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COMPLEXATION OF PAPAIN WITH STRONG POLYANIONS AND ENZYMATIC ACTIVITIES OF THE RESULTING COMPLEXES

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ABSTRACT

The complexation of papain (EC.3.4.22.2) with potassium poly(vinyl alcohol) sulfate (KPVS) and sodium poly(styrene sulfonate) (NaPSS) was studied at different pH levels by means of colloid titration. The enzymatic activities of the resulting complexes were also studied as a function of pH and temperature, using $N\alpha$-benzoyl-DL-arginine-*p*-nitroanilide as the substrate. The moles (M_S) of the sulfate or sulfonate groups in the polyelectrolytes that took part in the complexation with 1 g papain varied depending on pH due to a pH-induced change in the protonation of the protein basic groups: 11 amino (including one *N*-terminal), 2 imidazolyl, and 12 guanidyl groups. However, the curves of M_S vs pH were independent of neither the species of the polyelectrolytes nor the molecular weight of NaPSS. On the other hand, the pH–activity curve of papain was shifted toward the alkaline pH range upon complexation, which occurred only in a region of pH < 5.5, without an accordant shift of the optimum pH (near 6.5 for both native and complexed

enzymes). A loss in the enzyme activity due to complexation was observed of the magnitude of that of NaPSS-50 (MW = 1,260,000) > KPVS ≅ NaPSS-7 (MW = 68,000). The mechanism of formation and structure of the protein/polyelectrolyte complexes consisting of papain and KPVS or NaPSS are discussed on the basis of these results, particularly in terms of the effect of the hydrophobicity of polyion chains on the structure of the complexes.

INTRODUCTION

Proteins interact with polyelectrolytes in aqueous media to result in either soluble or insoluble complexes, the latter in the form of a complex coacervate or an amorphous precipitate. Such phenomena will not only be important in technological fields such as protein separation using polyelectrolytes, but will also provide a basis for a better understanding of the macromolecular interactions available in living systems. Many previous studies have thus focused on the formation mechanism and structures of protein/polyelectrolyte complexes (PPCs) [1–17].

It has become apparent from our previous studies [3–5, 13, 17] that the complexation of proteins with polyelectrolytes occurs mainly through the formation of salt linkages or ion pairs between oppositely charged groups. Furthermore, the formation reaction for salt linkages follows a "1:1 stoichiometric relationship," at least under conditions where the acidic or basic groups of the protein molecules are completely dissociated in the absence of neutral salts; i.e., pH < 3 or >12. In a specific pH region, however, some of the salt linkages in the stoichiometric PPCs are severed during the course of the addition of other polyanions or polycations and small bases or acids.

Very recently, the formation of PPCs has been studied using quasi-elastic light scattering and electrophoretic light scattering techniques [12, 16]. When the pH of the protein solutions was increased in the presence of polycations such as poly(diallyldimethylammonium chloride), several species of PPC with different Stokes diameters were detected, the constituents of which were presumably an intrapolymer complex and its aggregates.

The mechanism of PPC formation assumed from a summary of the above results may be depicted as in the schematic illustrations in Figs. 1a and 1b. One polyelectrolyte molecule forms an "intrapolymer" complex with many of the protein molecules until all of its polyion charges are stoichiometrically neutralized by the opposite charges of the proteins. After this, the resulting intrapolymer complexes interact with one another, yielding aggregates or coacervates. The structure of such an intrapolymer PPC is shown schematically in Fig. 1b, where the complex consists of a number of nonflexible and global protein molecules bridged or bundled by one loosely extended polyelectrolyte ion [17]. The salt linkages maintaining the structure of PPC as an amorphous precipitate seem to be very loose, because changes in pH or additions of other polyions sever some of the salt linkages [4, 13, 17, 19]. This looseness may make it possible for the protein and polyion molecules to undergo stoichiometric neutralization or 1:1 binding with oppositely charged groups through thermal motion [17].

