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Preparation and characterization of water-soluble chitin and chitosan derivatives

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Abstract

Chitosan was modified with poly(ethylene glycol)–aldehyde (PEG-aldehyde) of various molecular weights under the various molar ratios of PEG–aldehyde to chitosan. Then the prepared chitosan–PEG hybrid was converted to chitin–PEG hybrid by the acetylation with acetic anhydride. The solubility of various derivatives was investigated in three buffers of various pH. Some of these derivatives were soluble in 0.01 M phosphate buffer saline (PBS, pH = 7.2). The solubility in PBS was dependent on the degree of PEG substitution, the degree of acetylation, the molecular weight of PEG, and the weight ratio of PEG in chitin/chitosan–PEG hybrid. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: Chitin; Chitosan derivatives; PEG–aldehyde

1. Introduction

Chitin is an abundant biopolymer like cellulose and is distributed in the shell of crustacea, e.g. crab and shrimp, and the cuticle of insects and also in the cell wall of some fungi and microorganisms. Chitin consists of 2-acetamido-2-deoxy-(1-4)-β-D-glucopyranose residues (*N*-acetyl-D-glucosamine units) which has intra- and inter-molecular hydrogen bonds and is water-insoluble due to its rigid crystalline structure. Chitosan ideally consists of 2-amino-2-deoxy-(1-4)-β-D-glucopyranose residues (D-glucosamine units) and has no or a small amount of *N*-acetyl-D-glucosamine units, and is water-soluble as the salt with various acids on the amino group of D-glucosamine unit. Additionally, partially acetylated chitosan which has about 50% D-glucosamine unit is only able to dissolve in water (Aiba, 1989).

Chitin and chitosan have been used in agricultural, food, industrial, and medical fields. Recently they have been considered as biomaterials in fields such as biomedicine, pharmacology, and biotechnology due to their biocompatibility, biodegradability, and biological activities (Sugano et al., 1978Sugano et al., 1980; Kurita, 1986; Sashiwa et al., 1990; Chandy et al., 1990; Shigemasa et al., 1995).

There are also problems in using chitin and chitosan. One of them is that it is difficult to dissolve them in water and neutral pH range. So, various studies were conducted to make water-soluble derivatives of chitin and chitosan by chemical modification techniques. However, when chemical modifications change the fundamental skeleton of chitin and chitosan, the modified chitin and chitosan lose the original physicochemical and biochemical activities (Nishimura et al., 1986; Murata et al., 1989; Dutkiewicz et al., 1990; Tanigawa et al., 1992).

On the other hand, the modification with a polymer may have an advantage, because the modification with a hydrophilic polymer would be expected to result in hydrophilic chitin or chitosan while keeping the fundamental skeleton intact.

Some approaches for the graft copolymerization of hydrophilic polymer onto chitin and chitosan were reported as a technique to improve the affinity to water or organic solvents (Blair et al., 1987; Kurita et al., 1988; Yalpani et al., 1991; Aoi et al., 1994; Kurita et al., 1994; Hoffman et al., 1997).

Poly(ethylene glycol) (PEG) is a polymer widely used as a pharmacological product of preferable hydrophilicity and biocompatibility with low biodegradability. Harris et al. (1984) and Aiba (1993) have published the modification of chitosan with PEG. We have paid attention to improving the water-solubility of chitin and chitosan by the modification with high molecular weight PEG. We prepared the

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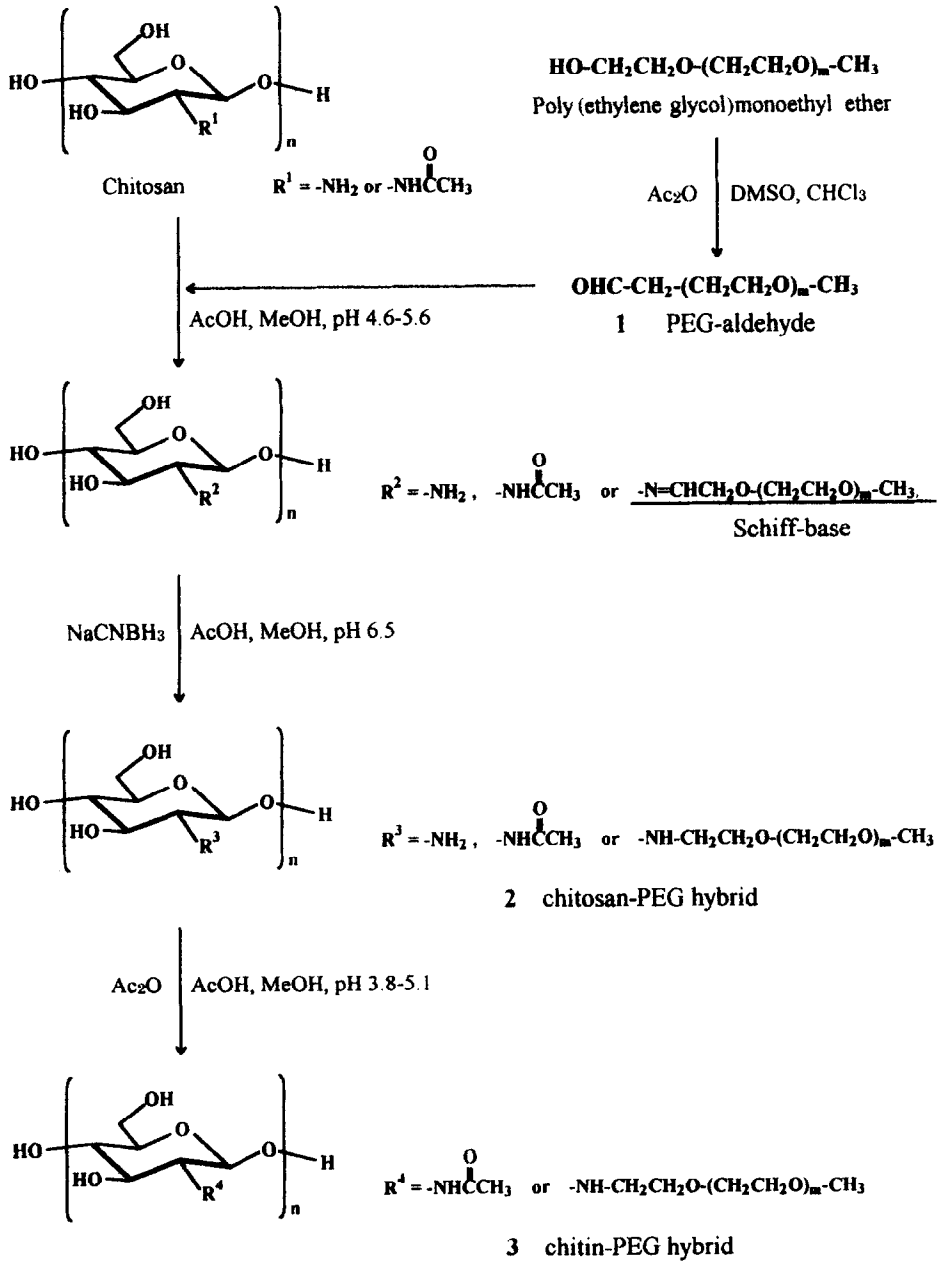

