Colloid Polym Sci 280:310–315 (2002)

ORIGINAL CONTRIBUTION

Hasan Ahmad
M. Okubo
Y. O. Kamatari
H. Minami

Structural conformation of biomolecules released from temperature-sensitive composite polymer particles – a study by circular dichroism

Received: 10 April 2001
Accepted: 13 June 2001

Part CLXXXII of the series "Studies on suspension and emulsion"

H. Ahmad (✉) · M. Okubo
Y. O. Kamatari · H. Minami
Department of Chemical Science and Engineering, Faculty of Engineering
Kobe University, Rokko, Nada
Kobe 657-8501, Japan

Present address: H. Ahmad
Department of Chemistry
University of Rajshahi
Rajshahi 6205, Bangladesh
e-mail: rajucc@citechco.net
Fax: +88-0721-750064

Abstract A conformational study of the biomolecules released from submicron- and micron-sized temperature-sensitive composite polymer particles by decreasing the temperature from 40 to 25 °C was done with circular dichroism and the results were compared with those of the native biomolecules. In almost all cases the biomolecules released retained their native conformational states.

Keywords Temperature-sensitive · Adsorption · Biomolecules · Conformation

Introduction

Temperature-sensitive polymers are considered to have many potential applications, such as in medicine, biotechnology, and environmental problems [1–5]. Recently many researchers have shown interest in poly(*N*-isopropylacrylamide) (poly-NIPAM) and pluoronic polymer because of their attractive temperature-sensitive properties and because they are considered to be applicable in drug delivery systems [6–10].

We found that poly(dimethylaminoethyl methacrylate) in an aqueous medium exhibited a similar temperature-sensitive property as poly-NIPAM and utilized it as a temperature-sensitive flocculant for the treatment of wastewater [11]. Recently we prepared submicron- and micron-sized temperature-sensitive composite polymer particles by seeded emulsion copolymerization of dimethylaminoethyl methacrylate (DM) and ethylene glycol dimethacrylate (EGDM) with polystyrene (PS) seed particles [12–17].

One of the important requirements for all carriers in drug delivery systems is that the biomolecules should retain their conformational state throughout adsorption/desorption steps because a conformational change would greatly reduce the activity. The conformational change is caused mainly by the hydrophobic interaction between the biomolecules and the surface of the particles [18, 19]. Additional factors, such as pH, temperature, ionic strength, and surface groups, were also considered to have effects [20–24].

In this work, we tried to detect any conformational change of the biomolecules released from the submicron- and micron-sized temperature-sensitive composite polymer particles by comparing the results with the native ones, using circular dichroism (CD) spectroscopy.

Experimental

Materials

Styrene was distilled under reduced pressure in a nitrogen atmosphere. DM and EGDM were of reagent grade and were used as received. 2,2′-Azobis(isobutyronitrile) (AIBN) and 2,2′-azobis(2-amidinopropane) hydrochloride (V-50) were recrystallized before use. Poly(vinylpyrrolidone) (PVP), tricaprylyl methyl ammonium chloride (aliquat 336), and poly(oxyethylene) sorbitan monooleate (Tween 80) were also of reagent grade. Lysozyme (LZ) and egg albumin (AL) supplied by Wako Pure Chemicals Co. and trypsin, type 1 (10,600 units/mg solid) supplied by Sigma Chemical Co. were preserved in a refrigerator and were used as received. Deionized water was distilled with a Pyrex distillation apparatus. The other chemicals used were of analytical grade.

Preparation of PS seed particles

PS seed particles (0.177 μm)were prepared by emulsion polymerization of 64 g styrene in the presence of nonionic emulsifier (Tween 80) with 0.256 g water-soluble cationic V-50 initiator in a nitrogen atmosphere. The polymerization was carried out at 60 °C for 24 h.

PS seed particles (1.77°μm) were prepared by dispersion polymerization of 40 g styrene in the presence of 1.6 g PVP and 0.4576 g aliquat 336 using 0.4 g AIBN as an oil-soluble initiator. The polymerization was carried out in a nitrogen atmosphere at a stirring rate of 80 rpm for 24 h. Here the conditions were changed a little from those reported by Almog et al. [25]. The seed particles were washed repeatedly to replace serum, i.e. the liquid part with distilled deionized water. The particles were observed with a JEOL JEM-2010 transmission electron microscope (TEM).