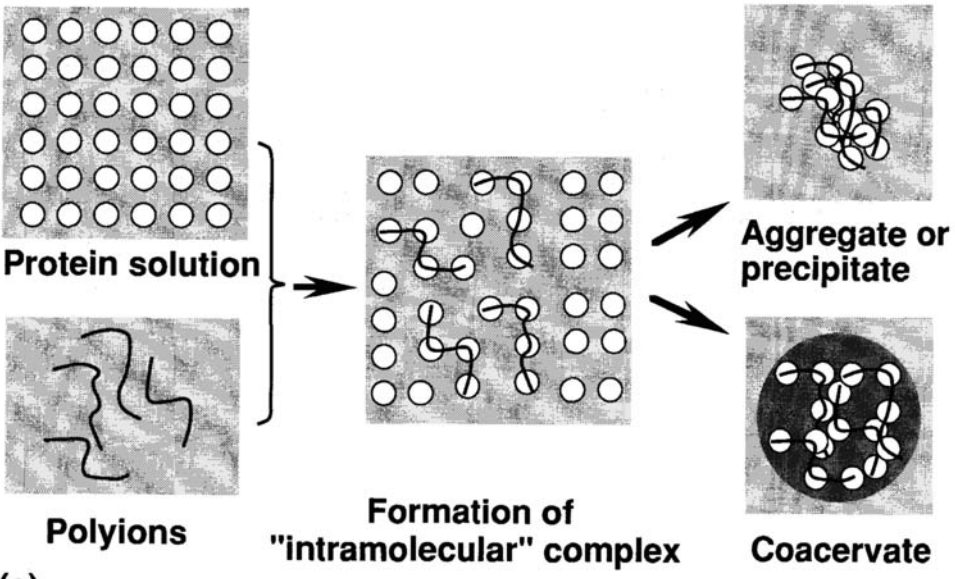


FIG. 1a. Schematic illustration of assumed mechanism of protein/polyelectrolyte complexation.

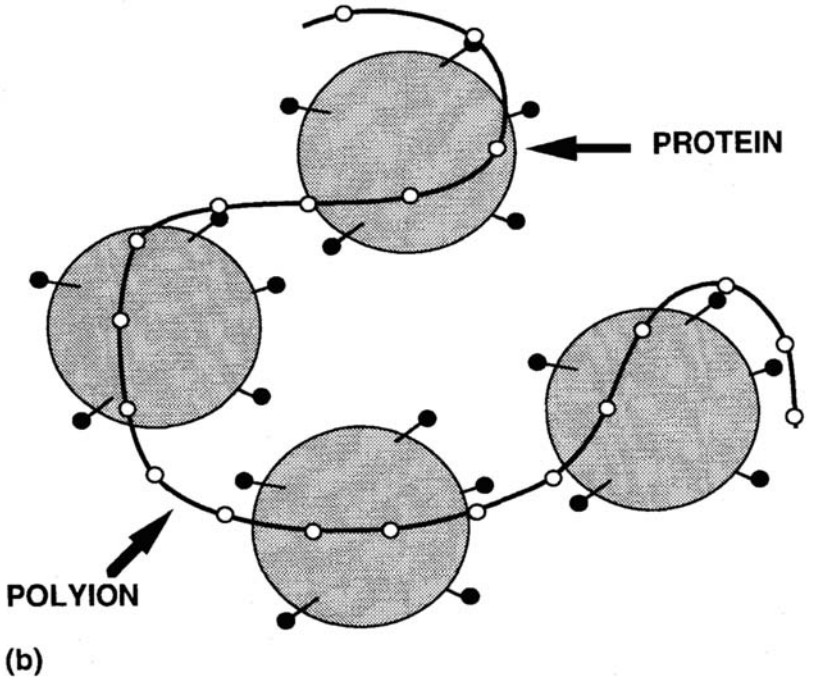


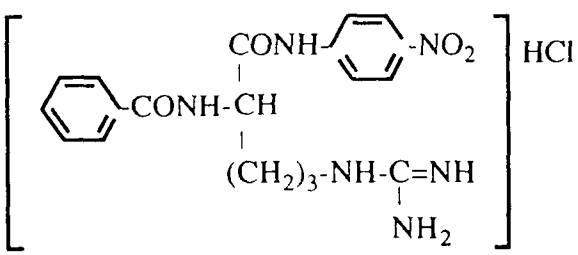
FIG. 1b. Structure of an intramolecular complex. The open and solid circles represent ionizable groups in the polyion and protein, respectively.

