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ORIGINAL CONTRIBUTION

Synthesis of poly[2-(perfluorooctyl)ethyl acrylate-*co*-poly (ethylene glycol) methacrylate] and its control of enzyme activity in supercritical carbon dioxide

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Abstract Poly[2-(perfluorooctyl)ethyl acrylate-*co*-poly (ethylene glycol) methacrylate], P(POA-*co*-PEGm) was prepared as a new surfactant for $scCO_2$. The random copolymer was obtained by the radical polymerization of 2-(perfluorooctyl)ethyl acrylate (POA) and poly(ethylene glycol) methacrylate (PEGm) in DMF. The molar ratio of the POA and PEGm units in the copolymer was POA/PEGm=0.972/0.028 by ^{1}H NMR. The molecular weight and molecular weight distribution were estimated by size exclusion chromatography to be Mn=133,000 and Mw/Mn=8.25, respectively. It was suggested that the copolymer formed micellar aggregates with the cores of the PEGm chains in $scCO_2$, based on the analyses of the copolymer in hexafluorobenzene by ^{1}H NMR and dynamic light scattering. The copolymer was soluble in $scCO_2$ and had a cloud point at a much higher pressure than the critical pressure. It was found that the copolymer solubilized CO_2-insoluble proteins such as bovine serum albumin and subtilisin Carlsberg in $scCO_2$. The solubility of the copolymer was not influenced by the presence of the proteins; however, the solubility decreased in the presence of a small amount of water along with the protein. The activity of the subtilisin slightly decreased when only placed in $scCO_2$, whereas a marked decrease in the activity was observed for the subtilisin in the presence of the copolymer in $scCO_2$. The subtilisin activity decreased as the CO_2 pressure increased.

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Keywords P(POA-*co*-PEGm) · Supercritical carbon dioxide · Cloud point · CO_2 pressure · Bovine serum albumin · Subtilisin Carlsberg · Enzyme activity · Micellar aggregates

Introduction

Carbon dioxide has many environmentally benign characteristics, such as being nontoxic and odorless and having recyclable properties. Carbon dioxide also has industrial utilities, such as being spontaneous and volatile with a mild critical point (31 °C, 73.8 bar). Based on these benefits, supercritical carbon dioxide ($scCO_2$) has attracted considerable attention as a substitute for organic solvents in industrial manufacturing. Examples include the extraction of natural products [1, 2] and transition metals [3], the separation of paraffin from crude oil [4], the coating of fluoropolymers [5] and metal powders [6], cleaning technologies [7], the dyeing of fibers [8, 9], the synthesis of aerogels [10, 11], the preparation of polymer foams [12, 13], enzymatic reactions [14, 15], and polymerizations [16, 17]. Many compounds like enzymes and commercial polymers are insoluble in nonpolar $scCO_2$. Accordingly, surfactants are often required to dissolve them for use in $scCO_2$. Some enzymatic reactions in $scCO_2$ are performed in micelles formed by surfactants. For instance, the reaction by cholesterol oxidase was promoted in the presence of the ammonium carboxylate perfluoroether surfactant [18]. The dispersion and emulsion polymerizations in $scCO_2$ always need surfactants to produce spherical particles. Based on the importance of the surfactant structure, a variety of surfactants have been developed for these polymerizations. Examples include poly(perfluoroalkyl acrylate) [19], poly (dimethylsiloxane) [20], and their block [21, 22] and

random copolymers [23, 24]. These surfactants were efficiently utilized in the dispersion polymerization of styrene and methacrylates to produce microspheres with a narrow particle size distribution.

We found that the random copolymer containing hydrophilic poly(ethylene glycol) and CO_2-philic perfluoroalkyl segments acted as a surfactant for proteins and controlled the enzyme activity in $scCO_2$. The copolymers consisting of PEG and perfluoroalkyl acrylate or methacrylate have already been prepared as block copolymers, such as poly[2-(perfluorooctyl)ethyl methacrylate]-*block*-oligo(ethylene glycol) methacrylate [25] and poly[2-(perfluorooctyl)ethyl acrylate]-*block*-PEG [26]. However, these block copolymers were not sufficiently investigated as surfactants. In general, random copolymers are more easily prepared and form larger sized micelles than block copolymers [27]. This paper describes the synthesis of the poly[2-(perfluorooctyl)ethyl acrylate-*co*-poly(ethylene glycol) methacrylate] random copolymer, P(POA-*co*-PEGm), the solubility behavior of the surfactant, and the control of the enzyme activity by the surfactant in $scCO_2$.

Experimental

Instrumentation

The ^{1}H NMR measurement was conducted using a Varian 300 FT NMR spectrometer. The size exclusion chromatography (SEC) was performed using a Tosoh GPC-8020 instrument equipped with a DP-8020 dual pump, a CO-8020 column oven, and a RI-8020 refractometer. Two polystyrene gel columns, Tosoh TSKgel GMH_{HR}-M, were used with hexafluoroisopropanol as the eluent at 40 °C. The scanning electron microscopy (SEM) measurements were made using a JEOL JSM-6300 electron microscope. The cloud point measurement was performed with a Nekken variable volume view cell (with a window made of tempered glass) equipped with an Eyela CCA-1110 cooler and a Nihon Seimitsu Kagaku NP-D-321 personal pump. The UV spectra were obtained using a Shimadzu UV-160A UV–vis recording spectrophotometer.