Seeded emulsion copolymerization of DM and EGDM

Seeded emulsion copolymerizations of DM and EGDM were carried out in the presence of 0.177-μm- and 1.77-μm-sized PS seed particles at 60 °C under the conditions listed in Table 1. The composite polymer particles produced were washed repeatedly by serum replacement with distilled deionized water to remove any traces of ionized salt. The conductance of the purified composite emulsion was found to be under 5 μS/cm.

The diameters of the composite polymer particles were 0.184 and 1.8 μm, respectively.

Adsorption of biomolecules

For each measurement, 10 ml purified emulsion was mixed with 10 ml biomolecule aqueous solution. The concentration of the

Table 1 Preparation of polystyrene (*PS*)/poly(dimethylaminoethyl methacrylate–ethylene glycol dimethacrylate) [*P(DM-EGDM)*] composite particles by seeded emulsion copolymerization with 2,2′-azobis(2-amidinopropane) hydrochloride (*V-50*) initiator

Ingredients	Submicron[a]	Micron[b]
PS emulsion (g)	83.33[c]	57.36[d]
DM (g)	11.81	5.085
EGDM (g)	0.37	0.16
V-50 (g)	2	1.2
Water (g)	700	294

[a]60 °C, 8 h, N_2, 100 rpm
[b]60 °C, 8 h, N_2, 80 rpm
[c]Polymer solid, 168 g/l
[d]Polymer solid, 105 g/l

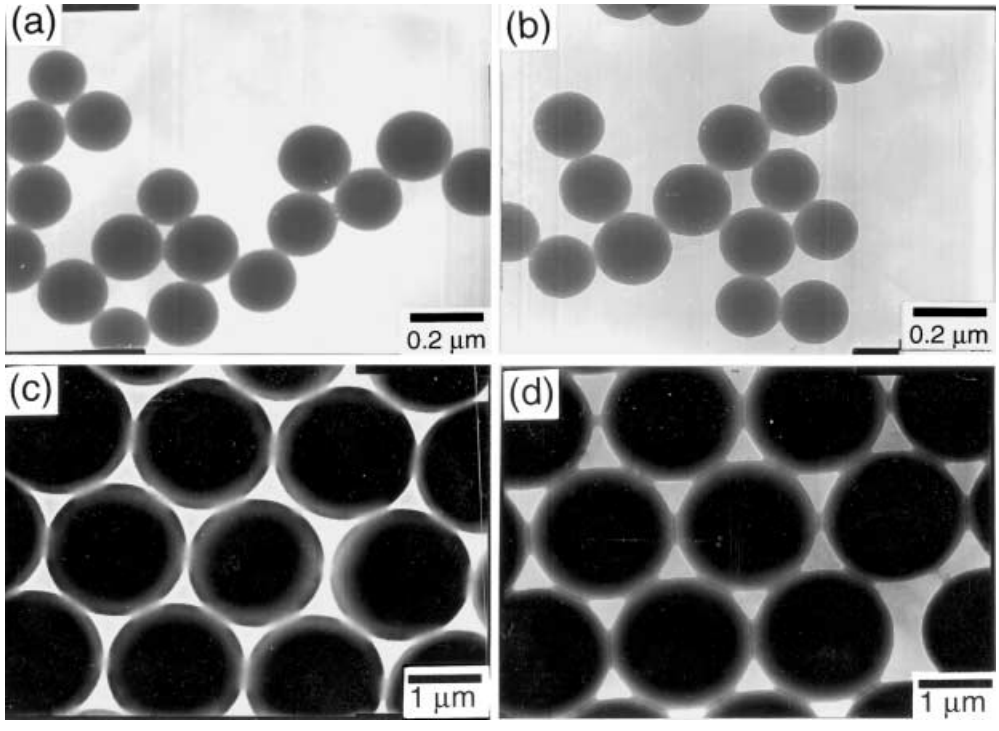

Fig. 1 Transmission electron microscopy photographs of **a** unwashed submicron-sized polystyrene (*PS*) seed particles, **b** PS/poly(dimethylaminoethyl methacrylate–ethylene glycol dimethacrylate) [*P(DM–EGDM)*] composite particles, **c** micron-sized PS seed particles and **d** PS/P(DM-EGDM) composite particles