Scheme 1. Preparation of chitin/chitosan–PEG hybrid.

chitosan modified with PEG (chitosan–PEG hybrid) in various reaction conditions and acetylated them to get the chitin modified with PEG (chitin–PEG hybrid) (Scheme 1). In this paper, we also report on the water-solubility of these derivatives.

2. Experimental

2.1. Materials

Chitosan was obtained from Kyowa Tecnos Co. The molecular weight (M_n) was 28 000 (determined by GPC based on pullulan as standard) and the degree of deacetylation was 86% (determined by ^{1}H-NMR). Poly-(ethylene glycol) monomethyl ethers (MeO–PEG) of various molecular weights were obtained from Aldrich Chemical Co. and PEG4000 (M_n 3000) was obtained from Tokyo Kasei Ltd. Other reagents were all chemical grade and used without further purification.

2.2. Preparation of PEG–aldehyde

PEG–aldehyde was prepared by the oxidation of PEG with DMSO/acetic anhydride (Harris et al., 1984). Acetic anhydride (5 ml) was added to MeO–PEG (M_n 2000, 10 g)

Table 1
Preparation and characterization of water-soluble chitin

Run No.	PEG[a]/M_n		DMSO/ml	$CHCl_3$/ml	Ac_2O/ml	Molar ratio of Ac_2O/–OH	Time/h	Temperature/°C	Yield/%	DC[b]
1-1	MeO–PEG	5000	25	10	4.1	20	12	r.t. (21)	87 ± 23[c]	0.83 ± 0.15[c]
1-2	MeO–PEG	2000	30	2	5.1	10	9	r.t. (21)	76 ± 17[c]	0.56 ± 0.03[c]
1-3	MeO–PEG	550	30	–	9.3	5	3	50	(100)[d]	1.07
1-4	PEG4000	3000	25	7.5	0.8	1.2	30	r.t. (18)	96	0.38

[a] MeO–PEG means polyethyleneglycol monomethylether. 10 g of PEG was used in each sample.
[b] DC means the degree of conversion from –OH to –CHO, and –CHO was estimated by Schales' method.
[c] Mean ± SD were calculated from $n = 3$.
[d] The product was not purified.

in 32 ml anhydrous dimethylsulfoxide containing 6% chloroform under an Ar atmosphere and the mixture was stirred for 9 h at room temperature (20°C). The reaction mixture was then poured into 400 ml diethylether. The precipitate was filtered with a paper filter (No.2) and reprecipitated twice from chloroform solution with diethylether. After drying, white powder (8.2 g) was obtained. In the case of MeO–PEG (M_n 550), the reaction mixture was neutralized with aqueous 1 M NaOH solution and applied to the next reaction without the precipitation with chloroform and diethylether. The reaction conditions and yields are summarized in Table 1. The degree of the conversion (DC) from hydroxy group to aldehyde group was estimated by Schales' method with the calibration curve of glutaraldehyde (Imoto et al., 1971). The reason why DC in the case of MeO–PEG (M_n 550) was over 1.0 might be due to the existence of PEG with two aldehyde groups on both ends.

2.3. Preparation of chitosan–PEG hybrid

The preparation of chitosan–PEG hybrid was performed by the method of Harris (Harris et al., 1984). Chitosan (0.5 g, 3 mmol as monosaccharide residue containing 2.6 mmol amino group) was dissolved in a mixture of aqueous 2% acetic acid solution (20 ml) and methanol (10 ml). A 7 ml aqueous solution of PEG–aldehyde (M_n 2000, 2.77 g, –CHO: 0.75 mmol) was added to the above chitosan solution and stirred for 30 min at room temperature. Then the pH of chitosan/PEG–aldehyde solution was adjusted to 6.5 with aqueous 1 M NaOH solution and stirred for 60 min at room temperature. At this time, the solution was still pale yellow and did not precipitate. $NaCNBH_3$ (0.476 g, 7.6 mmol) in 7 ml water was added to the reaction mixture dropwise for 20 min and the solution was stirred for 18 h at room temperature. The reaction

Table 2
Preparation of chitosan–PEG hybrid[a]

Run No.	PEG–aldehyde		Molar ratio		Chitosan–PEG hybrid		
	M_n	DC[b] of –CHO/–OH	–CHO of PEG/$-NH_2$ of chitosan	$NaCNBH_3$/–CHO of PEG	Yield[c]/%	DS[d]	Weight ratio PEG/hybrid[e]
2-1	5000	0.91	0.29	10	120	0.07	0.68
2-2	5000	0.68	0.19	10	106	0.08	0.71
2-3[f]	5000	0.68	0.10	10	44	0.05	0.60
2-4	2000	0.53	0.96	3	93	0.37	0.82
2-5	2000	0.53	0.48	5	107	0.16	0.66
2-6	2000	0.57	0.29	10	81	0.16	0.66
2-7[f]	2000	0.55	0.19	10	60	0.12	0.59
2-8[f]	2000	0.55	0.10	10	27	0.09	0.52
2-9	550	1.07	1.20	10	60	0.23	0.43
2-10[f]	550	1.07	0.60	10	27	0.16	0.35
2-11	550	1.07	0.30	10	79	0.05	0.14
2-12	3000	0.38	0.30	10	87	0.14	0.72