Materials

2-(Perfluorooctyl)ethyl acrylate (POA) was purified by passage through an alumina column to remove the inhibitor. Poly(ethylene glycol) methacrylate (PEGm) without an inhibitor was purchased from NOF and was used without further purification. The molecular weight and degree of polymerization of PEGm was estimated by ^{1}H NMR as Mn=936 and DP=19, respectively. Azobisisobutyronitrile (AIBN) was recrystallized in methanol. *N*,*N*-Dimethylformamide (DMF) was purified by distillation under reduced pressure over anhydrous magnesium sulfate, and further distillation over calcium hydride. Dimethyl sulfoxide (DMSO) was distilled under reduced pressure over calcium hydride. Bovine serum albumin (BSA) and subtilisin Carlsberg were purchased from Sigma and used without further purification. *N*-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (Suc-Ala-Ala-Pro-Phe-*p*NA) was purchased from Bachem. Hexafluorobenzene to be used as a solvent for ^{1}H NMR and light scattering measurements was distilled over calcium hydride. Trifluoroacetic acid and hexafluoroisopropanol were used without further purification.

Synthesis of P(POA-*co*-PEGm)

POA (2.465 g, 4.76 mmol), PEGm (0.588 g, 0.628 mmol), AIBN (26 mg, 0.158 mmol), and DMF (5 ml) were placed in an ampule. After the contents were degassed, the ampule was sealed in vacuo. The polymerization was carried out at 60 °C for 43 h and was terminated by cooling with liquid nitrogen. The reaction mixture was poured into 1 l of hexane to precipitate a polymer. The precipitate was dried in vacuo for several hours. The product was dissolved in hexafluoroisopropanol (10 ml) and precipitated in 1 l of water to remove the PEGm remaining unreacted. The precipitates were collected and dried in vacuo for several hours. P(POA-*co*-PEGm) (2.298 g) was obtained.

Light scattering measurements of P(POA-*co*-PEGm)

P(POA-*co*-PEGm) (24 mg) was dissolved in 3 ml of hexafluorobenzene. The solution was subjected to light scattering at θ=90 ° at 25 °C. Trifluoroacetic acid (44 μl) was added to the solution. The mixture was subjected to the light scattering at θ=90° at 25 °C. The hydrodynamic diameter of the copolymer was determined by the cumulant

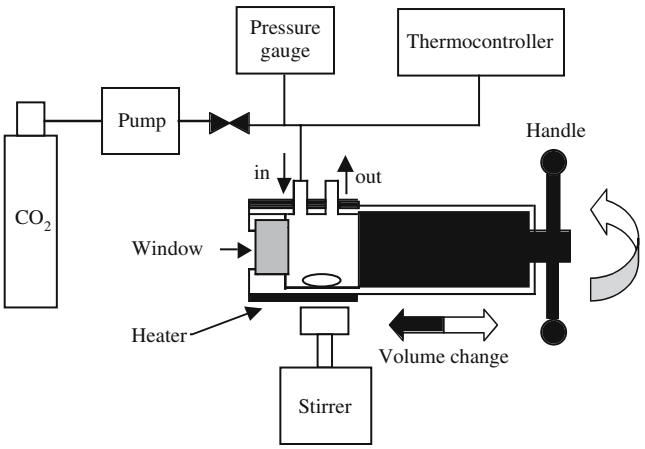

Fig. 1 A schematic of the experimental variable volume view cell

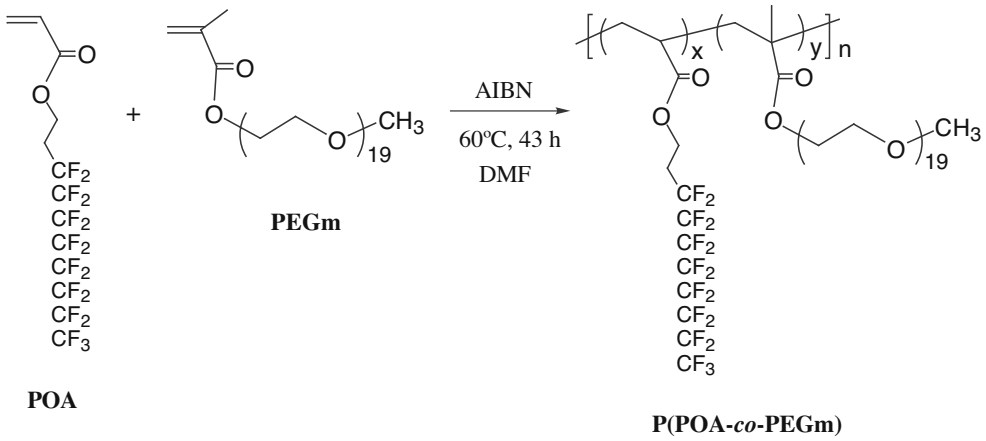

Fig. 2 Synthesis of the P(POA-*co*-PEGm) random copolymer

analysis. The distribution of the hydrodynamic diameter was obtained by the Marquadt analysis [28].