biomolecules in the aqueous solution was kept constant against the total surface area of the polymer particles. The pH value was adjusted immediately to the isoelectric point of the respective biomolecule to eliminate the effect of ionic interaction with the particle surface; however, for trypsin the pH value was adjusted to 7 because at this pH value trypsin retains a high specific activity for a long time [15]. The amounts of adsorption at 40 and 25 °C were measured after keeping the mixture at each temperature for 3 h, with the exception of the emulsion–trypsin mixture, which was kept for 45 min. The concentration of the biomolecules in the medium was measured using a UV spectrophotometer at 280 nm. The amounts of adsorption of different biomolecules on submicron- and micron-sized composite polymer particles are presented in Table 2 [13, 15].

Table 2 Amounts (mg/g) of biomolecules adsorbed on polymer particles. The initial protein concentration was 2 mg/m^2 + 0.5 g/l. The immobilization time was 45 min

Biomolecules	pH	Submicron-size		Micron-size	
		40 °C	25 °C	40 °C	25 °C
Lysozyme	11	20.3	16.5	3.6	1.4
Egg albumin	4.9	57.5	51.4	5.3	4.2
Trypsin	7	35.6	25.4	4.7	2.8

Structural conformation of the biomolecules released

For each measurement, 30 ml purified composite emulsion (particles – 30 g/l) was mixed with 10 ml biomolecule aqueous solution in which the concentration of the biomolecules was kept below the maximum amount of adsorption. The pH was immediately adjusted to the isoelectric point of the respective biomolecule, except for trypsin, where the pH was adjusted to 7. The mixture was kept at 40 °C for 3 h and then the composite particles loaded with adsorbed biomolecules were separated by centrifugation. The separated particles were redispersed in a fresh buffer solution and kept at 25 °C for 3 h, except for the polymer particles loaded with adsorbed trypsin, where the time was fixed at 45 min to minimize the loss of specific activity. Finally, the liquid part was separated

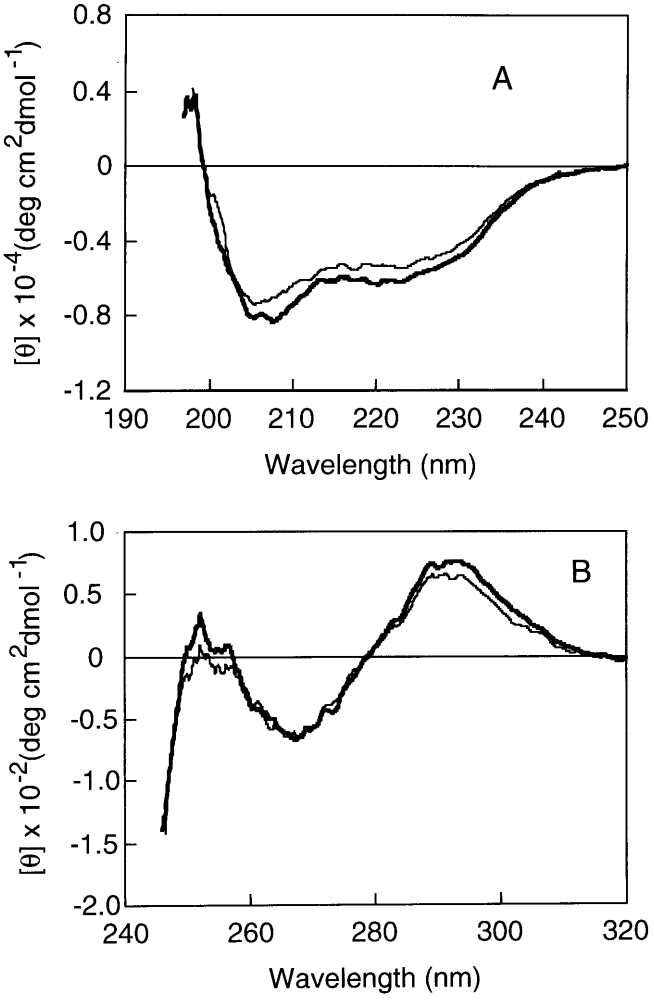

Fig. 2 Circular dichroism (*CD*) spectra of native lysozyme (*LZ*) (———) and LZ released from 0.18-μm-sized PS/P(DM–EGDM) composite particles at pH 11 (— — —) in **A** the far-UV and **B** the near-UV regions

