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ORIGINAL CONTRIBUTION

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Preparation and characterization of conjugates of silk fibroin and chitooligosaccharides

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Abstract With the aim of the functionalization of silk fibroin (SF), conjugates of SF and polycationic chitooligosaccharides (COS) were prepared by the chemical modification of SF with cyanuric chloride (CY)-activated COS (COS-CY). The ^{1}H NMR spectrum of the reaction product between a model compound D-glucosamine and CY suggested that the COS-CY modifier was synthesized by the reaction of the amino group and the terminal anomeric hydroxyl group in COS, with the chlorine atom of CY. The ^{1}H NMR spectrum and amino acid analysis of the conjugates (COS-CY-SF) clarified that the tyrosine and lysine residues of SF reacted with a second chlorine atom of the triazine ring of the modifier. On the basis of the results of the hexosamine determination and the amino acid analysis of COS-CY-SF, it is estimated that COS-CY-SF consists of 38 wt% COS, 8 wt% CY, and 54 wt% SF. The absorbance at 600 nm as a function of pH for COS-CY-SF and SF indicated that the introduction of a large amount of hexosamine made SF amphiphilic and more water-soluble at lower pH values. The COS-CY-SF conjugates retarded the growth of *Escherichia coli* after incubation for 24 h at a conjugate concentration of 0.6% (w/v), while SF did not retard the growth at a SF concentration of 0.7% (w/v).

Keywords Silk fibroin · Chitooligosaccharides · Conjugates · pH-dependent solubility · Bacterial growth retardation

Introduction

Silk fibroin (SF) is a structural protein created by the *Bombyx mori* silkworm and mainly consists of the Gly-Ala-Gly-Ala-Gly-Ser sequence [1, 2]. Although SF fiber has been used as a textile material for a long time, there have been recent attempts to utilize SF as biomaterials and medical materials in forms of membranes and/or gels, for example, enzyme-immobilization matrices [3, 4], cell culture substrates for fibroblast cells [5, 6], and an oral dosage form [7]. Moreover, because of its unique properties, such as antithrombogenicity [8] and high oxygen and water vapor permeabilities [9], SF is found to be applicable to vascular prostheses [8] and burn wound dressings [9]. However, as applied studies on SF are still limited at present, we intended to examine further the applicability of SF materials in biomedical and biotechnological fields by carrying out chemical modification of SF. We have already reported the chemical modifications of SF with poly(ethylene glycol) [10] and natural oligosaccharides [11]. Poly(ethylene glycol)-modified SF is considered to be applicable to antiadhesion membranes owing to very low attachment and growth of fibroblast cells [10].

Chitin and chitosan are abundant natural polysaccharides and the study of their utilization has been extensively carried out owing to their functionality in biotechnological and medical applications [12, 13]. In

particular, chitosan, which is positively charged under slightly acidic conditions and can complex with DNA through electrostatic interaction, has been recently used as a gene carrier for delivering DNA to cells or tissues [14, 15]. It has also been reported that water-soluble, lower analogues of chitin and chitosan, such as *N*-acetyl chitooligosaccharides (NACOS) [16, 17] and chitooligosaccharides (COS) [17, 18] are biologically active substances. Thus, we previously prepared the conjugates of SF and NACOS (NACOS-cyanuric chloride (CY)-SF) by the homogenous chemical modification of SF in aqueous solution [11].

In the present work, COS obtained by chemical [19] or enzymatic [18] hydrolysis of chitosan was chosen as a modifier oligosaccharide because of its high water-solubility and unique polyationic nature. It is considered that the conjugation with COS endows SF with polycationic nature and facilitates charge–charge interactions of SF with DNA or with cells bearing negative charges on their surface [20]. Therefore, such conjugates may have many applications in the field of gene delivery system or tissue engineering.

The conjugates of SF and COS (COS-CY-SF) were prepared by the reaction of amino acid residues of solubilized SF with CY-activated COS (COS-CY). To clarify the chemical structure of the COS-CY modifier and the COS-CY-SF conjugates, D-glucosamine (GlcN), which is a monomer unit of COS, was used as a model compound of COS and reacted with CY. The composition of the COS-CY-SF conjugates was determined by a modified Elson–Morgan procedure for the determination of hexosamines [21] and amino acid analysis. The COS-CY-SF conjugates obtained were also characterized by the pH-dependent solubility and the retardation effect on the growth of *Escherichia coli*.

