Polym. Bull. (2011) 67:741–751
DOI 10.1007/s00289-011-0500-5

ORIGINAL PAPER

Activities of lysozyme complexed with polysaccharides and potassium poly(vinyl alcohol sulfate) with various degrees of esterification

Daisuke Takahashi · Kaoru Uchida · Tsuyoshi Izumi

Received: 11 November 2010 / Revised: 15 December 2010 / Accepted: 6 May 2011 /
Published online: 15 May 2011

Abstract Enzymatic activities of complexes of lysozyme (Lyz) with polysaccharides and potassium poly(vinyl alcohol sulfate) (KPVS) with different degrees of esterification (D_e) were studied as a function of pH, substrate concentration, and temperature. Molar masses and radii of polyelectrolytes and complexes and fluorescence spectra of Lyz molecules after complexation were examined by means of dynamic and static light scattering and fluorescence spectroscopy measurements. The relative activities of KPVS–Lyz complexes toward *Micrococcus lysodeikticus* and the number of hydroxyl groups in the formed complex increased with the decrease in D_e in KPVS molecules, whereas molar masses and radii of the complexes decreased. Moreover, kinetic parameters and fluorescence spectral data analysis indicate that activities and microenvironments around the active sites in Lyz complexed with KPVS with low D_e (0.130) and chondroitin sulfate C were nearly equal to that of native Lyz. These results indicate that the decrease in activities through complexation is mainly caused by the steric hindrance between Lyz and substrate due to aggregation of complexes, not by conformational change in Lyz molecules. Therefore, polyelectrolytes with hydroxyl groups have great potential as enzyme immobilization matrixes.

Keywords Enzymatic activity · Lysozyme · Complex · Polysaccharide · Potassium poly(vinyl alcohol sulfate) · Degree of esterification

Introduction

Complexation of proteins with natural and synthetic polyelectrolytes is interesting in two respects. The first concerns the manner in which polymers interact with

D. Takahashi (✉) · K. Uchida · T. Izumi
Department of Industrial Chemistry, College of Industrial Technology, Nihon University, Narashino, Chiba 275-8575, Japan
e-mail: takahashi.daisuke@nihon-u.ac.jp

nonflexible protein molecules, an understanding of which could provide a better explanation for the interaction mechanism of polyelectrolytes with ionic colloidal particles. The second concerns the extent to which biochemical activity is maintained in the resulting complexes, the answer to which is central to the molecular design of composite protein-polymer systems, such as immobilized enzymes, as well as to the design of protein separation processes using water-soluble polymers.

A number of studies have dealt with the formation of polyelectrolyte–protein complexes (PPCs) in aqueous salt-free and salt-containing systems under different pH conditions [1–6]. In addition, biochemical methods such as the measurement of enzymatic activity have been employed in appropriate cases [7, 8]. The main conclusions derived from these previous studies may be summarized as follows: (i) in salt-free systems, protein molecules are complexed with flexible polyelectrolytes through the stoichiometric formation of ion pairs (or salt linkages) between oppositely charged groups; (ii) the distribution of dissociation groups (basic groups) in the protein contributes to the aggregation and stability of the complexes; (iii) in salt-containing systems, formed PPCs behave as free draining molecules during electrophoresis at higher ionic strength than 0.05; and (iv) there is an appreciable retention of enzymatic activities in the resultant complexes indicating that changes in the three-dimensional structure of the protein molecules through complexation are not so large as to cause a loss of original function.

In the present study, we investigated activities of complexes of Lyz with polysaccharides and potassium poly(vinyl alcohol sulfate) (KPVS) toward *Micrococcus lysodeikticus* and hexa-*N*-acetylchitohexaose as a function of pH, substrate concentration, and temperature to determine the effect of the hydroxyl groups in the polyelectrolytes. For this purpose, KPVS with different degrees of esterification (D_e), sodium dextran sulfate, and chondroitin sulfate C, which all have hydroxyl and sulfonate groups in their molecular structures, were chosen as polyelectrolyte samples. Molar masses and radii of the polyelectrolytes and complexes, and pH dependencies on fluorescence spectra of Lyz molecules through complexation were investigated by dynamic and static light scattering (DLS and SLS) and fluorescence spectroscopy. From these methods, it should be possible to determine how the linear charge density and the number of hydroxyl groups of the polyelectrolyte affect the activities of complexed Lyz. Through this, one may establish more efficient methods for the preparation of immobilized enzymes.