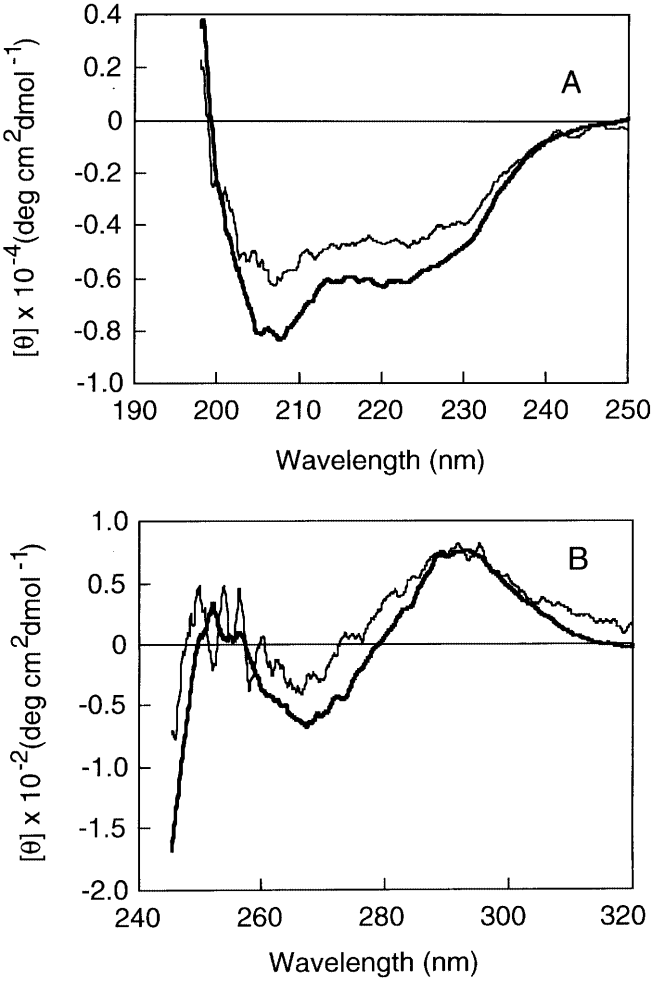

Fig. 3 CD spectra of native LZ (———) and LZ released from 1.8-μm-sized PS/P(DM–EGDM) composite particles at pH 11 (— — —) in **A** the far-UV and **B** the near-UV regions

from the composite particles by centrifugation and then by filtration with 0.2-μm microporous cellulose acetate filter to remove the wafting particles. The filtrate containing biomolecules released from the composite particles was subjected to CD measurement using a Jasco spectropolarimeter (J-720).

Results and discussion

TEM photographs of unwashed submicron- and micron-sized PS seeds, PS/poly(DM–EGDM) [P(DM–EGDM)] composite particles, are shown in Fig. 1. The TEM photographs of both composite particles indicate that no P(DM–EGDM) copolymer particles were produced during seeded emulsion copolymerization. These indicate that seeded emulsion copolymerizations proceeded mainly in PS seed particles.

The CD spectra of native LZ and LZ released from the submicron- and micron-sized temperature-sensitive PS/P(DM–EGDM) composite particles at pH 11 in the far- and near-UV regions are shown in Figs. 2 and 3. It is commonly known that in the far-UV region the ellipticity minima at 222 nm represents the α-helical secondary structure of the biomolecule [26, 27]. So the ellipticity minima in Figs. 2A and 3A indicate that the native LZ has a high degree of α-helical-rich secondary structure. The near-UV region represents the tertiary structure, i.e. the folding state of the segments of secondary structure which gives an idea about the microenvironment around different amino residues [28]. Figure 2B shows that the microenvironment around various amino residues, particularly tyrosine, tryptophane, phenyl alanine, histidine and cystine, of LZ released from the submicron-sized composite particles remained almost the same as the

