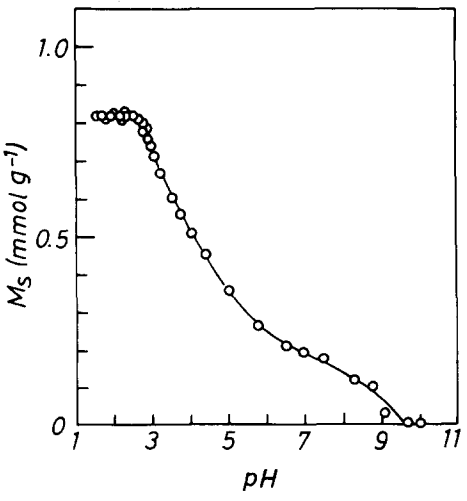

Figure 4 Colloid titration curve of trypsin with KPVS titrant

Table 1 Number of ionizable groups in bovine trypsin counted from the amino acid sequence reported by Walsh[11] and Mikeš *et al.*[10].[a]

Ionizable group	Amino acid residue with ionizable groups	Number	
		Ref. 11	Ref. 10
ε-Amino	Lysyl	14	14
α-Amino	N-terminal	1	1
Imidazolyl	Histidyl	3	3
Guanidyl	Arginyl	2	2
β-Carboxyl	Aspartyl	7	4
γ-Carboxyl	Glutamyl	4	2
α-Carboxyl	C-terminal	1	1
Phenolic OH	Tyrosyl	10	10
Mercapto	Cysteinyl	0	0

[a] Mercapto groups were omitted in counting the number of acidic groups because all are bound to each other by disulphide linkages

By using the obtained intrinsic M_s value, the number of basic groups responsible for the complexation can be estimated when the molecular weight of trypsin is known. The amino acid sequence of bovine trypsin, useful for determining the absolute value of its molecular weight, was first published by two different research groups: Walsh *et al.*[9] and Mikeš *et al.*[10]. At that time Walsh corrected part of the sequence data in their original publication[11]. The molecular weight of bovine trypsin determined on the corrected data of Walsh[11] was 23 268, although a slightly different value (23 253) was obtained from the data of Mikeš *et al.*[10]. The number of basic groups taking part in the complexation was thus estimated to be 19, with an error of ±0.4, when multiplying the molecular weight from Walsh's data by the intrinsic M_s value obtained. The deviation arising from the difference in the molecular weights was within the usual limit of the variation of the titration data. The estimated value of 19 differs by approximately one group from the number counted on the basis of the amino acid sequence, as shown in *Table 1*. However, as described below, enzymological studies of complexed trypsin indicated that one imidazolyl group in the active centre was free of salt linkages with KPVS. Taking into account this result and the precision of colloid titration, it is clear that salt linkage formation between KPVS and trypsin follows a stoichiometric relationship under conditions of complete protonation of all the basic groups in the enzyme.

Consideration of a difference in the contents of the ionizable groups between KPVS and trypsin is required for an understanding of the structure of the stoichiometric complex. From the ionizable group contents of KPVS (see Experimental section) and trypsin (see *Table 1*), it is apparent that the number of sulphate groups per molecule of KPVS is much larger than that of the basic groups per molecule of trypsin. This signifies that one molecule of KPVS forms a complex with many of the enzyme molecules until all of its negative charges are stoichiometrically neutralized with the positive charges of trypsin. It is thus reasonable to consider that the complex consists of numbers of non-flexible and global trypsin molecules bridged with loosely extended KPVS ions. Such a structure seems to be distinct from a ladder

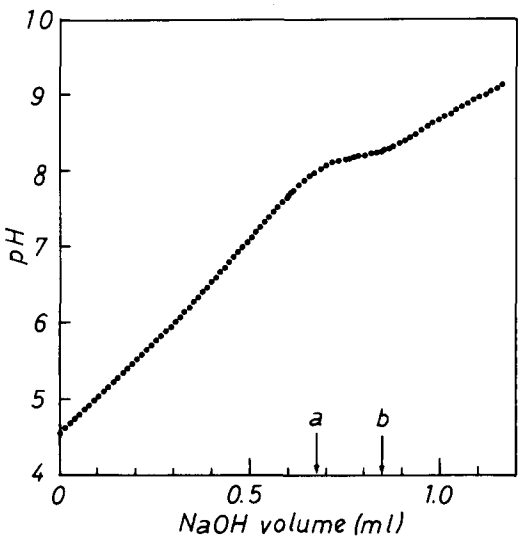

Figure 5 Potentiometric titration curve of complexed trypsin with 0.0190 N NaOH as the titrant. Arrow (a) (0.679 ml) and (b) (0.848 ml) on the abscissa indicate the neutralization points of 12 carboxyl groups and of 12 carboxyl groups plus 3 imidazolyl groups, respectively, which were calculated from Walsh's data shown in *Table 1*. The complex containing 25 mg of trypsin was subjected to titration which was done as described in the text

model which was originally proposed by Michaels for interpreting a stoichiometric complexation between oppositely charged flexible polyions[19].