Experimental

Chemicals

"Chitosan pentamer" (pentasaccharide of GlcN·HCl, $C_{30}H_{57}N_5O_{21}\cdot 5HCl$, 1,006) obtained from molecular weight of Seikagaku Co. (Tokyo, Japan) was used as COS. GlcN·HCl and CY were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetylacetone was supplied by Dojindo (Kumamoto, Japan). All the other chemicals were commercial reagent-grade products.

Preparation of the COS-CY-SF conjugates

COS-CY-SF conjugates were synthesized in a similar manner to the preparation of NACOS-CY-SF conjugates as previously reported [11]. A solution of 75 mg COS in 4.5 ml distilled water was prepared. To the solution, 30 mg CY in 1.5 ml 1,4-dioxane was slowly added at 4 °C over 15 min and the pH of the mixture was kept at about 9 by adding 20% (w/v) Na_2CO_3 solution. The mixture was stirred at 4 °C for 2 h and the pH was maintained at about 9 to prepare the COS-CY modifier.

B. mori cocoons were degummed with aqueous boiling 0.5% (w/v) Na_2CO_3 solution to remove sericin protein from SF fiber. An aqueous solution of SF was prepared by dissolving degummed SF fiber in 9 M LiBr aqueous solution and dialyzing the dissolved solution against distilled water [8, 10, 11, 22]. Although native SF has a molecular weight of 370 kDa [2], the LiBr-solubilized SF has a heterogeneous molecular weight in the range of 30 kDa to more than 200 kDa [22]. To the COS-CY solution, 3.2 ml 1.9% (w/v) SF aqueous solution containing 61 mg SF was added. The mixture was then incubated at 37 °C and pH 8.5 for 3 h in order to make SF react with the COS-CY modifier. The resulting mixture containing the COS-CY-SF product was dialyzed against distilled water using a Spectra/Por dialysis membrane (molecular-weight cutoff of 12,000–14,000, Spectrum Laboratories, Rancho Dominguez, Calif., USA) for 1 day to stop the reaction and separate unreacted reagents.

After the dialysis, a slightly turbid solution containing a small amount of white precipitate was obtained. This insoluble precipitate was estimated to be a cross-linked product of COS-CY, since a large amount of white precipitate appeared after further incubation of COS-CY at 37 °C and pH 8.5 for 3 h without adding SF solution. The white precipitate was easily separated by centrifugation at 3,000*g* for 10 min, and a supernatant containing only COS-CY-SF product was obtained. The supernatant was concentrated to about 4 ml using ultrafiltration with a Molecut-L TK-kit (Nihon Millipore, Tokyo, Japan). The concentrated COS-CY-SF solution was cast onto polyethylene film and dried at ambient relative humidity at room temperature [9, 10], and transparent COS-CY-SF films were then obtained. The COS-CY-SF films were further dried in a desiccator containing P_2O_5 for several days and 106 mg COS-CY-SF product was obtained.

The COS-CY-SF films were made water-insoluble by immersion in 90% (v/v) methanol/10% (v/v) water for 30 min [5, 9, 10]. After the water-insoluble COS-CY-SF films had been soaked in distilled water for 1 day, a weight loss of the COS-CY-SF films was scarcely observed. For comparison, blended films (the weight ratio of COS to SF was 0.67) were obtained by casting a mixture of aqueous SF solution and aqueous COS solution, and subsequently treating them with methanol aqueous solution as described earlier. After soaking in distilled water, the blended films showed a weight loss corresponding to the removal of COS from the not solubilized SF films.

Reaction of GlcN with CY

A solution of 216 mg (1 mmol) GlcN·HCl in 8 ml distilled water was cooled to 4 °C. To the solution, 461 mg (2.5 mmol) CY in 6 ml 1,4-dioxane was added at 4 °C over 30 min and the pH of the mixture was kept at about 9. The reaction mixture was stirred at 4 °C and pH 9 for 3 h. After the reaction, the reaction mixture was neutralized and centrifuged. The supernatant was evaporated and crude product GlcN-CY was obtained. To avoid the hydrolysis of GlcN-CY [11], this crude product was dissolved in D_2O and was used without purification for the measurement of the ^1H NMR spectrum of GlcN-CY.