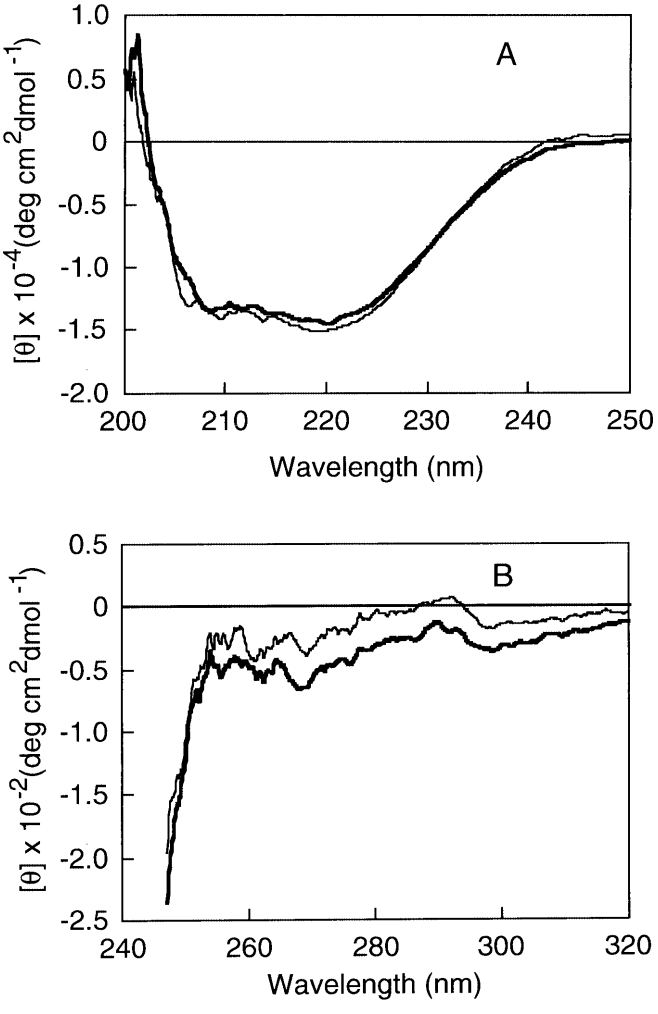

Fig. 4 CD spectra of native AL (———) and AL released from 1.84-μm-sized PS/P(DM–EGDM) composite particles at pH 4.9 (———) in **A** the far-UV and **B** the near-UV regions