Experimental

Materials

Lysozyme from chicken egg white (Lyz, purity 95%) was obtained from Sigma-Aldrich Japan Co., Japan. *Micrococcus lysodeikticus* (*M.l.*, Sigma-Aldrich Japan Co., Japan) and hexa-*N*-acetyl-D-glucosamine [$(GluNAc)_6$, Seikagaku Co., Japan, purity $\geq$ 95%] were used as bacterium and low molecular weight substrates, respectively.

KPVS, sodium dextran sulfate (Dex), and chondroitin sulfate C sodium salt (Chs) used as polyelectrolyte samples were obtained from Wako Pure Chemical Industries,

Table 1 Molar masses and radii of polyelectrolyte samples in 0.2 mol/dm^3 NaCl solution

Sample	$M_{PE} \times 10^{-4}$ (g/mol)	R_g (nm)	R_h (nm)	ρ (–)[a]	$A_2 \times 10^4$ (cm^3·mol/g^2)	D_e
KPVS (14/13)	8.10	23.4	17.5	1.34	4.13	0.130
KPVS (14/30)	9.39	34.6	16.8	2.06	8.45	0.301
KPVS (14/54)	13.2	39.4	17.5	2.25	9.50	0.540
KPVS (14/68)	15.3	39.4	16.8	2.34	8.89	0.680
KPVS (14/98)	22.6	42.3	16.9	2.50	8.87	0.978
Dex	68.5	171	52.0	3.29	70.0	–
Chs	1.80	32.1	8.0	4.01	6.30	–

[a] ρ value is calculated from the ratio of the radius of gyration (R_g) with hydrodynamic radius (R_h), i.e., $\rho = R_g/R_h$ (See details in [14])

Ltd., Japan. KPVS samples with different D_e were prepared via partial hydrolysis of KPVS (14/98) (degree of polymerization of 1,416; D_e of 0.978; M_w: 2.26 × 10^5) with HCl (see details in [5]). Molar masses and radii of polyelectrolyte samples are listed in Table 1, together with D_e in KPVS molecules. All other reagents were of analytical grade and used without further purification. All water used in this study was deionized, twice distilled, and filtered through a Gelman 0.22 μm filter.

DLS and SLS Measurements

DLS and SLS measurements were carried out with a Brookhaven system BI-200SM (Brookhaven Instruments Co., USA) equipped with a 256-channel digital autocorrelator (BI-2030AT) and a 2 W Ar laser (Stabilite 2017, Spectra-Physics Lasers). A 400-μm pinhole aperture was employed for the EMI photomultiplier tube, and decahydronaphthalene was used as the refractive index matching fluid to reduce stray light. We analyzed the autocorrelation functions with the CONTIN program [9] and estimated hydrodynamic radii (R_h) of polyelectrolytes and complexes. For SLS measurements, the optical alignment was ensured to less than 3% deviation from linearity in the $I\sin\theta$ versus θ plot over the range of $40° \leq \theta \leq 140°$. Each measurement was carried out for 1 s. We determined the Rayleigh ratio (R_θ) on the basis of the average of five such measurements. Changes in the refractive index with concentrations for various polyelectrolytes and Lyz were measured at 25°C with an Otsuka electrophotometric differential refractometer (model DRM-1021) (see details in our previous papers [4–6]).

For SLS and DLS measurements, complex solutions were prepared by mixing an equivalent amount of polyelectrolyte solution with 30 cm^3 of 0.1 g/dm^3 Lyz solution at pH 2.0. Free Lyz molecules were not detected by absorption spectra measurements.