Potentiometric titration curve

The acid groups, COOH and phenolic OH, remain in the stoichiometrically complexed enzyme, and they can be potentiometrically titrated with NaOH. The titration curve shown in *Figure 5* is characterized by two inflection points at titrant volumes of 0.68 and 0.86 ml. It is often said that such an inflection point appears in the potentiometric titration curves of polypeptides when conformational changes take place during the titration. However, analysis of the potentiometric data by a technique similar to that used in the previous section showed that 12 acidic groups are titrated up to the first inflection point. An error arising from a slight difference in the molecular weights determined was within the precision (±2%) of the titration used. The estimated number agrees with that of the carboxyl groups reported by Walsh (see *Table 1*).

Further analysis of the titration data showed that three groups were titrated between the first and second inflection points. Since the phenolic OH groups attached to polypeptides or proteins usually dissociate in the alkaline region pH >9.5–10 (see Reference 20), three titrated groups seem to be the imidazolyl group, i.e. one of them lies in the active centre and is free from the salt linkages and the others are bound to the KPVS ions. It is thus likely that the salt linkages between the imidazolyl and sulphate groups are severed during the deprotonation of the charged imidazolyl groups caused by an increase in pH. A similar result has been obtained in a complexation system composed of KPVS and human carboxyhaemoglobin[4].

Activity towards BANA

It is known that the active centre of bovine trypsin consists of three amino acid residues[14,15]: histidine, aspartic acid and serine. Also, their functional groups (imidazole, COOH and OH) cooperate in acylati deacylation as an intermediate step in the catal mechanism[14]. Thus studies on enzymatic properties complexed trypsin are useful for judging whether or the imidazolyl group in the active centre forms the linkage with KPVS. The pH–activity profiles of native and complexed enzymes using BANA are sho in *Figure 6*. The complexed trypsin is found to have appreciable retention of BANA-hydrolysing activ This finding indicates that the imidazolyl group in active site is free of the salt linkage with KPVS.

Detailed investigations of the results in *Figure 6* rev that in the acidic region the curve of complexed tryp shifts towards a higher pH range than the curve of native enzyme. A similar shifting of the pH-activity cu has been reported by Goldstein *et al.*[16], who stud effects of pH and ionic strength on the hydrolysis *N*α-benzoyl-L-arginine ethyl ester (BAEE) catalysed two modified trypsin derivatives, namely acetyltryp obtained from acylation of the ε-amino groups of tryp and water-insoluble trypsin in which the enzyme covalently bound via its ε-amino groups to the carbo groups of a 1:1 copolymer of maleic acid and ethyle For copolymer-bound trypsin, the magnitude of shifting was reduced with increasing ionic strength. Th they concluded that the pH–activity profile of tryp was altered by the effect of the electrostatic potential to the copolymer anions on the local concentration both hydrogen ions and positively charged substr molecules in the microenvironment of the bound enzy molecules. In our system, however, shifting of the acti curve was observed even at relatively high ionic stren (0.1). Also, the ionic strength did not have a la influence on the activity of the complexed enzyme (d not shown). These results are analogous to th for acetyltrypsin, in which the negative charges pre over the positive charges because of the acylation of amino groups. It, therefore, seems most likely that shifting observed here is not due to an electrostatic ef caused by the negative charges of the KPVS compon in the complex, but due to a stoichiometric neutralizat