Cloud point measurement

A schematic of the experimental variable volume view cell used for the cloud point measurement is shown in Fig. 1. P(POA-*co*-PEGm) (30 mg) was placed in the cell, then CO_2 liquefied with a cooler was added to it. The cloud point was defined as the point at which the contents of the cell turned opaque, indicating precipitation of the polymer from solution.

Preparation of subtilisin covered with P(POA-*co*-PEGm)

P(POA-*co*-PEGm) (30 mg) and the subtilisin (3.5 mg) were placed in the variable volume view cell, then CO_2 liquefied with a cooler was added to the cell. The mixture was kept in homogeneous solution under 172 bar at 35 °C for 1 h. The pressure of the solution was diminished to 22 bar lower than the cloud point pressure (160 bar), then the mixture was sprayed out into a polyethylene bag. The product (8 mg) was obtained.

SEM measurement

The subtilisin covered with P(POA-*co*-PEGm) was put on a carbon adhesive tape and was subjected to SEM measurement after coated with Pt.

Activity assay

A synthetic peptide, Suc-Ala-Ala-Pro-Phe-*p*NA (3.2 mg) was dissolved in distilled DMSO (0.5 ml). A Tris–HCl buffer (0.1 M, pH 8.0) was added to the mixture, and the solution was adjusted to 50 ml with a volumetric flask. This synthetic peptide solution was kept in an ice bath before use. Subtilisin Carlsberg (2.1 mg) was dissolved in a borate buffer (50 mM, pH 7.5) and was adjusted to 50 ml. The subtilisin solution was also kept in an ice bath before use. The subtilisin solution (10 μl) was added the peptide solution (3 ml) at 25 °C. The initial velocity of the hydrolysis of the synthetic peptide by the subtilisin was determined by measuring variability in the UV absorbance at 410 nm over time at 25 °C. The absorption at 410 nm was based on *p*-nitroaniline generated by the hydrolysis. The initial velocity was estimated for five different concentrations of the peptide solution: 1.281, 2.563, 4.100, 5.125, and 10.25 ($\times 10^{-5}$ M).

For activity assay of the subtilisin placed in $scCO_2$ under 172 bar at 35 °C for 1 h in the presence of the copolymer, the product (8 mg) obtained from the mixture in $scCO_2$ was dissolved in 19.9 ml of the borate buffer.

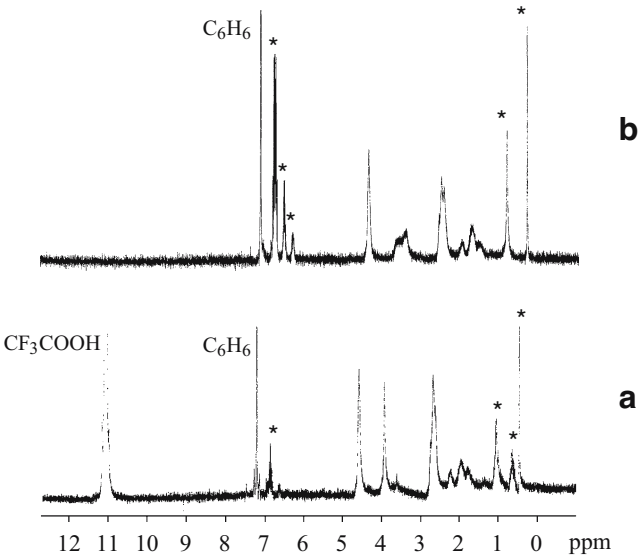

Fig. 3 ^{1}H NMR spectra of P(POA-*co*-PEGm) in the presence (**a**) and absence (**b**) of trifluoroacetic acid in C_6F_6. Lock solvent: C_6D_6. *Asterisk* indicates impurities in C_6F_6