Fluorescence spectra measurements

Fluorescence spectra of native Lyz and complex solutions at various pHs were measured with a Hitachi model F-4500 spectrophotometer (Hitachi High-

Technologies Co., Japan). Nitrogen gas was bubbled through sample solutions before spectroscopic measurements in order to minimize the effect of oxygen on the spectra. Each spectrum consisted of a mean value determined from five scans and was corrected for solvent contributions.

Activity measurements

Activity measurements of Lyz and various complexes toward two substrates were carried out by standard methods. For activity measurements, the Lyz concentration of complex solutions was adjusted to 0.01 g/dm^3 by dilution of the complex solutions prepared in a similar way as for DLS and SLS measurements with various buffer solutions, except for a temperature dependence of 0.005 g/dm^3. Several buffer solutions at an ionic strength of 0.1 were used: pH $<$ 5.8, acetate; pH 5.8–8.5, phosphate; pH $>$ 9.0, carbonate. After the dilution, free Lyz molecules were not detected in complex solutions by absorption spectra measurements. The relative activities of the complexes were calculated from relative values of the initial hydrolytic velocity of Lyz in complexes per initial hydrolytic velocity of native Lyz at optimum pH.

The hydrolytic reaction of *M.l.* was initiated by quick mixing of the *M.l.* solution (250 cm^3) with 25 cm^3 of the Lyz or complex solutions, which had been stored at specific temperatures from 20 to 45 °C. The absorbance at 450 nm of the solution was recorded versus reaction time using a Hitachi Model U-1000 spectrophotometer (Hitachi High-Technologies Co., Japan).

The hydrolytic reaction of $(GluNAc)_6$ was analyzed by the method of Imoto et al. [10]. To 1.0 cm^3 of $(GluNAc)_6$ solutions of different concentrations, 0.5 cm^3 of the Lyz or complex solutions was added and the solution was incubated for 40 min. at 45 °C. Next, 2.0 cm^3 of the color reagent solution were mixed with 1.5 cm^3 of sample solution and the mixture was incubated in boiling water for 15 min. After cooling, the absorbance at 420 nm of the sample solution was read versus water. The color reagent solution was prepared by dissolving 0.5 g potassium ferricyanide in 1.0 dm^3 of 0.5 M sodium carbonate solution. *N*-Acetyl-D-glucosamine (Seikagaku Co., Japan) was used to estimate the hydrolysis rate of $(GluNAc)_6$. The $(GluNAc)_6$ solution ranged in concentration from 2.0 to 12.0 $\times$ 10^{-5} mol/dm^3.

Results and discussion

Activities of KPVS–Lyz complexes with different D_e toward *M.l.*

The pH activity curves of KPVS–Lyz complexes with different D_e toward *M.l.* at 45 °C are shown in Fig. 1. Relative activities of various complexes are listed in Table 2, together with molar masses and radii of complexes obtained from SLS and DLS measurements. Whereas activities of complexed Lyz decreased compared to that of native Lyz, an increase in relative activity from 35.7 to 94.9% was observed with a decrease in D_e in KPVS molecules from 0.978 to 0.130. Similar high activity has been reported in a polyethylene glycol-pepsin complex system by Kokufuta