Enzymes could provide a good sample of proteins for the examination of such proposed models for the formation process and structure of PPCs; that is, some information on the structure of enzyme/polyelectrolyte complexes would be revealed through investigations of their possible catalytic activities toward different substrates after complexation with polyelectrolytes [13, 17, 18]. For these reasons we have studied the complexation of trypsin and potassium poly(vinyl alcohol) sulfate (KPVS) and the enzymatic activities of the resulting PPCs [13]. This study focuses upon the effect of the hydrophobicity of polyelectrolytes on complex formation and on the enzymatic activity of the complexes. Thus, in addition to KPVS, sodium poly(styrene sulfonate) (NaPSS) with different molecular weights was employed. Papain (EC.3.4.22.2), which is a typical protase like trypsin, was chosen because its amino acid sequence and enzymatic characteristics have been studied extensively [20, 21].

EXPERIMENTAL

Materials

The enzyme sample was obtained from a commercial source (Sigma Chemical Co., USA) and had an absolute molecular weight of 23,407 Daltons as estimated from the data on its amino acid sequence [21]. *N*α-Benzoyl-DL-arginine-*p*-nitroanilide (BANA) was also purchased from Sigma Chemical Co. for use as the substrate in the assay of enzyme activity. Potassium poly(vinyl alcohol) sulfate (KPVS) and sodium poly(styrene sulfonate) (NaPSS) were used as the polyelectrolyte samples. KPVS (nominal degree of polymerization, 1500) was the same as the sample used in the previous studies [3–5, 13], while the two NaPSS samples with molecular weights of 68,000 Daltons (NaPSS-7) and 1,260,000 Daltons (NaPSS-50) were commercial products (Wako Pure Chemical Co., Japan). The molecular weight of NaPSS was estimated by means of viscometric measurements [22].



BANA

Colloid Titration

The stoichiometry of the complexation was examined by colloid titration using an automatic recording titrator (see Fig. 2). An aqueous polyelectrolyte solution containing 0.0025 M (M = mol/dm^3) of ionizable groups in KPVS or NaPSS was used as the titrant after adjustment to the desired pH value. The end point of the titration was indicated by measuring the turbidity at 420 nm. The remaining protein concentration was also measured manually at several points during one titration using supernatant solutions from which PPC had been removed as follows: mild