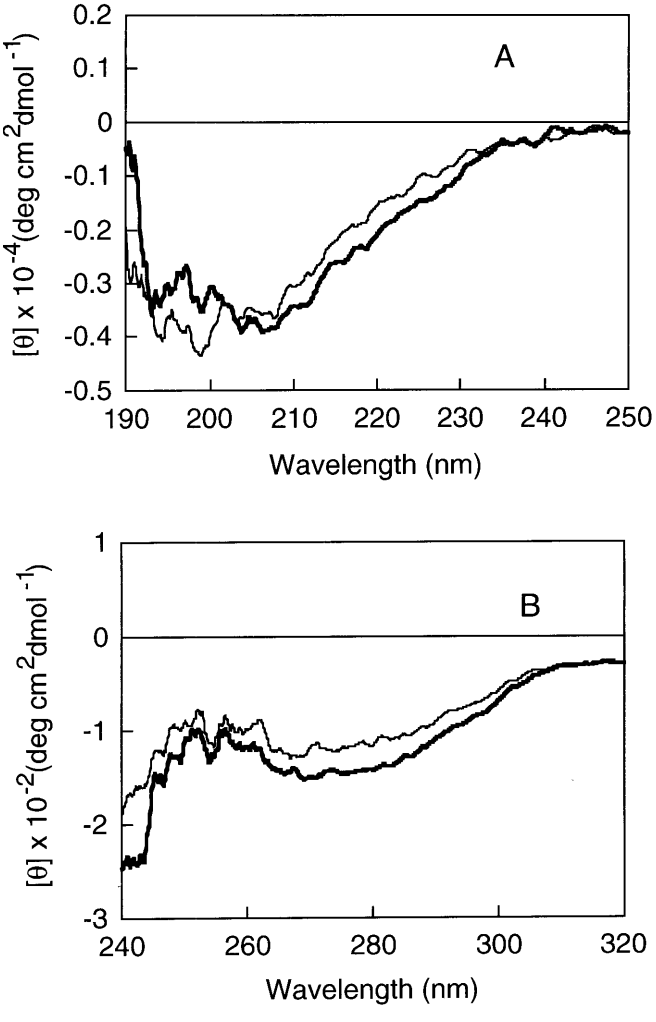

Fig. 5 CD spectra of native trypsin (———) and trypsin released from 0.184-μm-sized PS/P(DM–EGDM) composite particles at pH 7 (———) in **A** the far-UV and **B** the near-UV regions

native one. Figure 3 shows that LZ released from the micron-sized composite particles underwent only a slight conformational change with a slight decrease in the α-helical content. This indicates the possibility of a slight difference in the surface properties of the two composite particles prepared under different conditions. In comparison, in the case of hydrophobic PS particles we failed to obtain such CD spectra for released LZ because of the very low concentration. However, in the literature a complete loss in the molecular structure of some biomolecules, such as bovine serum albumin, fibrinogen and γ-globulin, released from hydrophobic polymer surfaces has been reported [29].

The CD spectra of native AL and AL released from submicron-sized temperature-sensitive composite particles at pH 4.9 in the far- and near-UV regions are shown in Fig. 4. In the far-UV region the ellipticity minima at 208 and 222 nm indicate a high degree of α-helix content in the native AL, which remained the same for the released AL. In the near-UV region the folding state of the α-helical-rich secondary structure of the AL released from the composite particles remained almost the same as the native AL.

The CD spectra of native trypsin and trypsin released from the submicron- and micron-sized composite polymer particles at pH 7 in the far- and near-UV regions are shown in Figs. 5 and 6. In both cases the released trypsin underwent a slight conformational change though in the case of the micron-sized temperature-sensitive composite particles the conformational change was more pronounced. In the near-UV region a slight decrease in the [θ] value indicates that the microenvironment around amino residues such as tyrosine, tryptophan and cystine changed only partially. This suggest that our previous results [15] in which the specific activity of adsorbed trypsin decreased slightly with time is due to the conformational change.

The CD spectra in the near-UV region of native trypsin and trypsin released from submicron-sized composite particles at pH 7 and 3 are shown in Figs. 7 and 8. In both cases, adsorption and desorption were carried out for 24 and 3 h. Figure 7 represents the case where the desorption at pH 7 was carried out simply by decreasing the temperature from 40 to 25 °C (below the lower critical solubility temperature). Figure 8 represents the case where the desorption was carried out by decreasing the pH from 7 to 3 without changing the temperature, which was fixed at 25 °C. Since the composite particle surface has tertiary amine groups due to the DM component, the particle surface should become comparatively hydrophilic at pH 3, which will initiate the desorption