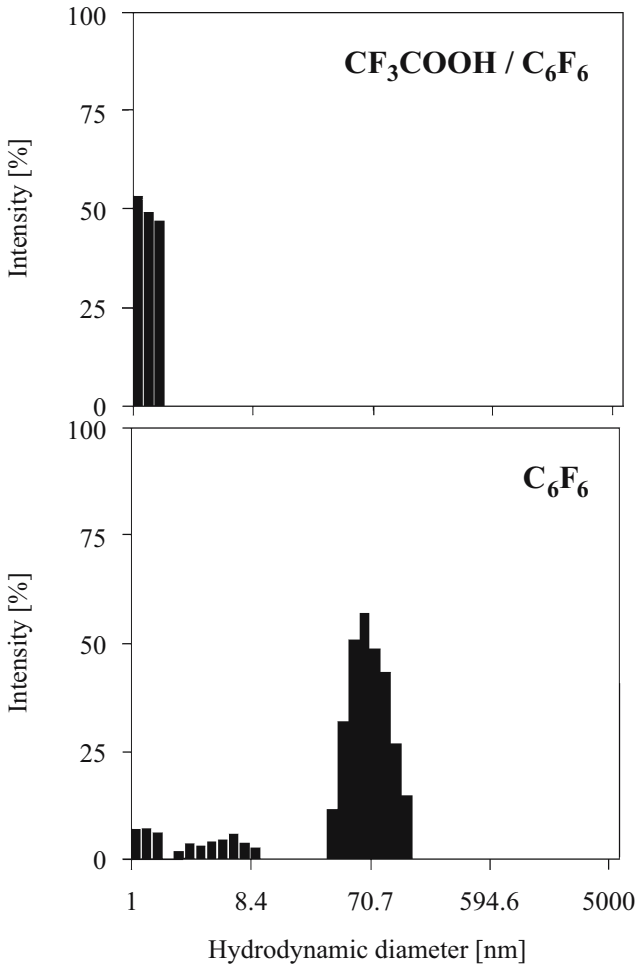

Fig. 4 Scattering intensity distribution of the hydrodynamic diameter of P(POA-*co*-PEGm) in the presence and absence of trifluoroacetic acid in C_6F_6. [copolymer], 8.00 g/l. [CF_3COOH], 0.190 mol/l

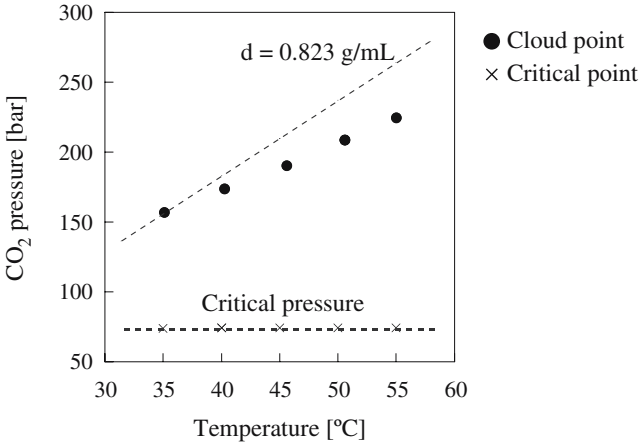

Fig. 6 Plots of the experimental cloud points of the copolymer at each temperature vs the CO_2 pressure. Copolymer, 30 mg. Initial volume, 4.17 ml

Results and discussion

The P(POA-*co*-PEGm) random copolymer was prepared by the radical copolymerization of POA and PEGm. The polymerization was carried out at a POA/PEGm feed ratio of 0.883:0.117 (mol/mol) in DMF using AIBN at 60 °C for 43 h (Fig. 2). The molecular weight and molecular weight distribution of the copolymer were estimated by SEC as Mn=133,000 and Mw/Mn=8.25, respectively, based on poly(methyl methacrylate) standards. Figure 3a shows the ^{1}H NMR spectrum of the copolymer. The spectrum was obtained in hexafluorobenzene in the presence of trifluoroacetic acid. Two different methylene protons in the POA units were observed at 4.58 and 2.64 ppm. The former was attributed to the methylene attached to the carboxyl group, and the latter to the methylene bonded to the perfluorooctyl group. The methylene protons of the main chain for the POA units were observed at 1.9–2.1 ppm, along with the