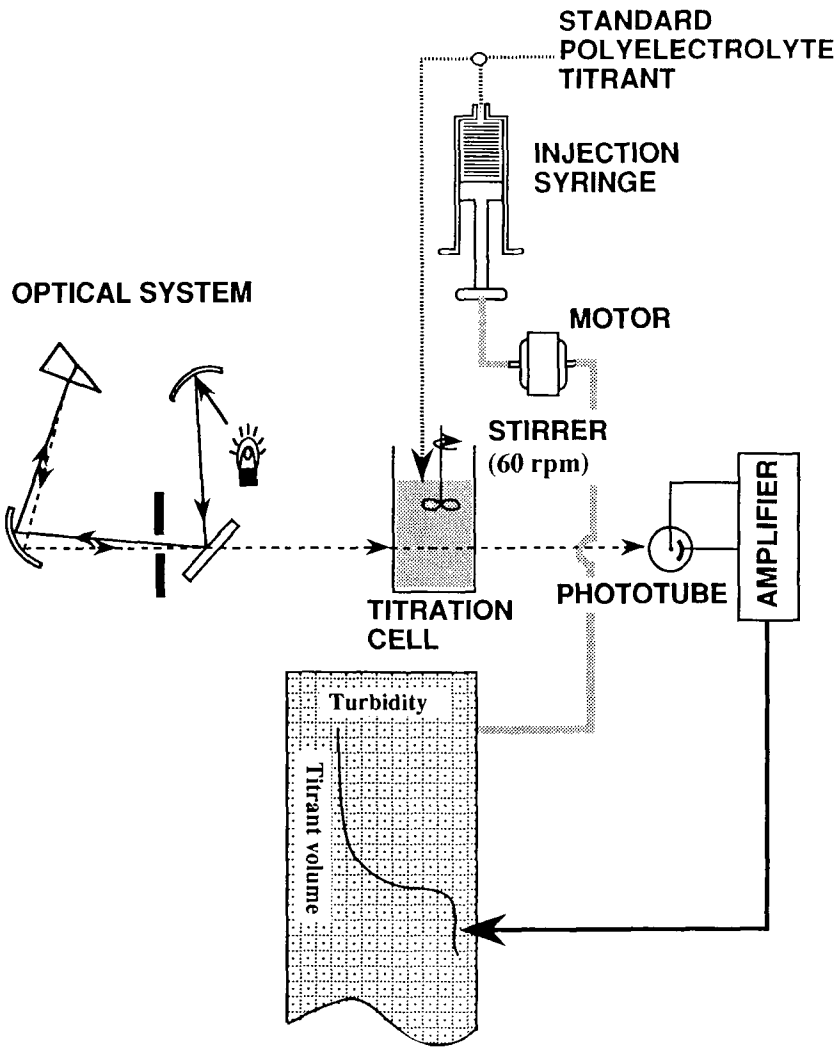


FIG. 2. Schematic illustration of turbidimetric titration apparatus used for colloid titration.

centrifugation of a protein/PPC mixture, followed by filtration with a membrane filter (0.22 μm pore size). In determining the remaining protein concentration, a calibration curve obtained by plotting the absorbance at 280 nm against the papain concentration was employed.

Enzymatic Assays

The activities of native and complexed enzymes were assayed at different pH values and temperatures, using BANA as the substrate. The assay of complexed papain was performed as follows: 1) aqueous papain solutions (30 mL including 3

mg protein) were adjusted to the desired pH levels and titrated with 0.0025 M of KPVS or NaPSS solution just until the end point of the titration in order to avoid the addition of an excess amount of polyelectrolyte; 2) the sample suspension obtained in 1) was mixed with 75 mL of the buffer solution with the same pH as that of the sample; 3) the sample prepared in 2) was mixed with 75 mL of the substrate solution which had previously been prepared by dissolving 87 mg BANA in the same buffer as used in 2). After this, *p*-nitroaniline derived enzymatically from BANA was monitored manually at regular intervals using a spectrophotometer (410 nm). The assay for the native enzyme was carried out using the same procedures described above, except that the sample solution was prepared by dissolving the enzyme in the same solvent as used in PPC preparation. Various buffer solutions at an ionic strength of 0.1 were used: pH < 5, acetate; pH 5–8, phosphate; pH > 8, carbonate.

RESULTS AND DISCUSSION

PPC Formation

Figure 3 shows the changes in turbidity and the remaining papain concentration which occurred with the addition of the standard titrant. As the titration proceeded, the sample began to become turbid, rapidly increasing in turbidity at a certain titrant volume, which indicated the end point of the titration, exactly as was performed in previous studies [3–5, 13, 17]. In the present study we further traced the turbidimetric titration curve by means of the remaining protein concentration. It was found that the remaining protein diminishes linearly as the titration proceeds, demonstrating that the formation of PPC takes place quantitatively during the

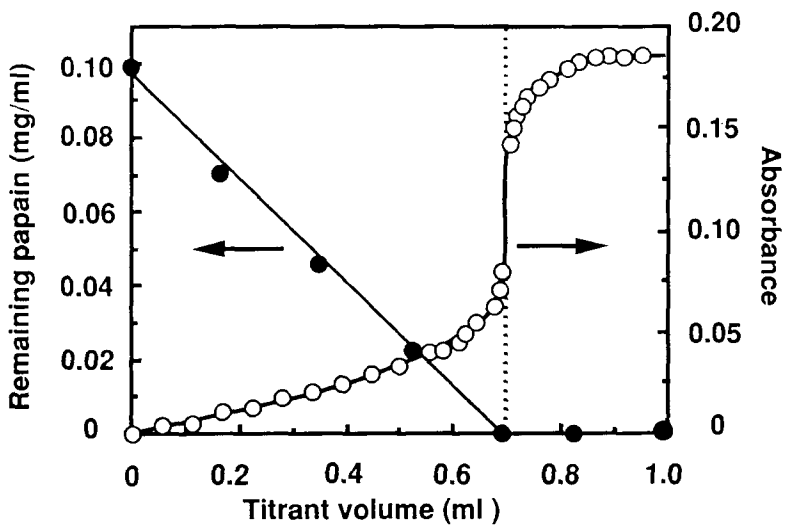


FIG. 3. Changes in turbidity and remaining papain concentration during the titration of aqueous papain solution with NaPSS-7 at pH 4.0. Protein concentration, 0.1 mg/mL; polyelectrolyte concentration, 2.5 mM; initial volume of the protein solution, 30 mL.