NMR measurements

^1H NMR spectra of COS-CY-SF, COS, and SF were obtained in D_2O at 400 MHz using a Brüker ASX400WB NMR spectrometer. ^1H NMR spectra of GlcN and GlcN-CY were recorded in D_2O at 300 MHz with a Varian 300BB NMR spectrometer. 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) was used as an internal standard. The sample of SF for analysis by ^1H NMR was prepared by dissolving the SF films obtained in a similar manner to that of COS-CY-SF films in D_2O [11]. Since the COS-CY-SF films were not completely soluble in water, the aqueous solution of

COS-CY-SF was exchanged with D_2O using ultrafiltration and the spectrum of COS-CY-SF was measured in a solution containing more than 99% (v/v) D_2O. The ^1H NMR spectra of COS-CY-SF and SF in D_2O were measured at a concentration of 1–2% (w/v).

Amino acid analysis

Each film sample (13 mg) of SF and COS-CY-SF was hydrolyzed using vacuum hydrolysis tubes and a heating module with an aluminum heating block (Pierce, Rockford, Ill., USA) under reduced pressure in 2 ml 6 N HCl at 110 °C for 20 h. The hydrolyzed samples were dried in a rotary evaporator at 40 °C, dissolved in 10 ml 0.02 N HCl, and filtered. The filtrate was applied to a Hitachi L-8500 type rapid amino acid analyzer.

Determination of COS content in the COS-CY-SF conjugates

A modified Elson–Morgan procedure for the determination of hexosamines developed by Neuhaus and Letzring [21] was used to determine the content of COS in the COS-CY-SF conjugates. In this procedure, the compound formed by GlcN and acetylacetone is extracted with isoamyl alcohol so that it can be separated from interfering substances. The final chromogen is then produced by adding Ehrlich's reagent to the alcoholic extract [21]. Therefore, this procedure minimizes interference from colored hydrolyzate and nonglucosamine substances that frequently react with Ehrlich's reagent. Since it is necessary to take into account the partial degradation of GlcN under hydrolysis, the hydrolysis product of COS was used as a standard instead of GlcN.

The film sample of COS-CY-SF (11.5 mg) and the powder of COS (10 mg) were hydrolyzed using vacuum hydrolysis tubes and a heating module under reduced pressure in 2 ml 4 N HCl at 110 °C for 15 h. The hydrolyzates were dried in a rotary evaporator at 40 °C. These concentrates were diluted with distilled water and neutralized with 1 N NaOH. The sample solutions were diluted to contain 5–40 mg/l hydrolyzed COS and were used for the determination of the COS content.

The acetylacetone solution was prepared by adding 0.3 ml acetylacetone to 10 ml carbonate buffer (pH 10) containing 8 g Na_2CO_3 and 2.1 g $NaHCO_3$ in 100 ml. Ehrlich's reagent was prepared by dissolving 0.4 g *p*-dimethylaminobenzaldehyde in a mixture of 1.5 ml concentrated HCl and 13.5 ml isoamyl alcohol. Acetylacetone solution (1 ml) was added to a 1 ml sample solution in a test tube. After mixing, the tubes were stoppered and heated for 20 min at 100 °C. After cooling, 5 ml isoamyl alcohol was added and the tubes were shaken vigorously for 2 min. The layers were separated on standing. Ehrlich's reagent (1 ml) was added to 4 ml of the alcoholic extract in a test tube, and the mixture was thoroughly shaken. After 15 min, the absorbances at 530 nm were measured. All the measurements were made against a reagent blank prepared by substituting 1 ml distilled water for the sample. The absorbances were converted to micrograms of COS by means of a standard curve. The standard curve was prepared by using 1 ml aliquots of the standard solutions containing 5–40 mg/l hydrolyzed COS, and a straight line was obtained.

pH-dependent solubility

The pH of 1.9 ml 1% (w/v) aqueous COS-CY-SF and SF solutions was adjusted to 12 with 1 N NaOH. The absorbance of the solutions was monitored at 600 nm at ambient temperature using a JASCO V-550 UV/vis spectrophotometer while the pH of both samples was lowered through addition of 1 N HCl [23]. The samples were stirred slowly during the addition of the HCl and were allowed to equilibrate for 3 min before each measurement.

Effect of COS-CY-SF on the growth of *E. coli*

The growth of *E. coli* JCM109 in the nutrient broth containing COS-CY-SF was examined to evaluate the effect of COS-CY-SF on the bacterial growth. *E. coli* JCM109 was grown for 24 h on nutrient agar slants. The lawn of bacteria grown on the nutrient agar was scraped with a platinum loop, and it was suspended in 5 ml nutrient broth. *E. coli* JCM109 was grown for 24 h at 37 °C in nutrient broth under rotary shaking of 80 rpm and washed with saline solution by centrifugation, and then the bacterial suspension for the experiment was prepared.