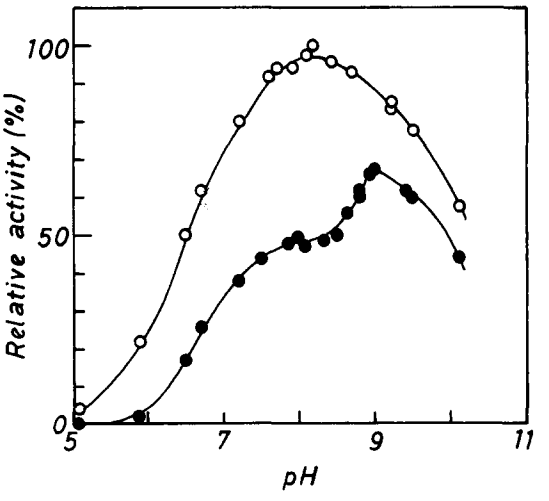

Figure 6 pH–activity curves of native (○) and complexed trypsin determined at 30°C by using a 2.66 mM BANA solution. Rel activity was calculated from the initial rate of BANA hydrolysis, 100% activity refers to the hydrolysing rate (4.61×10^{-2} mM mi by the native enzyme at pH 8.18

of the positive charges of trypsin molecules by complexing with KPVS.

Another important feature of *Figure 6* is that in a narrow pH range from 8 to 9 a rapid increase in the complexed enzyme activity was observed, whereas the native enzyme activity gradually decreased. In this pH range the cleavage of the salt linkages of KPVS with two imidazolyl groups (not members of the active site) is indicated when combining results from both the potentiometric titration and the activity measurement. Such a cleavage could cause a restoration of a complexation-induced and altered conformation of trypsin molecules, which results in a possible recovery of their enzymatic activity.

The results obtained in this section show that the complexation of trypsin with KPVS little affects the active centre of the enzyme. However, the pH–activity curve is changed mainly because the basic groups in the enzyme form salt linkages with the KPVS ion and are not capable of resulting in cationic charges via their dissociation. In addition, such salt linkages maintaining the bridged structure of the complex are not so rigid that a pH change brings about their partial cleavage, accompanying the recovery of the complexed enzyme activity via the restoration of an altered conformation of trypsin lying in the complex.

Michaelis–Menten constants

In the copolymer-bound trypsin system reported by Goldstein *et al.*[16], a strong electrostatic field developing from the negative charges of the copolymer was imposed on the enzyme molecules. This influences not only the pH–activity curve but also the Michaelis–Menten constant (K_m), which was evaluated by using $N\alpha$-benzoyl-L-arginine amide as a substrate. The kinetic studies were thus made to confirm the previously described results concerning the complexed trypsin activity. Since the cleavage of the salt linkages between the imidazolyl groups in trypsin and the sulphate groups in KPVS has been known to occur in the region pH >8, the Lineweaver–Burk plots were examined at pHs 6.7 and 10.1 (*Figure 7*). The values of a maximal hydrolysing rate (V_{max}) attained at very high substrate concentrations and of K_m were then estimated by the intercepts of each straight line on the Y- and X-axes, respectively. The results obtained are summarized in *Table 2*. It is found that the complexation causes a change in V_{max} but not in K_m. Therefore, the binding affinity of trypsin for the substrate is found to be little altered by complexing with KPVS. Hence, this supported the previous results that the complexation did not affect the state of the active centre in the enzyme. On the other hand, the observed difference in V_{max} between the native and complexed enzymes could be related to the shifting of the pH–activity curve described in the previous section.

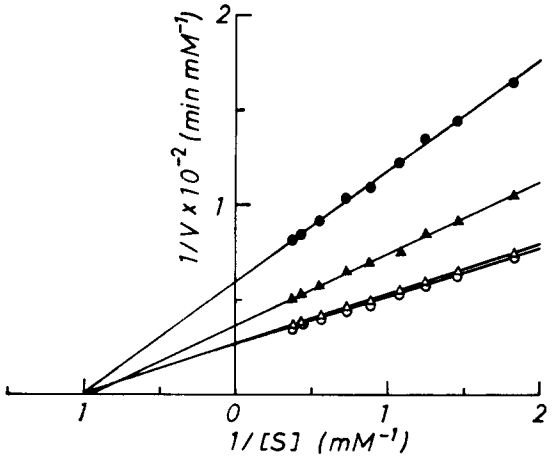

Figure 7 Lineweaver–Burk plots of native (○, △) and complexed trypsin (●, ▲) at pH 6.7 (○, ●) and pH 10.1 (△, ▲). The initial hydrolysing rate (V) of BANA was measured at 30°C using substrate solutions ranging in concentration ([S]) from 0.55 mM to 2.66 mM

Table 2 Values of K_m and V_{max} for the native and complexed enzymes estimated at pH 6.7 and 10.1 from the Lineweaver–Burk plots in *Figure 7*[a]

Enzyme	pH	K_m (mM)	10^3 V_{max} (mmol min^{-1} (mg enzyme)$^{-1}$)
Native	6.7	1.01	0.967
	10.1	0.981	0.922
	8.15	0.939	1.59
Complexed	6.7	1.00	0.413
	10.1	1.08	0.686