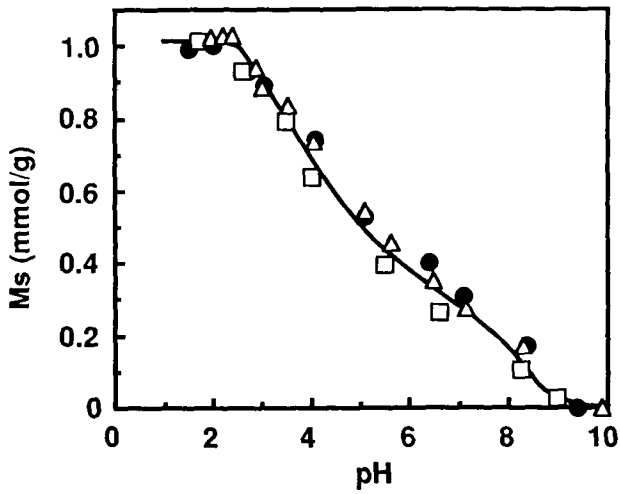


FIG. 4. Colloid titration curves of papain with KPVS (●), NaPSS-7 (△), and NaPSS-50 (□).

titration process. Also demonstrated was the fact that all of the protein molecules are complexed with the added polyions, because the remaining protein concentration was nearly zero at the end point indicated by the rapid increase in turbidity.

Taking the above results into consideration, we estimated the number of moles (M_S) of the sulfate groups in KPVS or the sulfonate groups in NaPSS that took part in the complexation with 1 g of papain as a function of pH. The colloid titration curves were then constructed by plotting M_S versus pH (Fig. 4). It is of interest that M_S is independent of the polyelectrolyte titrants employed, and in particular of the molecular weight in the case of NaPSS.

Since no protonation of the polyanions used here occurred at the pH levels studied, the curves of M_S vs pH can be interpreted in connection with an increase in the protein charges with decreasing pH via the protonation of the following basic groups of papain: 11 amino (including one *N*-terminal), 2 imidazolyl, and 12 guanidyl groups. Dividing the sum of these numbers of the basic groups by the absolute molecular weight (23,407), we obtained a total content of 1.07 mmol/g of the papain basic groups. This value is in agreement with the M_S value (1.01 ± 0.06 mmol/g) at pH < 3 of the colloid titration curves. Thus, it can be said that the complexation of papain with the three polyelectrolytes used takes place through a 1:1 stoichiometric neutralization or salt-linkage formation between the opposite charges of the protein and polyelectrolyte, at least in the region of pH < 3, where all the basic groups were completely protonated. Such complexation is influenced by neither of the species of the polyelectrolytes (KPVS and NaPSS) nor by the molecular weights of NaPSS (NaPSS-7 and NaPSS-50).

It is not easy to judge whether or not the formation of salt linkages obeys a 1:1 stoichiometric relationship at pH > 3. Although the number of charges bound to protein molecules can be estimated as a function of pH by means of potentiometric titration, polyions normally bring about acceleration of the dissociation of weakly acidic or basic groups attached to polymers; for example,

$$\text{⊢}(H_3C)_3N^+{}^-Cl + HOOC\text{⊣} = \text{⊢}(H_3C)_3N^+{}^-OOC\text{⊣} + H^+ + Cl^-$$

and

$$\text{⊢}_2OSO^-Na^+ + HO^-{}^+NH_3\text{⊣} = \text{⊢}_2OSO^-{}^+NH_3\text{⊣} + OH^- + Na^+$$