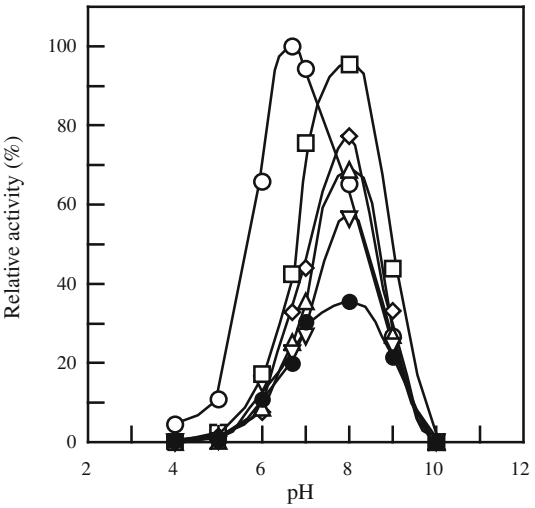

Fig. 1 pH activity curves of KPVS–Lyz complexes with various D_e toward *M.l.* at 45 °C. Symbols represent relative activities of complexed Lyz with KPVS with different D_e as follows: *open circle* native Lyz; *open square* KPVS (14/13)–Lyz complex; *open diamond* KPVS (14/30)–Lyz complex; *open triangle* KPVS (14/54)–Lyz complex; *inverted open triangle* KPVS (14/68)–Lyz complex; *filled circle* KPVS (14/98)–Lyz complex

et al. [11]. In the complexes with sodium polystyrene sulfonate (Scientific Polymer Products, Inc., USA, Mw 20.4×10^4), which has sulfonate and phenyl groups in its molecular structure, however, complexed Lyz exhibited a low relative activity of 41.0% (data not shown).

As can be seen in Table 2, relative activities of complexed Lyz decreased with an increase in M_x, R_g, and ρ values, but increased with an increase in the degree of aggregation (α) and the number of hydroxyl groups in the formed complex (n_{OH}) calculated from SLS data such as M_x, M_{PE}, and D_e. These results indicate polyelectrolytes with many hydroxyl groups, such as KPVS with low D_e (0.130), are efficient polymers for enzyme immobilized matrixes.

Moreover, pH activity curves of KPVS–Lyz complexes shifted to alkaline sides compared with that of native Lyz. Similar results were reported in activities of immobilized enzyme [12], KPVS–Trypsin complex [7], and KPVS–Papain complex [8]. In general, anions in enzymes were increased when the enzymes were bound to polyanionic carriers. Through incorporation of H^+ in immobilized regions, pH levels in immobilized regions were more acidified than in the bulk phase. Therefore, these alkaline shifts of the pH activity curves can be accounted for by the apparent decrease of pH values in immobilized regions as a result of H^+ incorporation by the excess negative charges.

Effect of complexation on the environment around aromatic amino residues in Lyz

Complexes containing KPVS with low D_e showed high activity, which is particularly interesting with respect to the preparation of immobilized enzymes. Moreover, the results discussed in "Activities of KPVS–Lyz complexes with different D_e toward *M.l.*" section suggest that complexed Lyz molecules are located in a more hydrophilic environment through complexation with the decrease in D_e, i.e., the increase in the n_{OH} values. Fluorescence spectra measurements were carried

Table 2 Relative activities of various KPVS–Lyz complexes toward *M.l.* at 45 °C and their values of molar masses and radii obtained from SLS and DLS measurements

Complex	Relative activity (%)	$M_x \times 10^{-7}$ (g/mol)	$M_x^0 \times 10^{-5}$ (g/mol)[a]	α (–)[a]	R_g (nm)	R_h (nm)	ρ (–)	$n_{OH} \times 10^{-5}$ (–)[b]
Open square KPVS (14/13)	94.9	6.69	2.15	312	66	50	1.32	9.80
Open diamond KPVS (14/30)	77.3	7.14	3.61	198	71	51	1.39	6.27
Open triangle KPVS (14/54)	68.8	7.29	6.30	116	75	52	1.44	3.11
Inverted open triangle KPVS (14/68)	56.4	7.33	7.83	94	77	52	1.48	1.89
Filled circle KPVS (14/98)	35.7	7.41	12.7	58	79	52	1.52	0.102

M.l. concentration (g/dm^3): 0.08

[a] The values of the weight average molecular weight of an intrapolymer PPC (M_x^0) and the degree of aggregation (α) were estimated from the following equations: $M_x = M_{PE} + n_B M_{Lyz}$ and $\alpha = M_x/M_x^0$, where M_{PE} is the weight average molecular weight of KPVS with different D_e, n_B is the average number of bound Lyz molecules per one polyion chain, and M_{Lyz} is the absolute molecular weight of Lyz (See details in Refs. [4] and [5])