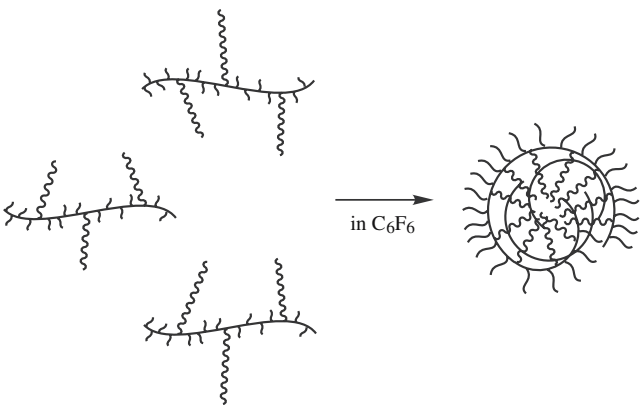

Fig. 5 Formation of micellar aggregates by the copolymer

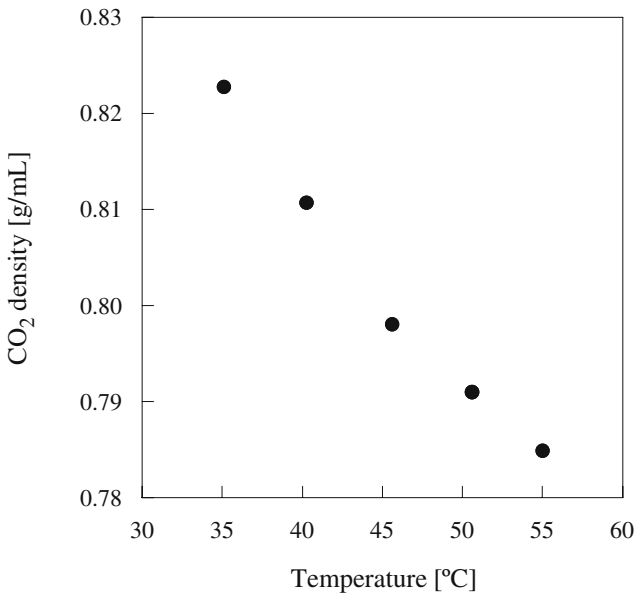

Fig. 7 Plots of the experimental cloud points of the copolymer at each temperature vs the CO_2 density. Copolymer, 30 mg. Initial volume, 4.17 ml

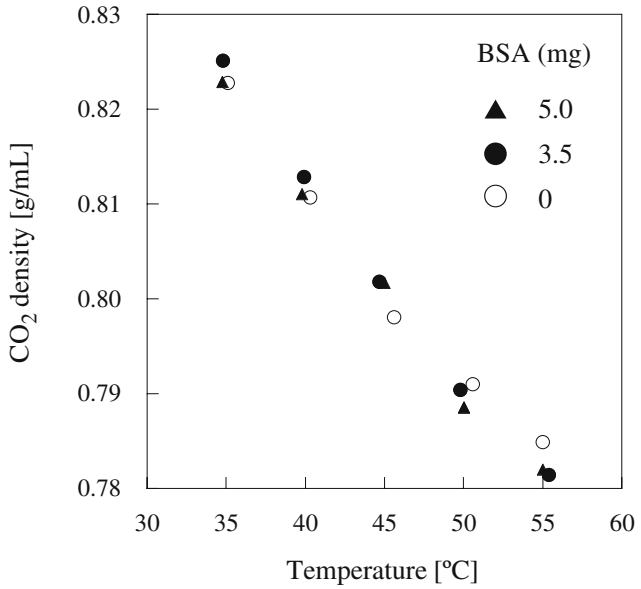

Fig. 8 Plots of the cloud points of the copolymer in the presence of BSA. Copolymer, 30 mg. Initial volume, 4.17 ml

methylene protons of the main chains of the PEGm units. The methyl protons attached to the main chains of the PEGm units were discerned at 2.1–2.3 ppm. The methylene protons based on the ethylene glycol units were observed at 3.95 ppm, while the methyl protons at the chain end of the PEG chain was evident at 3.59 ppm. The molar ratio of the POA and PEGm units in the copolymer was determined on the basis of the signal intensity at 4.58 and 3.95 ppm. The molar ratio of the units was estimated to be POA/PEGm= 0.972:0.028. A very small amount of PEGm polymerized with POA. However, it is possible that the PEG-rich copolymer was removed by reprecipitation into water along with the PEGm remaining unreacted. The PEG-rich copolymer may be produced as a block copolymer by the slow polymerization of PEGm at the end of the polymerization. During the terminal stage of the polymerization, the chain transfer occurs more often than the propagation, resulting in the formation of a block copolymer with a low molecular weight. Therefore, the PEG-rich block copolymer should be removed, and the obtained copolymer is expected as a random copolymer.

Hexafluorobenzene is regarded as a model compound of $scCO_2$ because it has solvent qualities similar to $scCO_2$ regarding the low polarity and ability to dissolve fluoropolymers. When the ^1H NMR measurement of the copolymer was performed in the absence of trifluoroacetic

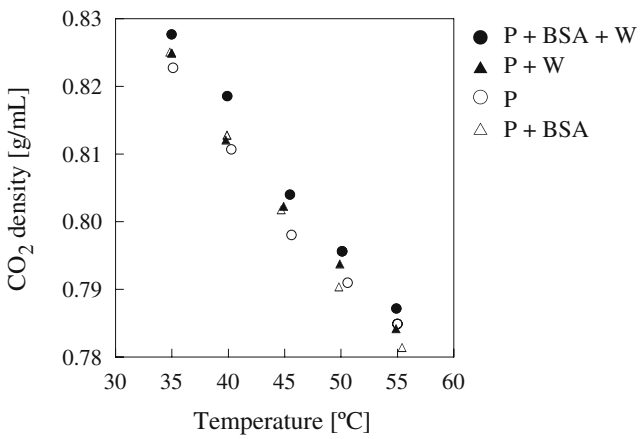

Fig. 9 Plots of the cloud points of the copolymer in the presence of BSA and water. *P*: copolymer (30 mg), *W*: water (3.5 mg), BSA (3.5 mg). Initial volume, 4.17 ml