Thus, we will engage in no further discussion of the stoichiometry of the salt-linkage formation between papain and KPVS or NaPSS at pH > 3. Nevertheless, from the colloid titration curves we can estimate the moles of the sulfate or sulfonate groups of the polyelectrolytes which were bound to the protein molecules at a given pH level.

pH–Activity Curves

The dependence of the initial BANA-hydrolyzing rates on pH for the native and complexed enzymes is shown in Fig. 5. Complexation brought about a loss in enzymatic activity, the magnitude of which was found to be larger for NaPSS-50 than for the others (NaPSS-7 and KPVS). This suggests that the activities of the PPCs consisting of papain and NaPSS depend on the molecular weight of the polyelectrolytes, although the complexation was not influenced by their molecular weights (see Fig. 4).

Another remarkable feature of the pH-activity curves is that in the acidic region, the curves of the complexed enzymes shift toward a higher pH range than for the curve of the native enzyme. A similar shifting of the pH-activity curve for PPC of trypsin with KPVS has been observed [13], a result which was explained by assuming that the negative charges in PPC prevail over the positive charges because of the complexation through a 1:1 stoichiometric neutralization between the oppositely charged groups.

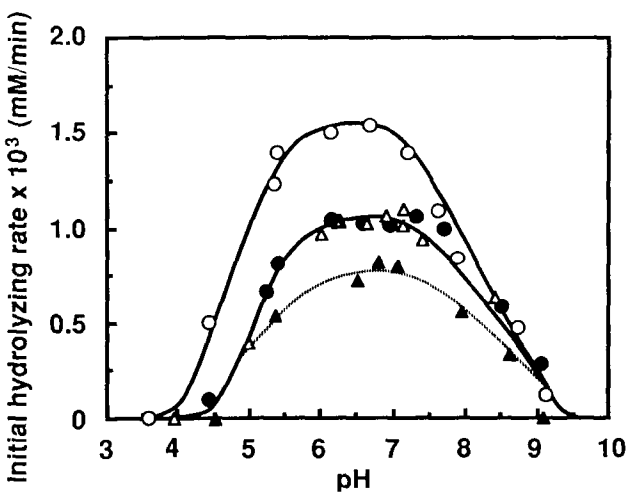


FIG. 5. pH-Activity curves of native papain (○) and its complexes with KPVS (●), NaPSS-7 (△), and NaPSS-50 (▲) at 35°C.

The activity measurements were carried out at the pH at which each PPC had been prepared; thus the number of the protein basic groups responsible for the complexation should vary depending on pH (see previous section). Strictly speaking, however, we cannot directly estimate the number of such basic groups from the M_S vs pH curves in Fig. 4. This is because the M_S values were obtained in a salt-free system (ionic strength $\approx$ 0) while the activity measurements were performed under an ionic strength of 0.1. In order to estimate the number of the basic groups neutralized by (or salt-linked with) the polyions within each PPC which had been subjected to the activity measurements, we are therefore forced to assume that the experimental procedures used in the activity measurements did not result in a dissociation of the polyions from PPCs. Under such an assumption, about 32% of the total basic groups take part in the complexation at the optimum pH (near 6.5) at which the loss in activity is about 20% for the KPVS or NaPSS-7 complexes and about 50% for the NaPSS-50 complex. In addition, at pH $<$ 5.5, where shifting of the pH–activity curves has been observed, more than 50% of the protein basic groups were neutralized by each polyelectrolyte.

Kinetic Behavior of Native and Complexed Papain

A lot of enzymes have been immobilized using a variety of polymer supports, the study of which has indicated that the effects of immobilization on the kinetic behavior of enzymes can be classified as follows (for example, see Ref. 19): 1) conformational and steric effects; 2) partitioning effects; 3) microenvironmental effects; and 4) diffusional or mass-transfer effects. In the case of PPCs consisting of enzymes and polyelectrolytes, complexation-induced changes in enzyme activity also fall into these four categories. Therefore, the examination of the kinetic behavior for the complexed papain provides useful information for clarifying the structure of PPCs.