[a] Chitosan was obtained from Kyowa Tecnos Co., $M_n = 28\,000$, the degree of deacetylation = 86% determined by ^{1}H-NMR. Reaction condition: solvent, 1:1 mixture of 2% acetic acid and methanol; pH = 6.5; temp., room temp.; reaction time, 18 h.
[b] DC means the degree of conversion from -OH to –CHO, and –CHO is estimated by Schales' method.
[c] Yield is indicated with the amount of recovered chitosan in chitosan–PEG hybrid calculated by DS. The case which is over 100% is considered by underestimation of DS.
[d] DS means the degree of substitution of PEG to monosaccharide residue of chitosan determined by ^{1}H-NMR.
[e] The weight ratio means the weight ratio of PEG in chitosan–PEG hybrid calculated using DS and M_n of PEG.
[f] Two fractions of chitosan–PEG hybrid were obtained. Upper line indicates water-soluble fraction and lower line indicates water-soluble fraction respectively.

mixture was dialyzed with dialysis membrane (Viskase Sales Co., Mw 12000 cut) against aqueous 0.05 M NaOH solution and water alternately. When the pH of the outer solution reached 7.5, the inner solution was centrifuged at 15 000 rpm (37 000 *g*) for 20 min. The precipitate and the supernatant were respectively freeze-dried and washed twice with 100 ml acetone. Unreacted PEG was removed by washing with acetone. After drying *in vacuo*, white powder from supernatant (1.2 g) and white powder from precipitate (4 mg) were obtained as water soluble and insoluble chitosan–PEG hybrid, respectively. The yield of water-soluble derivative was 60% by weight of chitosan and PEG–aldehyde (run No. **2-6** in Table 2). The reaction conditions are shown in Table 2.

2.4. Acetylation of chitosan–PEG hybrid

Chitosan–PEG hybrid was converted to acetylated chitosan–PEG hybrid (chitin–PEG hybrid in Scheme 1) by acetylation with acetic anhydride in acetic acid and methanol solvent (Hirano et al., 1976). The typical procedure is as follows. Acetic anhydride (0.22 ml) was added to a mixture of aqueous 1% acetic acid solution (10 ml) and methanol (40 ml) which contained chitosan–PEG hybrid (0.5 g, 1.0 mmol monosaccharide residue, 0.7 mmol $-NH_2$) and the mixture was stirred for 5 h at room temperature to give gel. After pH adjustment to 12.0 with aqueous 1 M NaOH solution, the reaction mixture was evaporated and dialyzed against water. When the pH of the outer solution became 7.5, the inner solution was freeze-dried to give 0.5 g of white powder (run No. **3–7** in Table 3). These results are shown in Table 3.

2.5. Infrared spectroscopy

IR spectra were recorded in KBr discs on a Shimazu FT-IR 4200 spectrometer under dry air at 20°C.

IR data of chitosan (KBr): 3400 (ν_{O-H}), 2900 (ν_{C-H}), 1660 (amide-I), 1560 (amide-II), 1070 cm^{-1} (ν_{C-O}). Chitosan–PEG hybrid (KBr): 3400 (ν_{O-H}), 2900 (ν_{C-H}), 1660 (amide-I), 1560 (amide-II), 1100 cm^{-1} (ν_{C-O}). Chitin–PEG hybrid (KBr): 3400 (ν_{O-H}), 2900 (ν_{C-H}), 1660 (amide-I), 1560 (amide-II), 1110 cm^{-1} (ν_{C-O}).

2.6. NMR spectroscopy

^{13}C- and ^{1}H-NMR spectra were recorded on a JEOL JNM-GX270 spectrometer. Samples were dissolved in D_2O containing one drop of 20wt% DCl/D_2O. In the case of a sample which did not dissolve by the addition of 20wt% DCl, the sample (30–240 mg) was dissolved in 0.8 ml of 20wt% DCl/D_2O at 0 or 80°C (Shigemasa et al., 1996).

The ^{1}H-NMR spectra were recorded at the concentration of 40–100 mg/ml, at 60 or 80°C. The ^{13}C-NMR spectra were recorded in only 20wt% DCl/D_2O at the concentration of 300 mg/ml, at 27°C. The chemical shifts were referenced from DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate).

The hydrolysis of the glycosyl linkage of chitosan in 20wt% DCl/D_2O decreases the viscosity of the sample solution and improves the sharpness of the peaks on NMR spectra. However, when the hybrid samples were dissolved in 20wt% DCl/D_2O at 80°C, the peak intensities of H-1 of monosaccharide residue and methyl protons of the *N*-acetyl group were not determined correctly. Therefore, the dissolution of hybrids in 20wt% DCl was performed at 0°C to minimize the hydrolysis of acetyl group and the degradation

Table 3
Acetylation of chitosan–PEG hybrid[a]

Run No.	Chitosan–PEG hybrid		Molar ratio	Time h	Temperature °C	Acetylated chitosan–PEG hybrid	
	M_n of PEG	DS[b]	$Ac_2O/-NH_2$ of hybrid			Yield[c]/%	DA[d]
3-1	5000	0.07	1.4	6	r.t. (20)	108	0.35
3-2	5000	0.08	2.8	12	r.t. (28)	101	0.37
3-3	5000	0.08	5.0	12	r.t. (28)	102	0.45
3-4	2000	0.37	3.9	12	r.t. (19)	92	0.64
3-5	2000	0.37	10.0	12	r.t. (28)	98	0.65
3-6	2000	0.16	2.2	12	r.t. (21)	97	0.70
3-7	2000	0.16	3.1	5	r.t. (20)	101	0.79
3-8	2000	0.16	5.1	12	r.t. (28)	100	0.65
3-9	2000	0.12	3.0	12	r.t. (28)	97	0.72
3-10	550	0.23	7.6	12	r.t. (28)	88	0.82
3-11	3000	0.16	2.4	2	r.t. (15)	101	0.80

[a] Reaction condition: chitosan–PEG hybrid (Table 2), 0.5 g; solvent, 1:4 mixture of 1% acetic acid and methanol; temp., room temp.; reaction time, 2–12 h.
[b] DA means the degree of substitution of PEG to monosaccharide residue of chitosan determined by ^{1}H-NMR.
[c] Yield is calculated from the amount of recovered chitosan in acetylated chitosan–PEG hybrid.
[d] DA means the degree of acetylation determined by ^{1}H-NMR.

of saccharide unit in the case of a sample which did not dissolve by the addition of 20wt% DCl. On the other hand, for the measurement of ^{13}C-NMR to confirm the bond between amino group of chitosan and PEG, it is not necessary to be concerned about the hydrolysis of acetyl group. The sample was treated at 80°C in 20wt% DCl/D_2O.