The pH of the aqueous sample (COS-CY-SF, SF, or COS) solution was adjusted to 7.0–7.2 with 2.5 N NaOH, and the sample solution was filtered through a Millex-GV13 membrane filter (0.22 μm, Nihon Millipore, Tokyo, Japan) for sterilization. To 0.9 ml of the sterile nutrient broth, the concentration of which was twice that of standard nutrient broth with the pH value adjusted to 7.0, 0.9 ml of the filtrate was added. Next, 50 μl of the bacterial culture was added to 1.8 ml of the medium. The initial bacterial concentration in the culture medium was adjusted to 10^5–10^6 CFU/ml. *E. coli* JCM109 was grown for 24 h at 37 °C in the nutrient broth containing the solubilized sample under rotary shaking of 80 rpm. Aliquots (50 μl) were taken periodically (0, 2, 4, 8, and 24 h) and diluted ten times with distilled water. The degree of growth was determined by measuring the turbidity of the diluted culture medium at 660 nm [18]. The nutrient broth which did not contain the test sample was used as a control.

Results and discussion

^1H NMR spectrum of the model compound GlcN-CY

The NMR spectra of GlcN before and after the reaction with CY were measured to determine the chemical structure of the COS-CY modifier and the COS-CY-SF conjugates. It is known that the signals of the anomeric proton (H-1) are at the lowest region of the spectrum of GlcN, whereas the signals of the methine proton bearing the amino group (H-2) are at the highest region [24]. Two doublets centered at 4.94 and 5.44 ppm were therefore assigned to the H-1 of the β and α forms of GlcN, respectively (Fig. 1). The H-2 signals of the β anomer and the α anomer of GlcN appeared as quartets centered at 3.00 and 3.29 ppm, respectively.

In the spectrum of the GlcN-CY crude product prepared by the reaction of GlcN and CY in the molar ratio of 1: 2.5, the original H-2 signals completely disappeared and no signal was observed in the upfield region from H-2 (Fig. 1). Consequently, it is estimated that the H-2 signals of GlcN-CY appeared at 3.4–4.4 ppm overlapping the signals of other methine and methylene protons, and that the H-2 signal of the β form of GlcN-CY shifted more than 0.4 ppm downfield compared to the corresponding one of GlcN. The downfield shifts of the H-2 can be explained by deshielding by the heterocyclic triazine ring of CY [25]. This means that the nucleophilic amino group of GlcN reacted with a chlorine atom of CY (Fig. 2).

In the region of H-1 signals, a weak signal at 5.33 ppm was observed; however, new signals showed

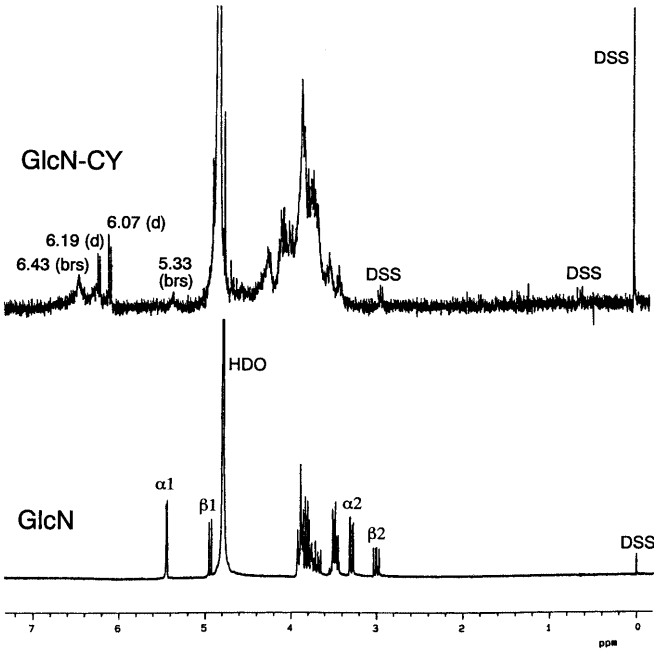

Fig. 1 ^{1}H NMR spectra of D-glucosamine (GlcN) and the GlcN-cyanuric chloride (CY) crude product in D_2O