[b] The average number of hydroxyl groups in KPVS molecules per one complex (n_{OH}) were estimated from the following equation: $n_{OH} = \alpha(n\ (1 - D_e) = M_x/\{(D_e/1 - D_e)\ 162.206 + 44.053\}$, where n is the average degree of polymerization of KPVS

out to determine the microenvironment around aromatic amino residues in complexed Lyz. The excitation wavelengths were chosen to be 280 and 305 nm, the regions in which absorption bands of aromatic amino residues such as Trp, Tyr, and Phe and Trp 108 in the active site of Lyz [13] are located. pH dependencies on fluorescence maximum wavelengths (EM_{max}) of various KPVS–Lyz complex solutions are shown in Fig. 2.

The EM_{max} values of all complexes showed blue shifts than that of native Lyz at pH lower than 11.0, but nearly equal to that of native one at pH 12 and 13 (see Fig. 2a). Similarly, the EM_{max} values of complexes excited at 305 nm showed blue shifts. However, the EM_{max} values of complexes increased and closed to that of native Lyz with the decrease in D_e in KPVS molecules (see Fig. 2b). Below pH 11.0, it was thought that fluorescent residues such as Trp, Tyr, and Phe in Lyz molecules were shielded from water molecules through binding to polyelectrolytes and became hydrophobic. Above pH 11.0, complexed Lyz molecules were denatured, similar to native Lyz. However, it is thought that the active site (Trp 108) of complexed Lyz was located in a microenvironment similar to that of native Lyz with the decrease in D_e in KPVS molecules, i.e., the increase in the n_{OH} values. Therefore, changes in activity of the complex with D_e in KPVS molecules were attributed to the ease of the hydrolytic reaction due to the increase of the hydrophilic environment around the active site of Lyz.

Activities of complexes of Lyz with polysaccharides and KPVS (14/98)

We next studied the hydrophilic effect of the hydroxyl group in polyelectrolytes on activities of the resultant complexes. Activities of complexes of Lyz with KPVS