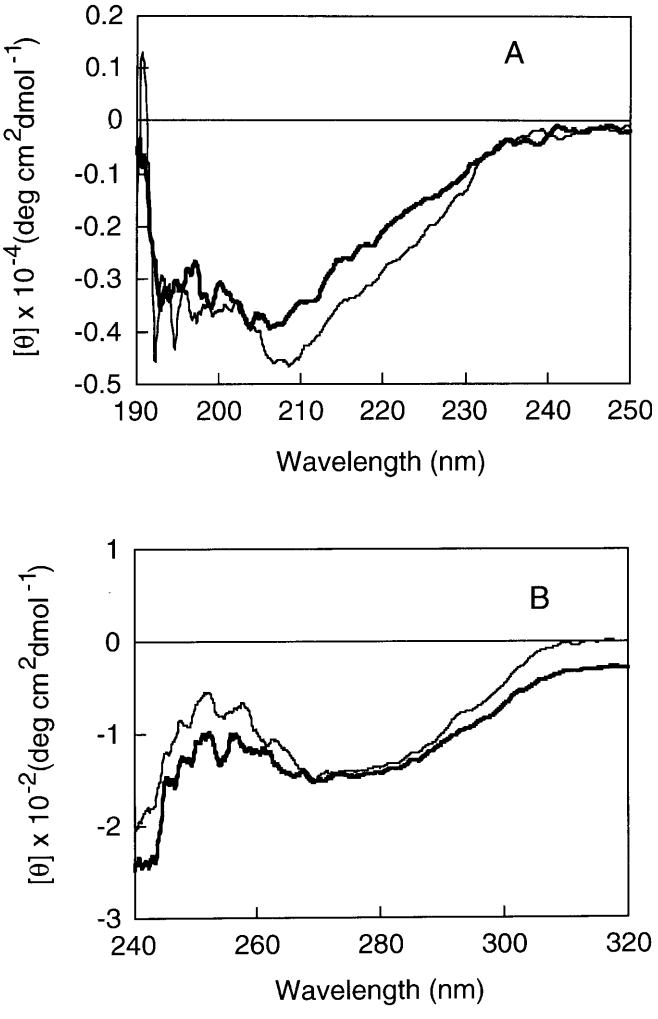

Fig. 6 CD spectra of native trypsin (———) and trypsin released from 1.8-μm-sized PS/P(DM–EGDM) composite particles at pH 7 (———) in **A** the far-UV and **B** the near-UV regions

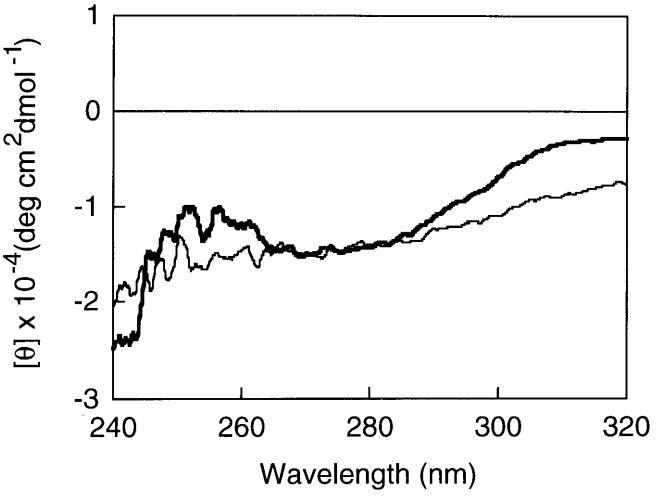

Fig. 7 CD spectra of native trypsin (———) and trypsin released from 0.184-μm-sized PS/P(DM–EGDM) composite particles in the near-UV region at pH 7 (———). Adsorption, 40 °C; 24 h

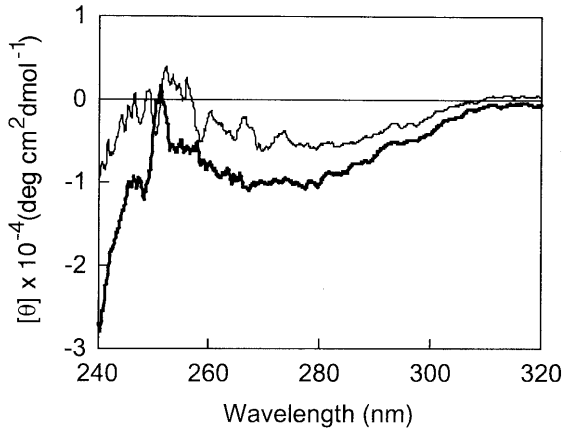

Fig. 8. CD spectra of native trypsin (———) and trypsin released from 0.184-μm-sized PS/P(DM–EGDM) composite particles in the near-UV region at pH 3 (———). Adsorption, 25 °C; 24 h

of the trypsin adsorbed at pH 7. Figure 7 shows that the structural conformation of the trypsin released at pH 7 was different from that of the native one. In comparison Fig. 5B shows that the structural conformation of the trypsin released, measured under the same conditions except for the adsorption time which was only 45 min, did not change so much. This indicates that the trypsin adsorbed for 24 h at 40 °C underwent conformational changes owing to the hydrophobic interaction with the particle surface. On the other hand Fig. 8 shows that the trypsin released underwent only slight conformational changes compared to native trypsin though the adsorption time was fixed at 24 h. This is because at 25 °C the particle surface is comparatively hydrophilic than at 40 °C and thereby decreased the hydrophobic interaction between the particle surface and adsorbed trypsin. At the pH values of 7 and 3 the structural conformations of the native trypsin were not the same. This is possibly due to the effect of the pH value [30]. This indicates that over longer periods of adsorption the conformational change of the biomolecule can be minimized by utilizing the pH-sensitive property of the PS/P(DM–EGDM) composite particles.