All the degree of substitution (DS) values shown in Table 4 were evaluated from the relative peak intensities between the methylene group of PEG (δ = 2.7–3.7 ppm) and H-1 (δ = 4.5–5.1 ppm) of monosaccharide residue in the ^{1}H-NMR spectra in D_2O containing one drop of 20wt% DCl/D_2O. Because the peak of PEG methylene was overlapped with those of H-2, 3, 4, 5, 6, and 6′ of monosaccharide residue, we used the following equations in order to evaluate DS:

$$\text{Corrected peak intensity of PEG methylene} = \frac{\text{whole intensity of 2.7 and 2.9 – 3.9 ppm}}{\text{H-1 intensity of 4.5 – 5.1 ppm}} - 6$$

$$\text{DS value} = \frac{\text{corrected peak intensity of PEG methylene}}{\text{number of protons in PEG}}$$

Here, 6 is the total protons of H-2, H-3, H-4, H-5, and 2 H-6. The number of protons in PEG is calculated from the number average of molecular weight (M_n) of PEG. As for the DS values of chitin–PEG hybrids, the DS values of chitosan–PEG hybrids before the acetylation were employed.

In the case of hybrids with low DS of high molecular weight PEG, the peak intensity of PEG methylene at 2.7 ppm was very small against the peak intensity of the other methylene protons of PEG at 3.7 ppm, and it was difficult to evaluate correct DS using the peak at 2.7 ppm. For this reason, this equation to evaluate DS was used.

The degree of acetylation (DA) was determined with the relative peak intensities between acetyl group (methyl protons, δ = 2.05 ppm) and H-1 of monosaccharide residue (δ = 4.5–5.1 ppm). Between the DA values of water-soluble

Table 4
Water-solubility of chitin/chitosan–PEG hybrid in AcOH buffer, PBS, and Na_2CO_3 buffer

Sample No.	PEG–aldehyde/ M_n	DS[a]	DA[b]	Weight ratio[c] PEG/ hybrid	Solubility[d]		
					0.1 M AcOH/AcONa buffer (pH = 4)	0.01 M PBS (pH = 7.2)	0.2 M Na_2CO_3/$NaHCO_3$ buffer (pH = 10)
Chitosan	–	0	0.14	0	+ + + (+ + +)	– (–)	– (–)
2-10-2	550	0.03	0.14	0.09	+ + +	–	+
2-11	550	0.05	0.14	0.14	+ + +	–	–
2-10-1	550	0.16	0.14	0.35	+ + + (+ + +)	± (+ + +)	+ + + (–)
2-9	550	0.23	0.14	0.43	+ + + (+ +)	+ + (+ +)	+ + (±)
3-10	550	0.23	0.82	0.39	+ + + (–)	+ (+)	+ + (–)
2-8-2	2000	0.05	0.14	0.37	+ + + (+ + +)	– (+ + +)	± (–)
2-8-1	2000	0.09	0.14	0.52	+ + + (+ + +)	+ + (+ + +)	+ + + (+ + +)[e]
2-7-1	2000	0.12	0.14	0.58	+ + +	+ + +	+ + +
3-9	2000	0.12	0.72	0.56	+	+	±
2-5	2000	0.16	0.14	0.66	+ + +	+ + +	+ + +
2-6	2000	0.16	0.14	0.66	+ + +	+ + +	+ + +
3-8	2000	0.16	0.65	0.63	+ + +	+ + +	+ + +
3-6	2000	0.16	0.70	0.63	+ + +	+ + +	+ + +
3-7	2000	0.16	0.79	0.62	+	±	±
2-4	2000	0.37	0.14	0.82	+ + +	+ + +	+ + +
3-4	2000	0.37	0.64	0.80	+ + +	+ + +	+ + +
3-5	2000	0.37	0.65	0.80	+ + +	+ + +	+ + +
2-12	3000	0.14	0.14	0.72	+	+	±
3-11	3000	0.14	0.80	0.68	+	+	±
2-3-1	5000	0.05	0.14	0.60	+ + +	+ + +	+ + +
2-1	5000	0.07	0.14	0.68	+ + +	+ + +	+ + +
3-1	5000	0.07	0.35	0.67	+ +	±	±
2-2	5000	0.08	0.14	0.71	+ + +	+ + +	+ + +
3-2	5000	0.08	0.37	0.69	+	+	+
3-3	5000	0.08	0.45	0.69	+ +	+	+

[a] DS means the degree of substitution of PEG to monosaccharide residue of chitosan determined by ^{1}H-NMR.
[b] DA means the degree of acetylation determined by ^{1}H-NMR.
[c] The weight ratio means the weight ratio of PEG in chitin/chitosan–PEG hybrid calculated using DS and DA values.
[d] The solubility was evaluated at the concentration 5 mg/ml in each buffer, and the solubility was checked after standing for 4 days. –, precipitate; ±, swelling; ±, gel or suspension; + +, partially soluble; + + +, soluble. The solubility in the parentheses ( ) was evaluated at the concentration of 5 mg/ml in aqueous 1% acetic acid solution, and the solubility was checked after the adjustment of pH to 4, 7, and 10 with aqueous 1 M and 0.1 M NaOH.
[e] A little precipitate appeared after standing at pH = 11.

chitosan hybrids evaluated from the ^{1}H-NMR spectra in D_2O with 1 drop 20wt% DCl and those in 20wt% DCl, there was no difference. On the other hand, the peak of H-2 proton which was observed at 2.70 ppm in D_2O, shifted to the lower field with the addition of 20wt% DCl, and reached to 3.31 ppm in 20wt% DCl.