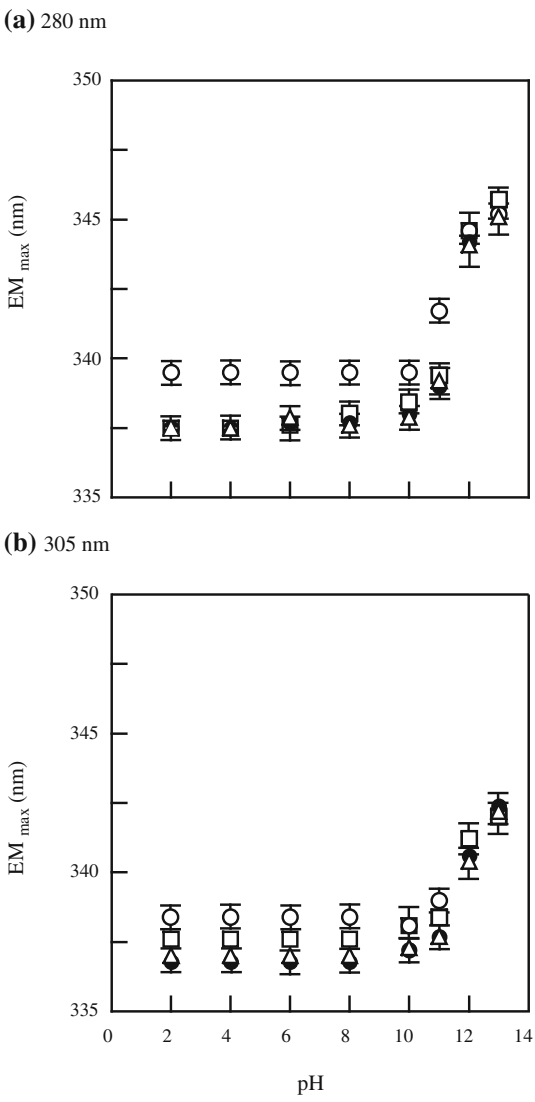

Fig. 2 Changes in EM_{max} values of various KPVS–Lyz complex solutions at 25 °C with pH. Fluorescence spectra measurements were carried out at **a** 280 nm and **b** 305 nm as excitation wavelengths. Symbols *open circle*, *open square*, *open triangle*, and *filled circle* represent EM_{max} values of native Lyz, KPVS (14/13)–Lyz complex, KPVS (14/54)–Lyz complex, and KPVS (14/98)–Lyz complex, respectively

(14/98), Dex, and Chs were examined as a function of pH, *M.l.* concentration, and temperature (Fig. 3). The values obtained from Fig. 3, such as optimum pH, relative activity, and kinetic parameters, are summarized in Table 3. Kinetic parameters such as apparent Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were estimated by the intercepts of each straight line on the *Y*- and *X*-axes in Lineweaver–Burk plots, respectively. The values of apparent activation energy (E) and frequency factor (A) were estimated from Arrhenius plots in the region in which the reaction velocity increased with temperature. The values of E_c/E and A_c/A were relative values of apparent activation energy and apparent frequency factor for complex and native Lyz, respectively.

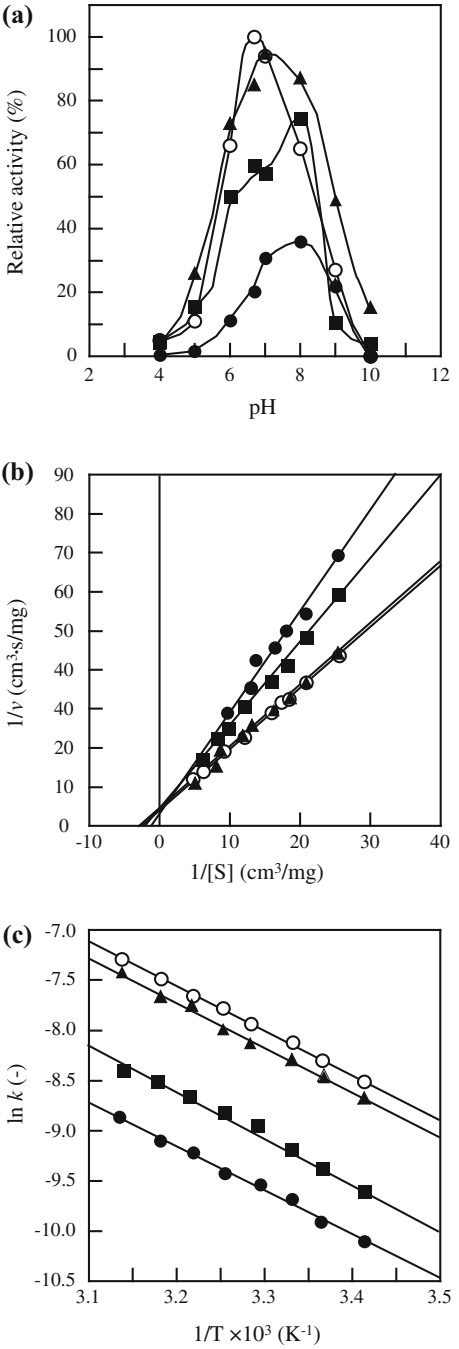

Fig. 3 Results of activity measurements of various complexes toward *M.l.* as a function of (a) pH, (b) *M.l.* conc., and (c) temperature. Symbols *open circle*, *filled circle*, *filled square*, and *filled triangle* represent native Lyz, KPVS (14/98)–Lyz complex, Dex–Lyz complex, and Chs–Lyz complex, respectively. Activity measurements were carried out under the following experimental conditions: **a** Lyz conc. 0.01 g/dm^3, *M.l.* conc. 0.08 g/dm^3, pH 4–10, temperature 45 °C; **b** Lyz conc. 0.01 g/dm^3, *M.l.* conc. 0.04–0.20 g/dm^3, optimum pH (6.7–8.0), temperature 45 °C; **c** Lyz conc. 0.005 g/dm^3, *M.l.* conc. 0.08 g/dm^3, optimum pH (6.7–8.0), temperature 20–45 °C