[a] Data for the native enzyme at pH 8.15 (optimal pH of bovine trypsin-catalysed BANA hydrolysis at 15°C) is cited for comparison from Reference 22

Activity towards casein

Another typical study on a water-insoluble trypsin has been reported by Haynes and Walsh[17], who prepared the trypsin by adsorbing the protein as a monolayer onto colloidal silica particles, followed by crosslinking with glutaraldehyde. They demonstrated that at pH 7.8 and ionic strength 0.1 (KCl), the insoluble enzyme obtained has about 80% retention of BAEE-hydrolysing activity compared with native trypsin. However, the activity towards casein (i.e. caseinolytic activity) was approximately 17% that of the native enzyme. The lowering of activity towards a high molecular weight substrate was interpreted in connection with both a lower availability of active sites and a lower efficiency at each site (they seem to arise from limiting substrate diffusion due to a steric hindrance effect of the supporting matrix). A similar conclusion has been drawn from enzymological studies of the copolymer-bound trypsin system with casein and haemoglobin as the high molecular weight substrate[21]. Taking these into account, a comparison of the pH–activity profiles obtained with BANA and casein serves to elucidate the structure of complexed trypsin.

Figure 8 shows the pH dependence of the caseinolytic activities of native and complexed trypsin. In contrast to the previous results[17,21], a large difference in the activities caused by molecular weight of substrate is not observed in our system (see *Figures 6* and *8*). Also, in the pH 8–9 range, where some of the salt linkages are severed, the fall in caseinolytic activity of complexed trypsin is much smaller than that of the native enzyme. This appears to be evidence supporting the recovery of enzyme activity via the restoration of complexation-induced conformational change of trypsin, although it was more clear when using BANA as the low molecular weight substrate (see *Figure 6*). Both results obtained here reveal that the usual diffusion limitation by polymeric substrates does not have a large influence on the complexed trypsin-catalysed degradation of casein. Consequently, the salt linkages

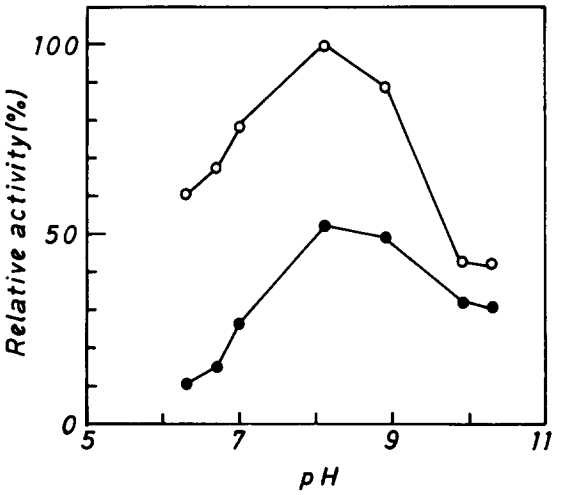

Figure 8 pH–activity curves of native (○) and complexed trypsin (●) determined at 30°C by using a 4% casein solution. Relative activity is represented by denoting by 100% activity the initial rate of casein digestion (0.14 absorbance change min^{-1}) by the native enzyme at pH 8.10

maintaining the structure of the complex are found to be very loose even if the enzyme was stoichiometrically complexed with KPVS.

CONCLUSIONS

The complexation of bovine trypsin with potassium poly(vinyl alcohol sulphate) (KPVS) has been studied as a function of pH by means of colloid titration. The resulting complex was examined by i.r. spectroscopy and potentiometric titration. The enzymatic activities of complexed trypsin towards *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BANA) as the low molecular weight substrate and casein as the high molecular weight substrate were also investigated. The major results are as follows.

Trypsin was complexed with KPVS via the salt linkages between the protonated basic groups and the sulphate ions. The complexation follows a stoichiometric relationship in the pH range where all the basic groups in the enzyme are protonated. The stoichiometric complex has an appreciable retention of catalytic activities towards both BANA and casein, and the activities of the low and high molecular weight substrates differ little. Thus the imidazolyl group in the active site of trypsin was found to be free of the salt linkage. In addition, the potentiometric titration curve showed that the salt linkages of KPVS with two imidazolyl groups, which are not members of the active site, are severed by increasing pH. We thus conclude that the salt linkages maintaining the structure of the complex are very loose even if the complex is stoichiometrically formed between trypsin and KPVS.

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