Chitosan–PEG hybrid in D_2O containing one drop of 20wt% DCl/D_2O, chitin–PEG hybrid in D_2O containing one drop of 20wt% DCl/D_2O, chitin–PEG hybrid in 20wt% DCl, and the starting chitosan in D_2O containing one drop of 20wt% DCl/D_2O showed broad signals on ^{1}H-NMR spectra and these data are described as follows:

^{1}H-NMR data of chitosan–PEG hybrid (D_2O): δ 4.60–4.82 (1 H, br.d., H-1), 3.82 (H-3, H-4, and H-6′), 3.67 (H-5, H-6, and $-OCH_2-$), 3.36 ($-OCH_3$), 3.09 (0.85 H, br.s., H-2), 2.69 (0.4 H, br.s., $-NH-CH_2-$), 2.04 ppm (0.4 H, br.s., $-COCH_3$), (30 H, total of H-3, H-4, H-5, H-6, H-6′, $-OCH_2-$, and $-OCH_3$). Chitin–PEG hybrid (D_2O): δ 4.58–5.05 (1 H, br.d., H-1), 3.92 (H-3, H-4, and H-6′), 3.68 (H-5, H-6, and $-OCH_2-$), 3.36 ($-OCH_3$), 3.28 (br., H-2), 2.70 (0.5 H, br.s., $-NH-CH_2-$), 2.03 ppm (2.1 H, br.s., $-COCH_3$), (35 H, total of H-2, H-3, H-4, H-5, H-6, H-6′, $-OCH_2-$, and $-OCH_3$). Chitin–PEG hybrid (20wt% DCl/D_2O): δ 4.80–5.17 (1 H, br.d., H-1), 3.99 (H-3, H-4, and H-6′), 3.72 (H-5, H-6, and $-OCH_2-$), 3.40 ($-OCH_3$), 3.31 (br., H-2), 3.07 (0.45 H, br.s., $-NH-CH_2-$), 2.38 ppm (2.2 H, br.s., $-COCH_3$), (35 H, total of H-3, H-4, H-5, H-6, H-6′, $-OCH_2-$, and $-OCH_3$). Chitosan (D_2O): δ 4.50–4.80 (1 H, br.d., H-1), 3.94 (br.s., H-3, H-4, and H-6′), 3.78 (br., H-5, H-6), 3.22 (0.85 H, br.s., H-2), 2.05 ppm (0.42 H, br.s., $-COCH_3$). The relative peak intensity was calculated from the standard peak intensity of H-1 as 1 proton (1 H). Additionally, the peak intensities of H-2, H-3, H-4, H-5, H-6, and H-6′ were not estimated on the spectrum of chitosan–PEG hybrid and chitin–PEG hybrid, because the peak of PEG methylene was overlapped with those of H-2, H-3, H-4, H-5, and 2 H-6 of monosaccharide residue. Here, H-2 means only H-2 of D-glucosamine unit.

^{13}C-NMR data of chitosan–PEG hybrid (20wt% DCl/D_2O): δ 99.1 (C-1), 58.0 (C-2), 73.1 (C-3), 78.9 (C-4), 76.7 (C-5), 62.6 (C-6), 23–24 (degraded $-COCH_3$), 72.0 ($-OCH_2-$), 49.2 ($-NHCH_2CH_2O-$), 67.2 ppm ($-NHCH_2CH_2O-$) (Loubaki et al., 1991; Holme et al., 1992).

2.7. Evaluation of water-solubility of chitin/chitosan–PEG hybrid

The water-solubility of chitosan or chitosan–PEG hybrid was evaluated in 0.1 M acetic acid/sodium acetate buffer (pH = 4.0), 0.01 M phosphate buffer saline (PBS, pH = 7.2), and 0.2 M sodium carbonate/sodium hydrogen carbonate buffer (pH = 10.0). A sample was soaked in each buffer at the concentration of 5 mg/ml and the solubility after 4 days was observed.

The relative solubility to pH was investigated for chitosan–PEG hybrids and chitin–PEG hybrid which were insoluble in PBS. Chitosan–PEG hybrids, chitin–PEG hybrid, and chitosan were dissolved in aqueous 1% acetic acid at a concentration of 5 mg/ml. Then the pH of each solution was changed with the addition of aqueous 1 M and 0.1 M NaOH solution from 4 to 11. The appearance of the precipitate was observed.

3. Results and discussion

3.1. Preparation of PEG–aldehyde

Poly(ethylene glycol) monomethyl ethers were used as PEG source in order to avoid the crosslinking reaction by bifunctional PEG–aldehyde. The DMSO/acetic anhydride oxidation reactions of various PEG are summarized in Table 1. The degree of conversion from hydroxy group to aldehyde group (DC) determined by Schales' method (Imoto et al., 1971) means the average number of aldehyde groups in one molecule of PEG.

MeO–PEG of higher molecular weight indicated lower reactivity against DMSO/acetic anhydride oxidation reaction (Table 1). In addition, the excessive reaction conditions (high temperature, high molar ratio of acetic anhydride to hydroxy group, and long reaction time) resulted in the decrease of the conversion as Harris et al. (1984) had already reported.