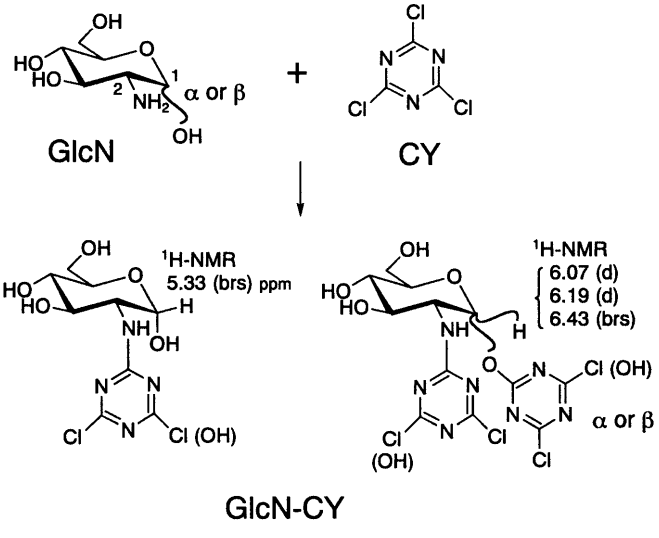

Fig. 2 Reaction of GlcN with CY

up at a lower field. In our previous study, large downfield shifts were observed for the H-1 of the reaction product of *N*-acetyl-GlcN (GlcNAc), which is a monomer unit of NACOS and was used as a model compound of NACOS, with CY compared to the corresponding ones of standard GlcNAc [11]. This downfield shift suggested that the anomeric hydroxyl group of GlcNAc was the reaction site with CY [11]. On the basis of the previous findings, the signals at 6.05–6.45 ppm were assigned to the H-1 of GlcN-CY, the anomeric hydroxyl group of which reacted with CY. These results reveal that not only the amino group but also the anomeric hydroxyl group in GlcN and COS reacted with CY (Fig. 2). Not two signals but three signals appeared in the interval 6.05–6.45 ppm for H-1 of the α and β forms of GlcN-CY. This fact indicates that the chlorine atoms of GlcN-CY were partially or completely hydrolyzed under the basic reaction condition. The signal at 5.33 ppm was assigned to the H-1 of the α anomer of GlcN-CY bearing a substituted amino group and a free anomeric hydroxyl group (Fig. 2), because the deshielding effect of the triazine ring bound to the amino group on the H-1 of GlcN-CY was probably small and the H-1 of GlcN-CY bearing a free anomeric hydroxyl group was thought to be almost intact.

The ^{1}H NMR spectrum of GlcN-CY confirms that both the amino group and the anomeric hydroxyl group of GlcN reacted with CY, and suggests that the COS-CY modifier was prepared by the reaction of a terminal anomeric hydroxyl group in COS with CY as well as by the reaction of plural amino groups in COS with CY. COS-CY was prepared by adding excess CY to COS in a molar ratio of 2.2:1 at 4 °C. Johnson et al. [26] reported that the first chlorine atom of CY reacts at 4 °C, whereas the second one reacts at 25 °C. Owing to such a low reactivity of the second chlorine atom of CY at 4 °C, we conclude that COS is not cross-linked by CY but is disubstituted by CY under the condition for the preparation of COS-CY.

Chemical structure and composition of COS-CY-SF

The ^{1}H NMR spectra of COS-CY-SF, SF, and COS are shown in Fig. 3. Compared with the spectrum of SF [11], the signals of COS-CY-SF became broader because the resonances of SF overlapped with those of the introduced COS. The new broad signals in the interval 3.25–3.8 ppm were attributed to H-2 and H-5 methine protons and H-6 methylene protons of COS. In the interval 3.8–4.0 ppm, the signals for SF and the signals for H-3, H-4, and H-6 of COS overlapped with each other and these signals became broader. These results suggest the successful introduction of COS into SF.

In the spectrum of COS-CY-SF, a broad peak appeared at about 7.2 ppm, while original peaks at 6.79 and 7.06 ppm attributed to the aromatic protons of the tyrosine residue in SF [11] disappeared. This downfield shift of the tyrosine residue was similarly observed for NACOS-CY-SF in our previous study [11]. These downfield shifts were caused by the deshielding of the triazine ring of CY [25] and support the fact that the phenolic hydroxyl group of the tyrosine residue in SF reacted with the CY-activated modifier [11].