From these results it was clarified that the biomolecules released from both submicron-and micron-sized temperature-sensitive PS/P(DM–EGDM) composite polymer particles by decreasing the temperature from 40 °C to below the lower critical solubility temperature almost retained their native conformational structures. This was possible owing to the weak hydrphobic interaction of the biomolecules with the composite particle surface. This suggest that our temperature-sensitive composite particles will be useful as carriers for the separation of biomolecules.

Acknowledgements The authors would like to thank. K. Akasaka of the Faculty of Science, Kobe University, for his kind help with the CD measurements.

References

1. Monji N, Hoffman AS (1987) Appl Biochem Biotechnol 14:107
2. Chen GH, Hoffman AS (1993) Bioconjugate Chem 4:509
3. Bae Y H, Okano T, Kim SW (1988) Makromol Chem Rapid Commun 9:185
4. Hirokawa Y, Tanaka T (1984) J Chem Phys 81:6379
5. Dong LC, Hoffman AS (1986) J Controlled Release 4:223
6. Yang HJ, Cole CA, Monji N, Hoffman AS (1990) J Polym Sci Polym Chem Ed 28:219
7. Bae YH, Okano T, Hsu R, Kim SW (1987) Makromol Chem Rapid Commun 8:481
8. Schild HG, Tirrel DA (1989) Polym Prepr Am Chem Soc Div Polym Chem 30:342
9. Kawaguchi H, Fujimoto K, Mizuhara Y (1992) Colloid Polym Sci 270:53
10. Hoffman AS, Chen G, Wu X, Ding Z, Kabra B, Randeri K, Schiller E, Ron E, Peppas N, Brazel C (1997) Polym Prepr Am Chem Soc Div Polym Chem 76:271
11. Matsumoto T, Nakamae K, Okubo M, Sue M, Shimao M, Komura M (1974) Kobunshu Robunshu 31:669
12. Okubo M, Ahmad H (1995) Colloid Polym Sci 273:817
13. Okubo M, Ahmad H (1996) Colloid Polym Sci 274:112
14. Okubo M, Ahmad H (1996) J Polym Sci Polym Chem Ed 34:3147
15. Okubo M, Ahmad H (1998) J Polym Sci Polym Chem Ed 36:883
16. Okubo M, Ahmad H (1999) Colloids Surf A 153:429
17. Okubo M, Ahmad H, Suzuki T (1998) Colloid Polym Sci 276:470
18. Kamei S, Okubo M, Matsuda T, Matsumoto T (1986) Colloid Polym Sci 264:743
19. Tan JS, Martic PA (1990) J Colloid Interface Sci 136:415
20. Goldstein L (1972) Biochemistry 11:4072
21. Valenzuela P, Bender ML (1971) Biochim Biophys Acta 250:538
22. Gatfield IL, Stute R (1972) FEBS Lett 28:29
23. Okubo M, Takeya T, Mori K, Kamei S, Matsumoto T (1983) Kobunshi Ronbunshu 40:801
24. Norde W, Lyklema J (1978) J Colloid Interface Sci 66:254
25. Almog Y, Reich S, Levy M (1982) Br Polym J 14:131
26. Frencesco R D, Pastore A, Vecchio G, Cortese (1991) Biochemistry 30:143
27. Buck M, Radford SE, Dobson CM (1993) Biochemistry 32:669
28. Shiroya T, Yasui M, Fujimoto K, Kawaguchi H (1995) Colloids Surf B 4:275
29. Soderquist ME, Walton AG (1980) J Colloid Interface Sci 75:386
30. Goto Y, Takahashi N, Fink AL (1990) Biochemistry 29:3480