Enzymic catalysis in homogeneous systems generally obeys the following Michaelis-Menten scheme:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_{+2}} \mathrm{E} + \mathrm{P} \tag{1}$$

where E denotes the enzyme, S the substrate, ES the enzyme/substrate complex, P the product from the substrate, and k the first-order rate constant for the corresponding process. The rate of this enzymic reaction, V, can be given by

$$V = \frac{k_{+2}[\mathrm{E}]_f[\mathrm{S}]_f}{K_m + [\mathrm{S}]_f} = \frac{V_{max}[\mathrm{S}]_f}{K_m + [\mathrm{S}]_f} \tag{2}$$

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \tag{3}$$

$$V_{max} = k_{+2}[\mathrm{E}]_f \tag{4}$$

where $[\mathrm{E}]_f$ and $[\mathrm{S}]_f$ represent the bulk concentrations of the enzyme and substrate, respectively, and V_{max} is the saturation rate (or maximum velocity) of the enzymic reaction. Also, K_m is the Michaelis constant and is equal to $[\mathrm{S}]_f$ when $V = V_{max}/2$.

Assuming that heterogeneous catalysis by complexed enzymes obeys the Michaelis–Menten scheme and also that all of the effects described above are negligible, the rate of the complexed enzyme reaction, V', which is actually determined by measuring the changes in the bulk concentrations of the substrate and the product, should be equal to V in Eq. (2). However, this is not the case in general [19]; for example, in the presence of concentration gradients, enzymes at different local positions within PPCs seem to exhibit different activities. As a rule, the observed V', which is an "overall" rate taken to be the sum of all "local" rates, involves one or more of the effects caused by complexation. The most important and interesting question is how to estimate complexation-induced effects from the dependence of V' on the bulk concentrations of the substrate. "Apparent" (or "effective") values for the saturation rate (V_{max}^{app}) and Michaelis constant (K_m^{app}) were thus estimated from the dependence of V' on bulk substrate concentrations under conditions in which the other factors remained fixed, as well as in studies of the effects of inhibitors in homogeneous enzymatic catalysis.

Figure 6 shows Lineweaver–Burk plots for native and complexed papain at pH 6.5, near the optimum pH, under the conditions at which the activity loss is about 20% for the complexes with KPVS and NaPSS-7, and also about 50% for the complex with NaPSS-50. A good linear relationship was observed in each plot

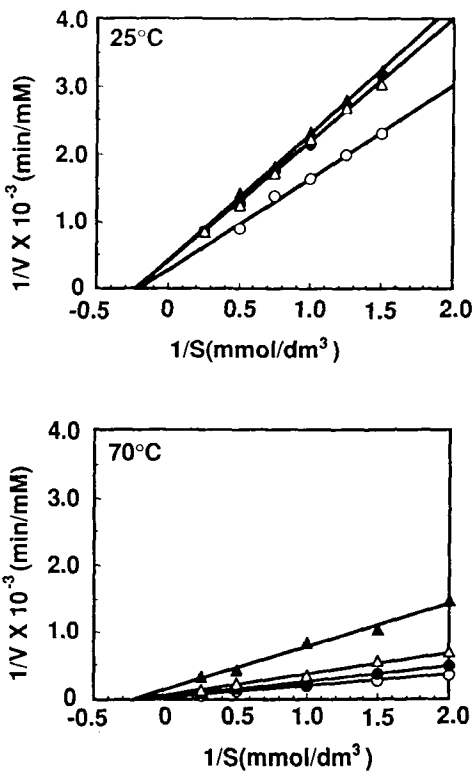


FIG. 6. Lineweaver–Burk plots for native papain (○) and its complexes with KPVS (●), NaPSS-7 (△), and NaPSS-50 (▲) at pH 6.5.

for the native and complexed enzymes at 25 and 70°C, strongly suggesting that the complexed enzyme catalysis obeys the Michaelis–Menten scheme. Thus, the value of V_{max} (for native enzyme) or V_{max}^{app} (for complexed enzymes) can be obtained from the intercepts on the Y-axis of each straight line, while the K_m or K_m^{app} values are obtained from the intercepts on the X-axis. The results are summarized in Table 1.