In order to investigate other reaction sites, the amino acid analyses of SF before and after the modification

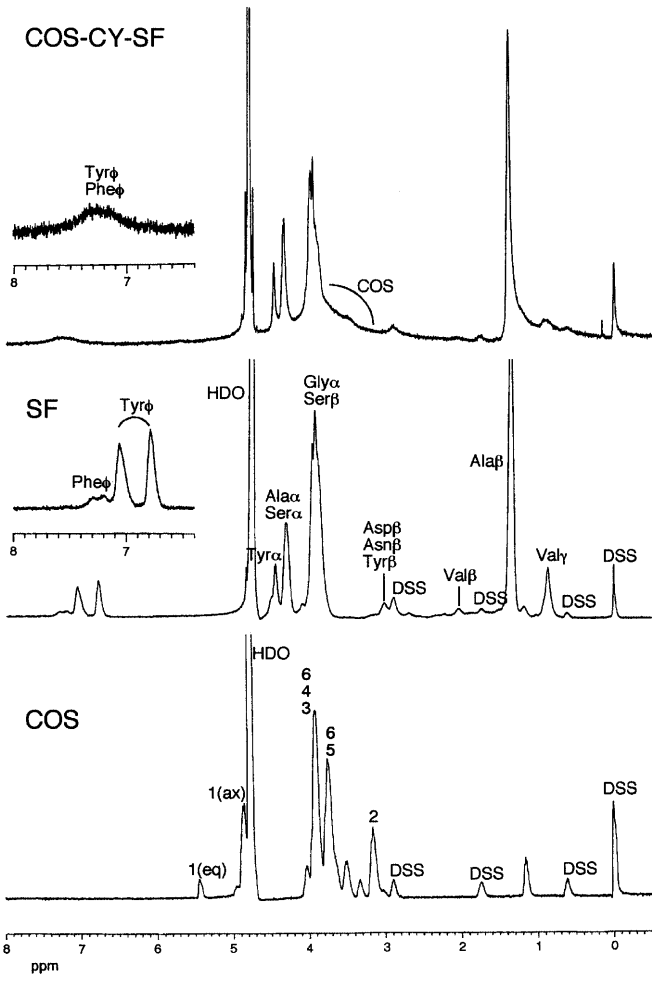

Fig. 3 ^{1}H NMR spectra of chitooligosaccharides (COS), silk fibroin (SF), and COS-CY-SF in D_2O

were carried out. The lysine content in COS-CY-SF was 0.12 mol%, while the content in SF was 0.32 mol%. A considerable decrease in the lysine content in COS-CY-SF means that the ϵ-amino group of the lysine residue reacted with the chlorine atom of the triazine ring of the modifier, and the bond between the ϵ-amino group and the triazine ring was partially hydrolyzed under hydrolysis conditions [11]. There was no significant decrease in other amino acid contents of COS-CY-SF compared with those of SF. The tyrosine contents in COS-CY-SF and SF were 4.9 and 5.2 mol%, respectively. A significant reduction in the tyrosine content was not observed for COS-CY-SF in the same manner as for NACOS-CY-SF [11]. In our previous investigation, it was demonstrated that since the ether linkage between the triazine ring and the hydroxyl group of the tyrosine residue was weaker than the linkage between the triazine ring and the amino group, the ether linkage was completely cleaved during the hydrolysis for amino acid analysis [11].

^{1}H NMR measurement of GlcN-CY and amino acid analysis of COS-CY-SF suggest the following reaction mechanism and the chemical structure of COS-CY-SF (Fig. 4). The COS-CY modifier was prepared by the reaction of the amino groups and the terminal anomeric hydroxyl group in COS with a chlorine atom of CY, after which a second chlorine atom of the COS-CY modifier reacted with the phenolic hydroxyl group of the tyrosine residue and ϵ-amino group of the lysine residue in SF.

The amino acid analyses of the same weight samples of SF and COS-CY-SF indicated that COS-CY-SF contains 54% amino acids of SF. This means that the content of SF in COS-CY-SF is 54 wt%. From 60 mg SF starting material, 106 mg COS-CY-SF product was obtained. Thus, the content of SF in COS-CY-SF is roughly calculated to be 58 wt%. This value does not greatly differ from the value obtained from the amino acid analysis. A modified Elson–Morgan procedure was applied to the determination of the content of COS in COS-CY-SF. By using the calibration curves for the hydrolyzed COS, it is evaluated that COS-CY-SF contains 38 wt% COS. According to the results of the amino acid analysis and the hexosamine determination, it is estimated that COS-CY-SF consists of 38 wt% COS, 8 wt% CY, and 54 wt% SF, and hence the weight ratio of COS to SF in the conjugates is calculated to be 0.70 (i.e., 38/54). We confirm that the reaction sites of SF are the tyrosine and lysine residues, the contents of which are 5.2 and 0.32 mol%, respectively. The average molecular weight of the amino acid unit constituting SF is 75 [11], and the molecular weight of COS is 1,006. If the weight of the SF starting material is assumed to be unity, the weight ratio of COS to SF in the conjugates is