3.2. Preparation of chitosan–PEG hybrid

According to Harris et al. (1984), the products modified with PEG were insoluble in water, and the degree of substitution (DS) of PEG was not over 0.1. We considered that the precipitation of chitosan in the reduction process of schiff-base by $NaCNBH_3$ would suppress the smooth reduction of schiff-base (Scheme 1). The excessive $NaCNBH_3$ rapidly changes the pH of the solution to alkali at which chitosan is not soluble in the aqueous solvent. The neutralization before the reduction of schiff-base would decrease the waste of $NaCNBH_3$ reacting with proton and cause the smooth transfer of the solution pH with the dropwise addition of aqueous $NaCNBH_3$ solution. Furthermore, neutral pH suppresses the degradation of schiff-base (Yao et al., 1994). The free PEG was hardly separated from the reaction mixture by the precipitation with organic solvent and the dialysis against water, which was reported by Harris et al. (1984). PEG (M_n 5000) in the aqueous solution could go through the dialysis membrane (cut-off molecular weight 12 000) easily, but free PEG could not be separated from the mixture of PEG, chitosan, and chitosan–PEG hybrid by the dialysis. Chitosan–PEG hybrid did not precipitate from aqueous solution by the addition of organic solvent like methanol and acetone, because chitosan–PEG hybrid had the high hydrophilicity and some of chitosan–PEG hybrids

dissolved in water. However, free PEG was well removed from the freeze-dried reaction mixture of PEG, chitosan, and chitosan–PEG hybrid by washing with acetone. In the model experiment, after washing well the mixture of chitosan and PEG with acetone, the amount of residual PEG analysed by ^{1}H-NMR was slight and was calculated as DS < 0.01. These improvements increased DS of PEG and gave purified chitosan–PEG hybrids.

The IR spectra of chitosan–PEG hybrid and unmodified chitosan are shown in Fig. 1. The IR spectrum of unmodified chitosan showed amide-I band (1660 cm^{-1}), amide-II band (1560 cm^{-1}), and C–O stretching band (1070 cm^{-1}). By using these absorptions, the degree of deacetylation of this chitosan was determined to be 85% which almost coincided with the value of 86% from the ^{1}H-NMR spectrum bands (Shigemasa et al., 1996). In the IR spectrum of chitosan–PEG hybrid, C–O stretching band of chitosan overlapped with C–O stretching band (1110 cm^{-1}) of PEG.

Concerned with the bond between chitosan and PEG, several related –NH–CH_2CH_2O– groups were assigned by the ^{1}H-NMR peak at near 2.45 ppm for *N*-(2-hydroxy-3-trimethylammoniopropyl) chitosan and the ^{13}C-NMR peak at 45.5–50.5 ppm for *N*-(2-hydroxy-3-trimethylammoniumpropyl) chitosan and *N*-(2-glycosyloxyethyl) chitosan, as reported by Loubaki et al. (1991) and Holme et al. (1992). Actually, the peaks which correspond to the signal of –NHCH_2CH_2O– appeared at 2.7 ppm and 49.2 ppm on ^{1}H- and ^{13}C-NMR spectra of chitosan–PEG hybrid prepared in this paper, respectively (sample No. **2-5** in Fig. 2 and sample No. **2-4** in Fig. 3). The reaction conditions and DS of PEG were summarized in Table 2.

DS value was dependent on the molar ratio of PEG–aldehyde to chitosan. The excess PEG–aldehyde to chitosan may give high DS (Holme et al., 1992), but high DS might lose the properties as chitosan. Some reaction conditions gave two fractions as water-soluble and water-insoluble fraction at the purification step. The higher molar ratio gave only water-soluble chitosan–PEG hybrids of high DS. On the other hand, lower molar ratio gave only water-insoluble chitosan–PEG hybrids of low DS, regardless of the PEG molecular weight. In the middle molar ratio, furthermore, both water-soluble and -insoluble hybrids were obtained. The modified reduction process of Harris et al. (1984) which was completed at neutral pH with the dropwise addition of $NaCNBH_3$, brought about the increasing DS, but was not able to give the narrow distribution of DS. Owing to the high viscosity of reaction mixture, the solution was not mixed enough, and the DS value would distribute widely. Especially, the bubbles of H_2 gas generated from H_2O and $NaCNBH_3$ decreased the stirring efficiency. This would be resolved by improving the solvent system. In order to get the narrow distribution of DS, it would be most important to control or improve the reduction process of schiff-base between chitosan and PEG–aldehyde.

3.3. Acetylation of chitosan–PEG hybrid

Chitosan–PEG hybrid gave a gel in aqueous acetic acid and methanol solution by the addition of acetic anhydride. Concerning this point, Hirano et al. (1976) reported a similar phenomena in the *N*-acetylation of chitosan with acetic anhydride in the same solvent. The reaction conditions and

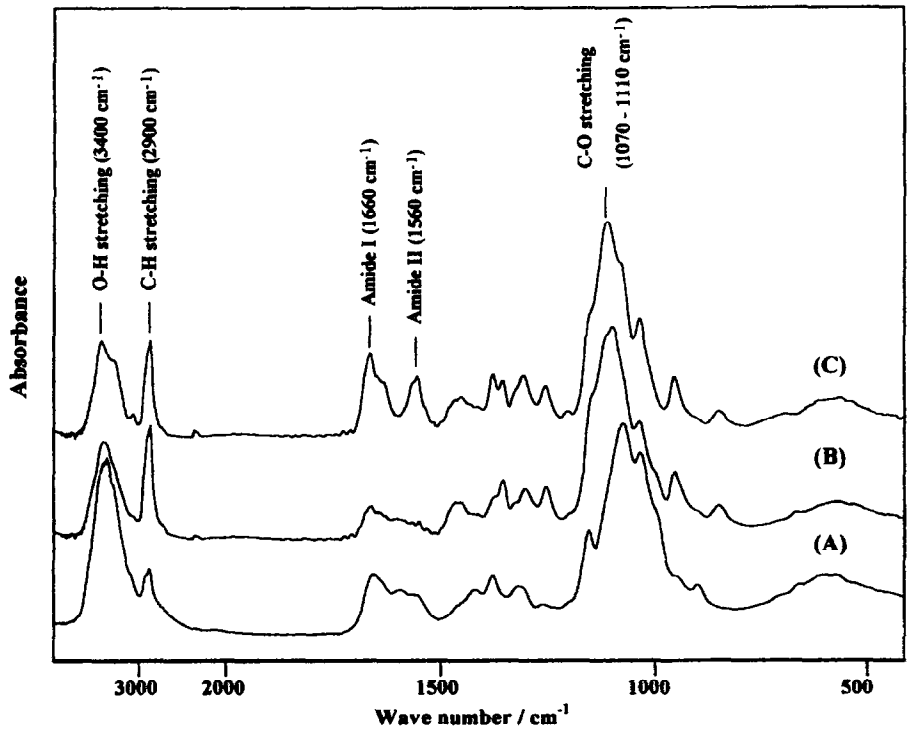

Fig. 1. IR spectra of chitosan (A), chitosan–PEG hybrid (B), and chitin–PEG hybrid (C).