The values of K_m^{app} at 25 and 70°C for the papain complex with KPVS are found to be very close to the K_m values for the native enzyme. This is similar to the results for PPC of trypsin and KPVS in the previous study [13]. In the case of the complexes with NaPSS, on the other hand, the K_m^{app} values are slightly smaller than the K_m values, especially at 70°C. In general, K_m^{app} increased when enzymes were immobilized; $K_m^{app} > K_m$, due to diffusional or mass-transfer effects (see above). However, the results for the complexed papain (and also the complexed trypsin) showed either an unaltered or reduced K_m^{app} value; this is consistent with the model shown in Fig. 1 because it appears that diffusional or mass-transfer effects may be neglected in PPC, which may be taken as the aggregate of intrapolymer complexes consisting of several protein molecules loosely bundled with one polyion chain. A slight decrease in K_m^{app} for the NaPSS complexes seems to be due to an increase in local substrate concentrations through partitioning effects. There are two possibilities for interactions between BANA in the bulk phase and NaPSS within PPC aggregates: 1) the Coulomb forces of a positive charge of BANA (see the structure in Experimental Section) with the negative charges residing in the NaPSS component of PPC; 2) a hydrophobic interaction between BANA and PPC. However, the former hypothesis may not be entertainable since no decrease in K_m^{app} was observed in the KPVS complex, which supports the model in Fig. 1, these facts indicating that all of the polyion charges are stoichiometrically neutralized by the opposite charges of the proteins. As a result, it appears that the observed decrease in K_m^{app} for the NaPSS results from a hydrophobic interaction between BANA in the bulk phase and NaPSS within PPC aggregates, both of which contain hydrophobic styrene groups.

From Table 1 it became apparent that the complexation leads to a decrease in the saturation rate ($V_{max}^{app} < V_{max}$); this is particularly marked in the case of the NaPSS complexes, especially at 70°C. Such a decrease in the saturation rate is generally related to conformational and steric effects; i.e., a decrease in $[E]_f$ or in

TABLE 1. Michaelis Constants and Saturation Rates (or Maximum Velocities) for Enzymatic Hydrolyses of BANA at pH 6.5 by Native and Complexed Papain

	K_m^{app}, mM		V_{max}^{app}, mM/min	
Papain sample	25°C	70°C	25°C	70°C
Native	5.0[a]	6.7[a]	3.6[b]	40[b]
Complexed with KPVS	5.1	6.7	2.8	31
Complexed with NaPSS-7	4.6	5.8	2.6	18
Complexed with NaPSS-50	4.5	4.4	2.4	6.9

[a]Denotes K_m.
[b]Denotes V_{max}.

k_{+2}. One might expect from Eq. (3) that a decrease in k_{+2} should bring about a decrease in K_m^{app}. However, there was no change in Michaelis constants between the native enzyme and the complexed enzyme with KPVS. Therefore, a loss in the activity caused by the complexation of papain with KPVS is attributable to a decrease in the effective enzyme concentration resulting from a conformational change of part of the enzyme molecules within PPCs.

In the case of the papain complexes with NaPSS-7 and NaPSS-50, one cannot immediately reach the same conclusion, because the complexation has resulted in a lowering of K_m^{app}. However, a detailed investigation of temperature effects has shown that the ratio of the saturation rates at 70 and 25°C, $(V_{max}^{app})_{70}/(V_{max}^{app})_{25}$ or $(V_{max})_{70}/(V_{max})_{25}$, is about 11 for both native papain and its complex with KPVS, while $(V_{max}^{app})_{70}/(V_{max}^{app})_{25}$ is 7 for the NaPSS-7 complex and 3 for the NaPSS-50 complex. In general, k_{+2} increases with a rise in temperature. It is very difficult for us to assume that the magnitude of such increases varies depending upon the molecular weight, when the enzymes form PPCs with polyelectrolytes with the same chemical structure. Accordingly, we believe that a decrease in the effective enzyme concentration resulting from a conformational change of part of the papain molecules is a dominant factor leading to a decrease in V_{max}^{app}. We also believe that the strong hydrophobicity of NaPSS, the level of which may be enhanced with a rise in temperature and/or an increase in molecular weight, causes a large reduction in V_{max}^{app} for PPC consisting of papain and NaPSS-50 at 70°C.

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