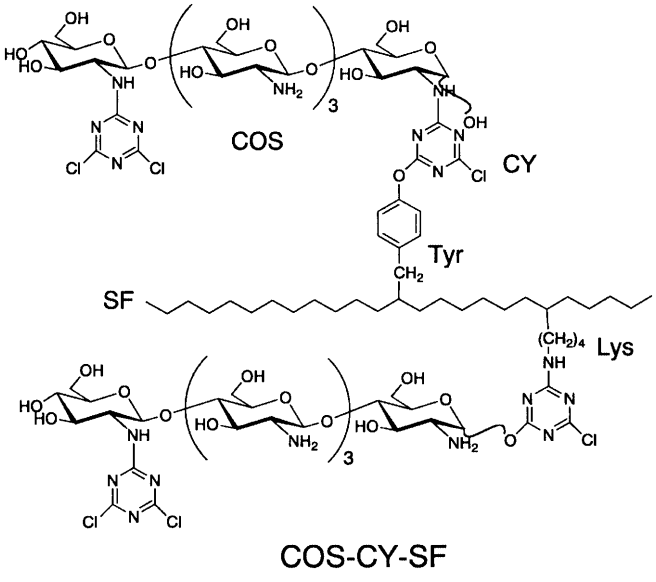

Fig. 4 Chemical structure of COS-CY-SF

calculated to be 0.74 [i.e., (1/75)×0.055×1,006]. Therefore, the experimental value obtained from amino acid analysis and hexosamine determination is consistent with the expected one.

pH-dependent solubility of COS-CY-SF

During purification of COS-CY-SF, it was noted that COS-CY-SF and SF exhibited differences in the pH-dependent solubility. The absorbance at 600 nm as a function of pH for COS-CY-SF and SF is shown in Fig. 5. Prior to precipitation, the SF solution showed a small increase in the optical density owing to the partial acid titration of the ϵ-amino group of the lysine residue and the phenolic hydroxyl group of the tyrosine residue. When the value of pH decreased from 5 to 2 and the carboxyl groups of the aspartic acid residue and the glutamic acid residue were titrated, it was found that SF precipitated and finally gelled exhibiting similar behavior to that of artificial protein comprising repeats of the sequence –(Ala–Gly)$_3$–Pro–Glu–Gly– [23]. In contrast to the SF solution, the COS-CY-SF solution indicated a slight increase in the optical density at pH 7–9 owing to the acid titration of the amino group of COS. The introduction of a large amount of positively charged hexosamine under acidic conditions made SF amphiphilic and more water-soluble at lower pH values.

Retardation effect of COS-CY-SF on the growth of *E. coli*

Uchida et al. [18] and Sudarshan et al. [27] reported that water-soluble chitosan and COS inhibited the growth of many bacteria including Gram-positive and Gram-negative ones. Further, Uchida et al. reported that COS, which consisted of the oligomer mixture of GlcN from tetramer to heptamer, inhibited the growth of *E. coli* at a concentration higher than 0.3% (w/v). The inhibitory effect at a COS concentration of 0.5% (w/v) was stronger than that at 0.3% (w/v) [18]. We also studied the effect of COS-CY-SF on the growth of *E. coli*. This approach was to investigate the change of the functional property of SF by the chemical modification with COS.

Because 0.7% (w/v) SF solution showed a similar increase in the optical density to that of the control until 24 h, it is obvious that SF did not affect the growth of *E. coli*. In the case of 0.6% (w/v) COS-CY-SF solution, an increase in optical density was not observed in the range of 8–24 h, whereas the increase in optical density for COS-CY-SF was similar to that for the control and SF until 8 h. For an alternative comparison, the relative growth ratio, R, is defined by the following equation:

$$R = (S_{24} - S_0)/(C_{24} - C_0),$$

where S_t and C_t are the optical densities at incubation time t for the test sample and the control, respectively. The R values for each sample are given in Fig. 6. The R value for COS-CY-SF was much lower than unity, while the value for SF was close to unity. This suggests that COS-CY-SF retarded the growth of *E. coli* after incubation for 24 h, while SF did not. Judging from these results, it can be considered that the COS moiety introduced was responsible for the retardation effect of COS-CY-SF on the bacterial growth. In agreement with the results obtained by Uchida et al. [18], it was confirmed in our experiments that COS retarded the growth of *E. coli* after incubation for 24 h at a concentration of