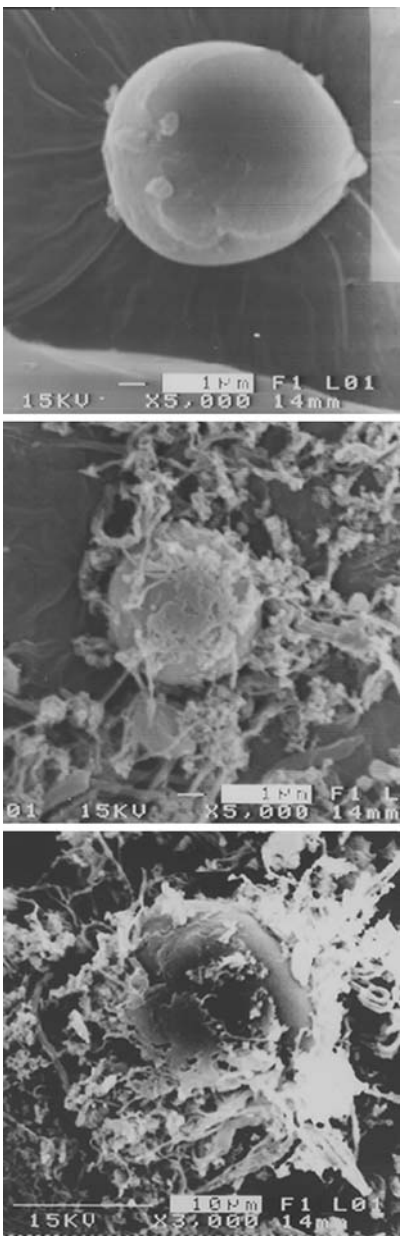

Fig. 10 SEM images of BSA obtained from the heterogeneous state in $scCO_2$ in the presence of the copolymer and water. *P*: copolymer (30 mg), *W*: water (3.5 mg), BSA (3.5 mg)

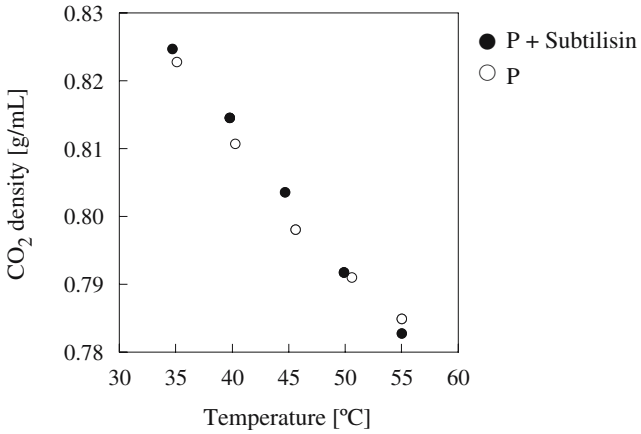

Fig. 11 Plots of the cloud points of the copolymer in the presence of the subtilisin. *P*: copolymer (30 mg), subtilisin (2.0 mg). Initial volume, 4.17 ml

acid, the ethylene protons attributed to the PEG chains were observed as a short signal, accompanied by a decrease in the signal intensity (Fig. 3b). In addition, the signals of the POA units slightly changed in the absence of the acid. These results suggest that the PEG chains aggregated in the hexafluorobenzene and were covered with the POA segments. Dynamic light scattering demonstrated that the copolymer formed aggregates in the absence of trifluoroacetic acid in hexafluorobenzene. Figure 4 shows the scattering intensity distributions of the hydrodynamic diameter of the copolymer in the absence and presence of trifluoroacetic acid. The hydrodynamic diameters were determined by the cumulant analysis. The hydrodynamic diameter of the copolymer was estimated to be 3.3 nm in the presence of the acid, indicating that the copolymer existed as isolated copolymers in its presence. In its absence, the diameter was 41.3 nm. The copolymer should form micellar aggregates in hexafluorobenzene by association of the insoluble PEG chains (Fig. 5). The formation of micellar aggregates by the copolymer in hexafluorobenzene suggests that the copolymer is expected to serve as a surfactant in $scCO_2$.

The copolymer was easily dissolved in $scCO_2$. We explored the solubility behavior of the copolymer in $scCO_2$ by measuring the cloud point of the copolymer using a variable volume view cell. Figure 6 shows the relationship between the CO_2 pressure and temperature at the cloud point of the copolymer. The copolymer had a cloud point much higher than the critical pressure. At a pressure lower than the cloud point pressure, the copolymer was not completely dissolved and formed two phases as a liquid and solid. The cloud point pressure increased with an increase in the temperature. This phenomenon is natural, as the CO_2