Table 3 Results of activities and kinetic parameters of various complexes toward *M.l*

Complex	Optimum pH (–)[a]	Relative activity (%)[a]	K_m (g/dm^3)[b]	V_{max} (g/dm^3·s)[b]	E_c/E (–)[c]	A_c/A(–)[c]
Open circle native Lyz	6.7	100	0.41	0.25	1.00	1.00
Filled triangle Chs	7.0	94.9	0.38	0.24	1.01	1.04
Filled square Dex	8.0	74.4	0.42	0.20	1.05	0.74
Filled circle KPVS (14/98)	8.0	35.7	0.48	0.19	1.05	0.39

M.l. concentration (g/dm^3): [a] 0.08; [b] 0.04–0.20; [c] 0.08

From Fig. 3a, two important characteristics were observed: (i) in the Chs complex system, the optimum pH was slightly shifted to the alkaline side than that of native Lyz after the complexation (Lyz at pH 6.7 and Chs–Lyz complex at pH 7.0) and the activity of complexed Lyz was nearly equal to that of native Lyz, with higher activity on the alkaline side at pH 6.7; (ii) pH activity curves of the Dex–Lyz and KPVS (14/98)–Lyz complexes, however, shifted to alkaline sides, in contrast with that of native Lyz, and the relative activities of the complexes decreased.

As shown in Table 3, values of K_m, V_{max}, E_c/E, and A_c/A in the Chs complex system were nearly equal to the native ones. However, V_{max} and A_c/A values in the Dex and KPVS (14/98) complex systems decreased, corresponding to the decrease in relative activities, whereas E_c/E values were not changed. In addition, the K_m value in the Dex–Lyz complex was nearly equal to that of the native one, but increased in the KPVS (14/98)–Lyz complex. This result indicates that, in the Chs–Lyz and Dex–Lyz complexes, neither the binding affinity of Lyz for *M.l.*, nor the three-dimensional structure of Lyz, changed by complexation because the E_c/E and K_m values do not change even after complexation.

In the KPVS (14/98)–Lyz complex, the increase in the K_m value and the decrease in values of V_{max}, A_c/A, and relative activity suggested a decrease in either the affinity between the Lyz and *M.l.* or the concentration of actual Lyz molecules due to steric hindrance through complexation.

Activities of KPVS (14/98)–Lyz complexes toward $(GluNAc)_6$ at 45 °C were measured as a function of pH and $(GluNAc)_6$ concentration. From pH dependencies on activities, KPVS (14/98)–Lyz complex toward $(GluNAc)_6$ showed higher activity (57.0%) than that of KPVS (14/98)–Lyz complex toward *M.l.* (35.7%) and the optimum pH (5.0) was not shifted after complexation. Furthermore, V_{max} and K_m values of complexed Lyz toward $(GluNAc)_6$ decreased to 0.88 mol/dm^3 min and 7.32 mol/dm^3 compared with those of native Lyz (2.01 mol/dm^3 min and 7.78 mol/dm^3).

These results indicate that the salt linkages maintaining the structure of the complex with Chs, Dex, and KPVS with lower D_e are very loose and changes in the three-dimensional structure of the Lyz molecules through complexation are not so large as to cause a loss of hydrolytic activity. Moreover, the decrease in A_c/A and V_{max} values are thought to be mainly attributed to the decrease in the concentration of actual Lyz molecules in the enzymatic reaction due to steric hindrance between Lyz and the substrate.

Conclusions

We studied activities of various polyelectrolyte–Lyz complexes towards two substrates as a function of pH, substrate concentration, and temperature. Molar masses and radii of formed complexes and changes in microenvironment around aromatic amino residues of Lyz through complexation were evaluated by means of DLS, SLS, and fluorescence measurements.