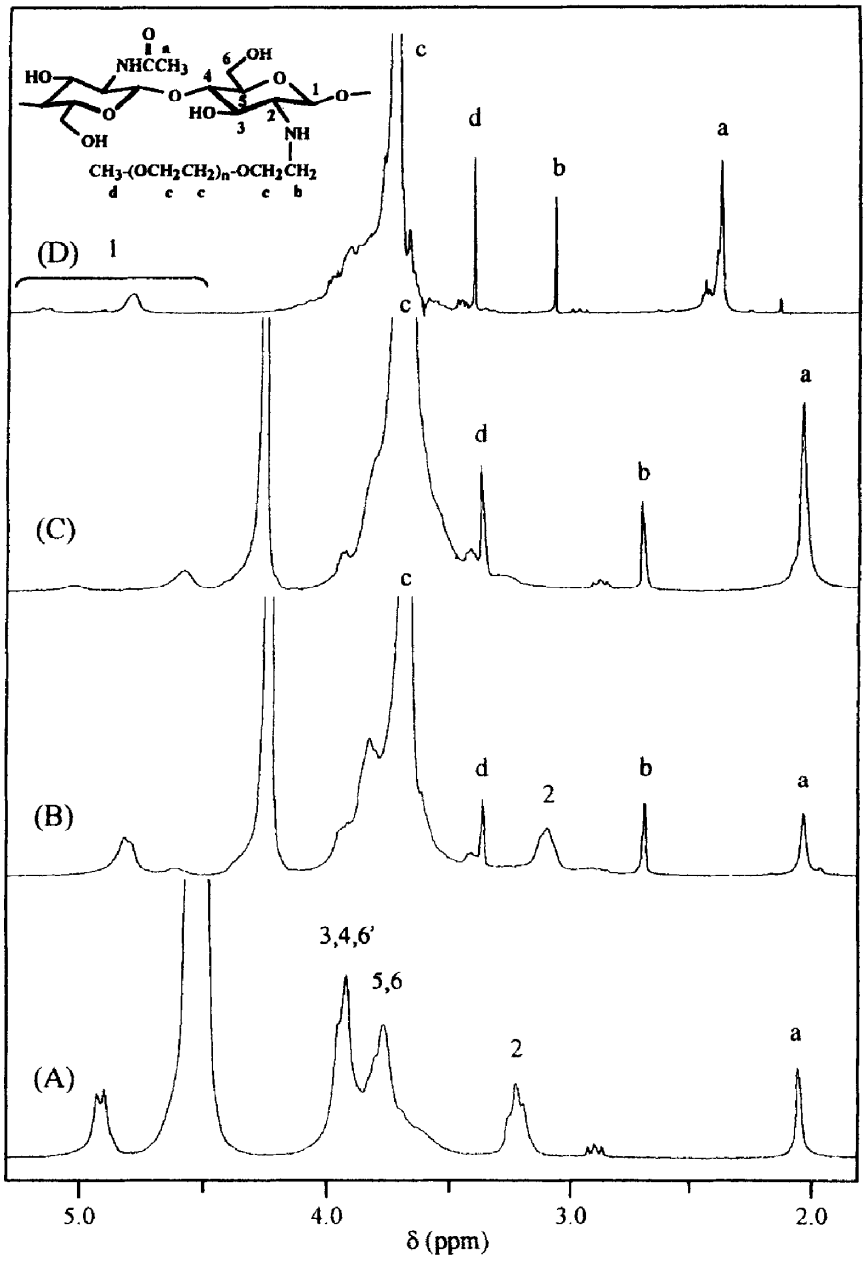

Fig. 2. ^{1}H-NMR spectra of chitosan (A), chitosan–PEG hybrid (B), chitin–PEG hybrid (C) in D_2O containing 1 drop 20wt% DCl/D_2O, and chitin–PEG hybrid (D) in 20wt% DCl/D_2O (270 MHz, 333 K).

the results are summarized in Table 3. Some products were over 100% for the yield. These errors were dependent on the accuracy of the evaluation of DS and the degree of acetylation (DA) by ^{1}H-NMR. The ^{1}H-NMR spectrum of chitin–PEG hybrid (sample No. **3-6** in D_2O contaning 1 drop 20wt% DCl and in 20wt% DCl) is shown in Fig. 2. The IR spectrum of acetylated chitosan–PEG hybrid is shown with the spectra of its source chitosan–PEG hybrid and chitosan in Fig. 1 shows the increase of the peak intensities of amide I (1660 cm^{-1}) and amide II (1560 cm^{-1}) band by the formation of acetamide group on the D-glucosamine unit.

It was obvious that the steric hindrance due to high molecular weight PEG disturbed the acetylation of their hybrids with high molecular weight PEG (M_n 5000, run **3-1**, **3-2**, and **3-3**). The molar ratio of acetic anhydride to amino group of chitosan–PEG hybrid seems to be important to control the DA value, but in these reaction conditions (Table 3) the molar ratio scarcely affected DA. In the case of run **3-4** and **3-5**, the gelation did not occur because of the good solubility of these chitosan–PEG hybrids in the reaction solvent. These products, furthermore, could dissolve in this reaction solvent. The DA value of these products reached a maximum limited DS value. So the solubility of hybrids in reaction solvent also influenced the DA value in addition to the molar ratio and the steric hindrance.