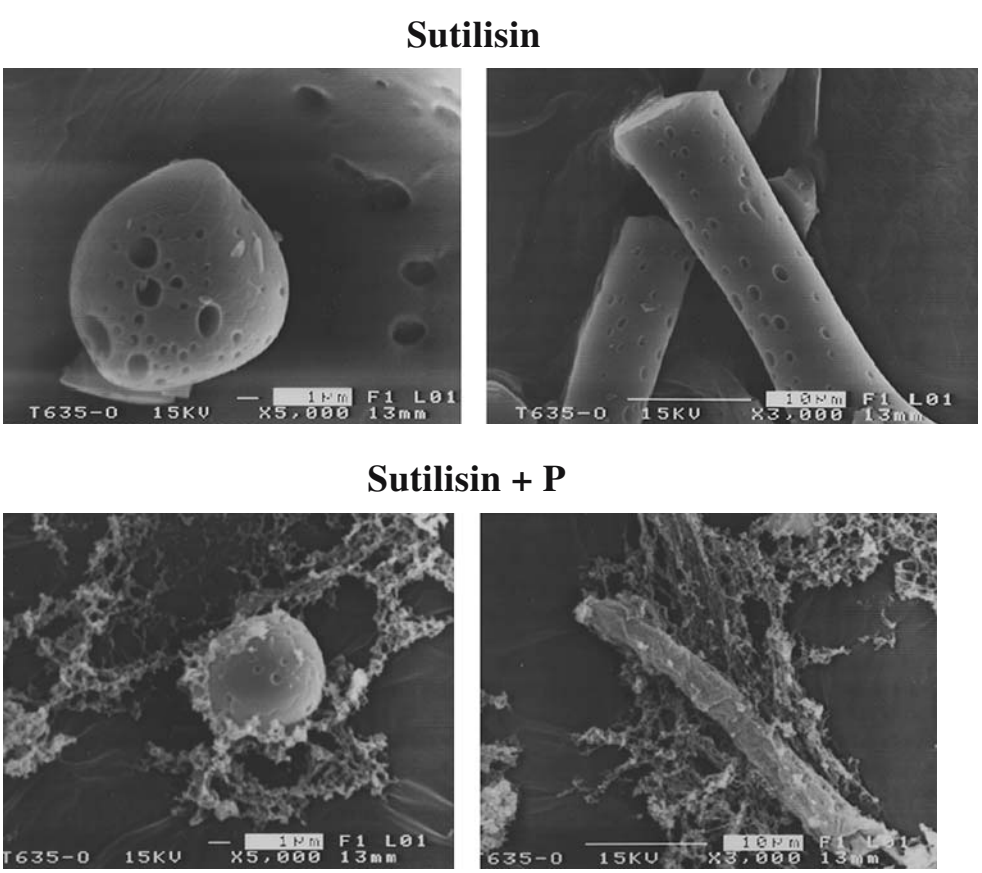

Fig. 12 SEM images of the native subtilisin and subtilisin obtained from the heterogeneous state in $scCO_2$ in the presence of the copolymer. *P*: copolymer (30 mg), subtilisin (2.0 mg)

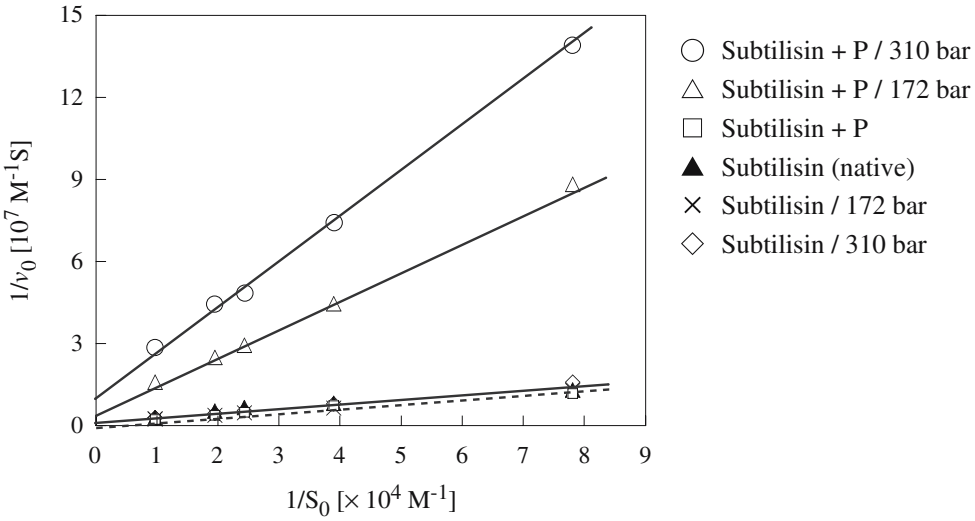

Fig. 13 The Lineweaver–Burk plots of the subtilisins placed under the different conditions. *P*: copolymer 25 °C, pH 8.0

pressure increases with an increase in temperature at an identical CO_2 density. However, as the temperature increased, the difference was greater between the cloud point pressure and the pressure at the same CO_2 density of 0.823 g/ml. From a plot of the cloud points versus the CO_2 density, the CO_2 density at the cloud point decreased as the temperature increased (Fig. 7). The solubility of the copolymer in $scCO_2$ increased with an increase in the temperature.