Activities of the formed complexes depended on the kind of polyelectrolyte. Activities of KPVS (14/13)–Lyz and Chs–Lyz complexes were nearly equal to that of native Lyz. From kinetic parameters and fluorescence spectral data, the decrease in activities of complexed Lyz was mainly caused by the steric hindrance between Lyz and the substrates through aggregation of complexes, not by conformational changes in Lyz molecules. Furthermore, as can be seen in K_m and E_c/E values in Table 3 and Fig. 2, the affinity between *M.l.* and Lyz was not affected for the reason that Lyz molecules located in the hydrophilic environment through complexation with polyelectrolytes having many hydroxyl groups in spite of the disadvantage for steric hindrance. These results demonstrated that polyelectrolytes with many hydroxyl groups in their molecular structures, such as KPVS with low D_e and Chs, have good potential as matrixes for immobilized enzymes. This information will aid in the process design for protein separation by polyelectrolytes and molecular designs of protein-polymer composites such as immobilized enzymes and drug delivery systems.

References

1. Nakagaki M, Sano Y (1972) Light-scattering studies of the interaction of sodium chondroitin sulfate C with bovine serum albumin. Bull Chem Soc Jpn 45:1011–1018
2. Kokufuta E, Shimizu H, Nakamura I (1981) Salt linkage formation of poly(diallyldimethylammonium chloride) with acidic groups in the polyion complex between human carboxyhemoglobin and potassium poly(vinyl alcohol sulfate). Macromolecules 14:1178–1180
3. Xia J, Dubin PL, Dautenberg H (1993) Light scattering, electrophoresis, and turbidity studies of bovine serum albumin-poly(dimethyldiallylammonium chloride) complex. Langmuir 9:2015–2019
4. Tsuboi A, Izumi T, Hirata M, Xia J, Dubin PL, Kokufuta E (1996) Complexation of proteins with a strong polyanion in an aqueous salt-free system. Langmuir 12:6295–6303
5. Takahashi D, Kubota Y, Kokai K, Izumi T, Hirata M, Kokufuta E (2000) Effects of surface charge distribution of proteins in their complexation with polyelectrolytes in an aqueous salt-free system. Langmuir 16:3133–3140
6. Matsunami H, Kikuchi R, Ogawa K, Kokufuta E (2007) Light scattering study of complex formation between protein and polyelectrolyte at various ionic strengths. Colloids Surfaces B 56:142–148
7. Kokufuta E, Takahashi K (1990) Stoichiometric complexation of bovine trypsin with potassium poly(vinyl alcohol sulfate) and enzymatic activity of the complex. Polymer 31:1177–1182
8. Izumi T, Hirata M, Takahashi K, Kokufuta E (1994) Complexation of papain with strong polyanions and enzymatic activities of the resulting complexes. J Macromol Sci A31(1):39–51
9. Provencher SW (1979) Inverse problems in polymer characterization; direct analysis of polydispersity with photon correlation spectroscopy. Macromol Chem Phys 180:201–209
10. Imoto T, Yagishita K (1971) A simple activity measurement of lysozyme. Agric Biol Chem 35:1154–1156
11. Kokufuta E, Nishimura H (1991) Complexation of pepsin poly(ethylene glycol). Polym Bull 26:277–282

12. Goldstein L, Levin Y, Katchalski E (1964) A water-insoluble polyanionic derivative of trypsin. II. Effect of the polyelectrolyte carrier on the kinetic behavior of the bound trypsin. Biochemistry 3:1913–1919
13. Ikada K, Hamaguchi K (1972) A tryptophyl circular dichroic band at 305 μm of hen egg-white lysozyme. J Biochem 71:265–273
14. Konishi T, Yoshizaki T, Yamakawa H (1991) On the "universal constants" ρ and Φ of flexible polymers. Macromolecules 24:5614–5622