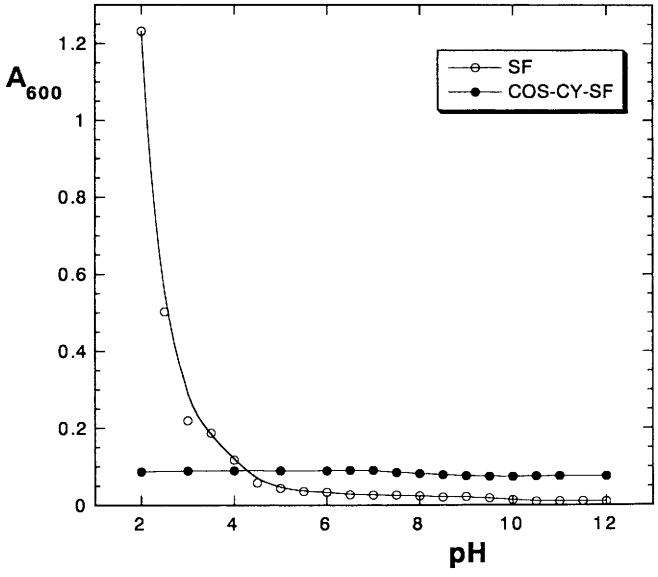

Fig. 5 Absorbance at 600 nm as a function of pH for SF and COS-CY-SF

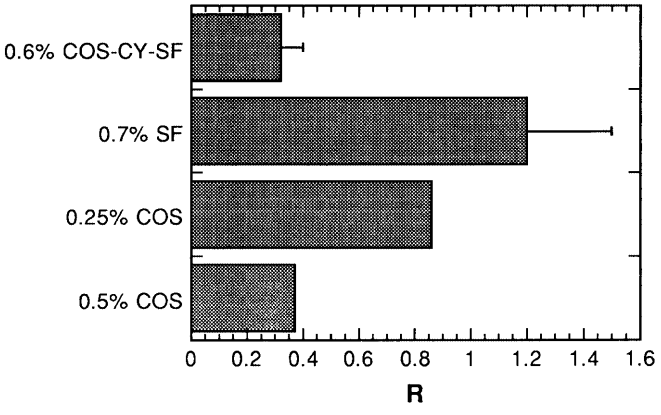

Fig. 6 Relative growth ratio, R, for COS-CY-SF, SF, and COS after incubation for 24 h. R is defined as $R=(S_{24}–S_0)/(C_{24}–C_0)$, where S_t and C_t are the optical densities at incubation time t for the test sample and the control, respectively. The R value for the control is defined as unity. The R values for COS-CY-SF and SF are mean values ± standard deviation of experiments performed in duplicate

0.5% (w/v), while COS did not retard the growth at a concentration of 0.25% (w/v) (Fig. 6). However, 0.6% (w/v) COS-CY-SF solution containing 0.23% (i.e., 0.6×0.38) (w/v) COS exhibited the retardation effect.

One probable explanation for the bacterial growth retardation of chitosan is that the polycationic chitosan owing to the amino groups interacts with the negatively charged bacterial cell surface and/or cell membrane to alter the cell permeability [28, 29]. Since COS molecules are immobilized onto SF proteins in COS-CY-SF, the concentration of COS and the density of positive charge are locally high in the vicinity of COS-CY-SF molecules and the electrostatic interaction with the cell surface is enhanced. Consequently, it is considered that the electrostatic interaction of the cell surface with COS-CY-SF is stronger than that with COS at the same concentration of COS. Chan et al. [30] recently demonstrated new insights into the physicochemical interactions between a model membrane and chitosan that might aid the development of a novel membrane perturber for gene delivery. As the possibility of the interaction between the COS-CY-SF conjugates and the bacterial cell surface and/or cell membrane has been indicated in our study, polycationic COS-CY-SF might be used as a gene carrier or a membrane perturber in a gene delivery system.

Conclusions

The conjugates of SF and COS were prepared by the chemical modification of amino acid residues in SF with CY-activated COS. The COS-CY-SF conjugates obtained consisted of 38 wt% COS, 8 wt% CY, and 54 wt% SF. The introduction of a large amount of COS made SF amphiphilic and more water-soluble at lower pH. Moreover, the COS moiety introduced was responsible for the retardation effect of COS-CY-SF on the growth of *E. coli*. The enhanced retardation effect of COS-CY-SF compared with COS was attributed to a local high density of polycations of the COS immobilized onto SF molecules. Further studies are underway to evaluate the improvement of the functional property of SF by the modification with oligosaccharides.

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