While PEG is CO_2-phobic, this polymer has a low toxicity for living tissue. Many water-soluble biopolymers such as BSA and protease are insoluble in $scCO_2$. Accordingly, they should be taken into the PEG cores of the P(POA-*co*-PEGm) micelles when placed in $scCO_2$. BSA and subtilisin were completely dissolved in $scCO_2$ in the presence of the P(POA-*co*-PEGm) copolymer. Figures 8 and 9 shows the cloud point behavior of the copolymer in the presence of BSA. The cloud points were slightly shifted to the lower side of the CO_2 density by BSA at higher temperatures, although they made negligible changes at the lower temperatures. When a small amount of water was added to the mixture, the cloud points clearly shifted to the higher side of the CO_2 density. This shift of the cloud points to the higher CO_2 density indicates that the solubility of the copolymer in $scCO_2$ decreased in the presence of BSA and water. In the absence of BSA, water had a negligible effect on the cloud points of the copolymer. This suggested that the PEG chains have a loose interaction with BSA through a van der Waals attraction in the absence of water. On the other hand, the PEG chains should be tightly attached to BSA via the hydrogen bonding of water. SEM observations revealed that the copolymer was more tightly adsorbed on BSA in the presence of water as compared to that in the absence of water (Fig. 10). As can be seen, more of the copolymers gathered around BSA, and there are no spaces between the copolymer and BSA in the presence of water as compared with those in its absence. The water should relax the van der Waals repulsion between the PEG and BSA by hydrogen bonding.

The subtilisin also showed negligible changes in the cloud points of the copolymer in the absence of water

Table 1 The k_{cat}/K_M for the subtilisins placed under the difference conditions

Copolymer	Pressure/bar	$k_{cat}/K_M/M^{-1}s^{-1}$
−	–	1
−	172	0.914
−	310	0.716
+	–	1.05
+	172	0.129
+	310	0.0845

The subtilisins placed at 35 °C for 1 h in $scCO_2$

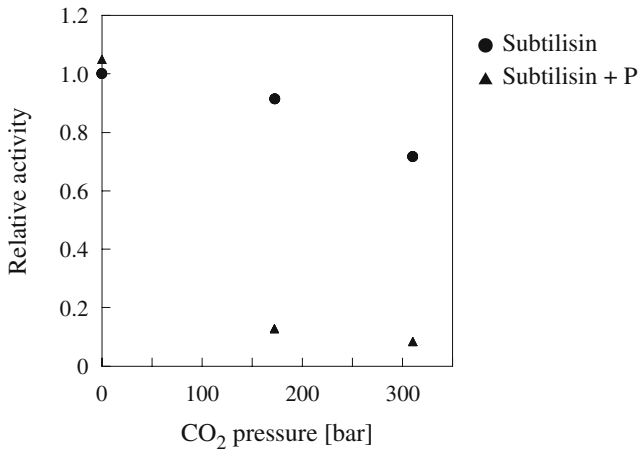

Fig. 14 Relative activity of the subtilisins placed under the different conditions. *P* Copolymer

(Fig. 11). This enzyme causes autolysis in water so that the cloud points were measured in the absence of water. As can be seen in Fig. 12, the subtilisin was also covered with the copolymer in $scCO_2$. We examined the activity of the subtilisin placed in $scCO_2$ in the presence and absence of the copolymer. The catalytic activity of the subtilisins was evaluated for the synthetic peptide, Suc-Ala-Ala-Pro-Phe-*p*NA, at 25 °C in Tris–buffer at pH 8.0. The Lineweaver–Burk plots of the subtilisins are shown in Fig. 13. The k_{cat}/K_M was estimated from the plots and summarized in Table 1. Based on the respective k_{cat}/K_M, the relative activity was determined for the subtilisins placed under the different conditions (Fig. 14). The presence of the copolymer did not affect the activity of the native under atmosphere. The activity somewhat decreased for the subtilisins placed solely in $scCO_2$. On the other hand, a marked decrease in the activity was observed for the subtilisins placed in the presence of the copolymer in $scCO_2$. The decrease in the activity was greater as the CO_2 pressure increased. This decrease in the activity should be caused by the copolymer covering the active site of the subtilisin in $scCO_2$.

Conclusion

The P(POA-*co*-PEGm) random copolymer was prepared by radical polymerization as a new surfactant for $scCO_2$. The copolymer was dissolved in $scCO_2$ at a much higher pressure than the critical pressure. The solubility of the copolymer in $scCO_2$ increased with an increase in the temperature. It was suggested that the copolymer formed micellar aggregates with the cores of the PEG chains in $scCO_2$ based on the dynamic light scattering studies of the copolymer in hexafluorobenzene. The copolymer solubilized the CO_2-phobic proteins of BSA and the subtilisin in $scCO_2$. The solubility of the copolymer was not influenced by the presence of proteins, while the solubility of the copolymer with BSA decreased in the presence of a small amount of water. This was accounted for by the fact that in the absence of water, the PEG chains had a loose van der Waals interaction with the proteins, whereas the PEG chains were tightly adsorbed on the surface of the protein via the hydrogen bonding of water. The activity of the subtilisin slightly decreased when solely placed in $scCO_2$. On the other hand, a marked decrease in the activity was observed for the subtilisin and the copolymer added to the $scCO_2$. The activity of the subtilisin decreased as the CO_2 pressure increased.

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