The products of run **3-4**, **3-5**, **3-7**, **3-10**, and **3-11** had few

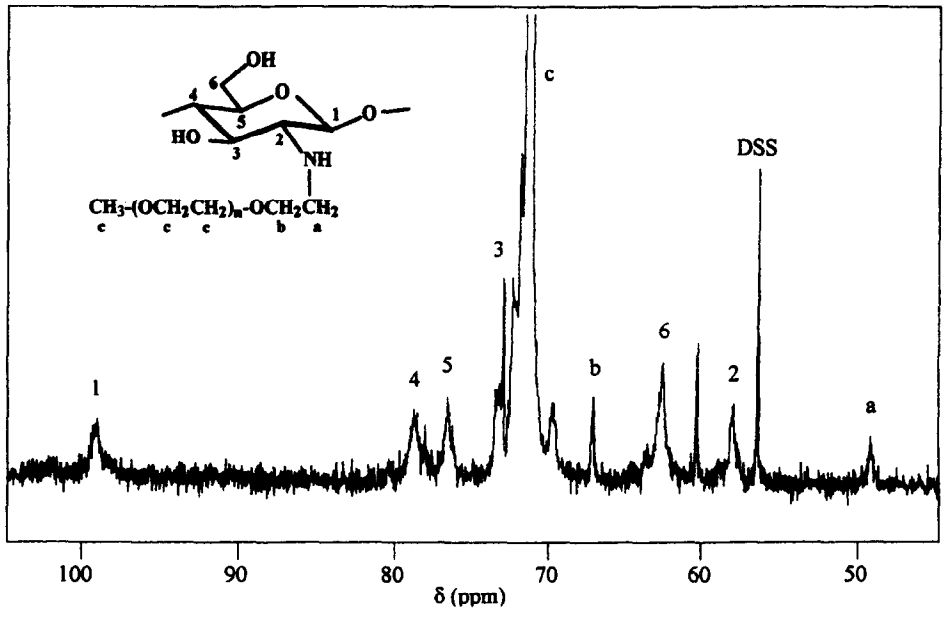

Fig. 3. ^{13}C-NMR spectrum of chitosan–PEG hybrid (270 MHz, 20wt%, DCl/D_2O, 300 K).

D-glucosamine units, and mainly consisted of *N*-acetyl-D-glucosamine and *N*-PEG-D-glucosamine units. They were defined as chitin–PEG hybrid in Scheme 1.

3.4. Water-solubility for chitin/chitosan–PEG hybrid

The water-solubility of chitin/chitosan–PEG hybrid was evaluated in various pH solutions. Their structure and water-solubility are summarized in Table 4. Almost all chitosan–PEG hybrids were soluble in acidic pH buffer. Furthermore, some hybrids dissolved in neutral and alkaline buffers. The good solubility in PBS would provide many possibilities for the application of chitin and chitosan in the biomedical field. The good solubility in alkaline buffer with retention of functional groups on the main skeleton of chitin and chitosan would progress the study for the chemical modification of chitin and chitosan. The modification with PEG improved the water-solubility of chitosan, and high molecular weight PEG (M_n 2000 and 5000) especially, gave remarkably high solubility. The hybrid modified

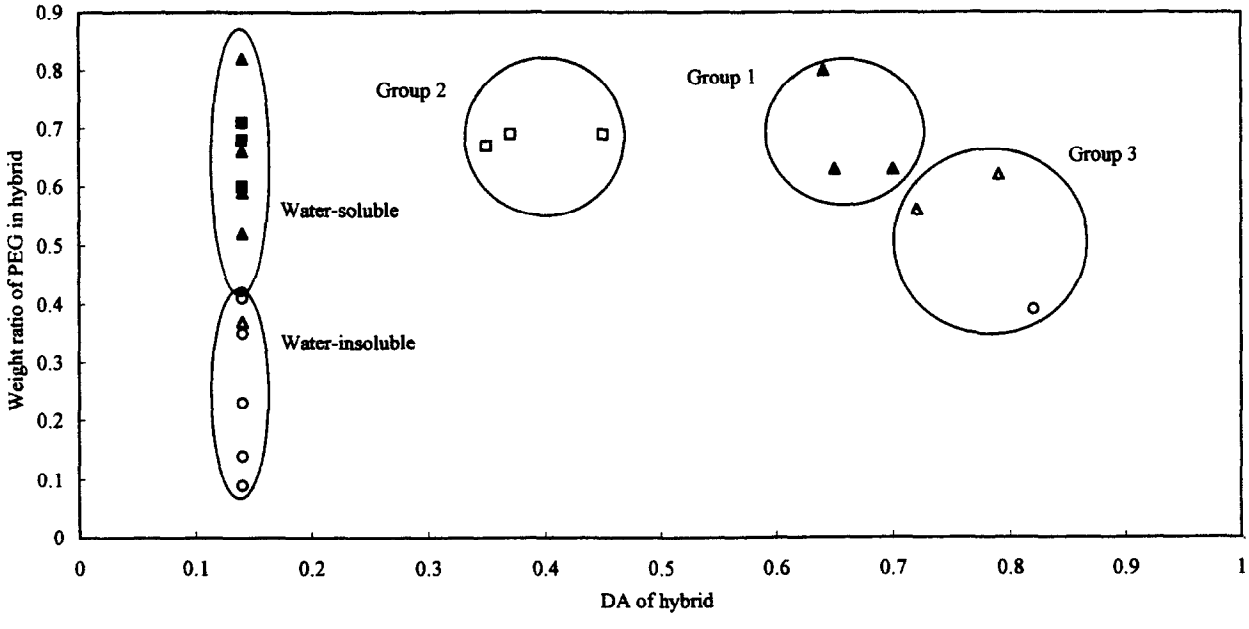

Fig. 4. Relationships among the weight ratio of PEG in various chitin/chitosan–PEG hybrids and the solubility of hybrids in PBS.
The water-solubility was estimated at concentration 5 mg/ml in 0.01 M PBS pH = 7.2 for 4 days at room temperature. Closed marks mean water-soluble and open marks mean water-insoluble. The water-insoluble marks include precipitate, suspension, swelling, and gel. ●○: MeO–PEG Mn = 550, ▲△: MeO–PEG Mn = 2000, ■□: MeO–PEG Mn = 5000. Weight ratio of PEG in chitosan–PEG hybrid was calculated by M_n of PEG, DA of chitosan, and DS determined by ^{1}H-NMR. DS means the degree of PEG introduction. DA means the degree of acetylation of chitin/chitosan–PEG hybrid determined by ^{1}H-NMR.

with PEG 4000 (M_n 3000) was dialdehyde and could make crosslinks between chitosan molecules was water-insoluble (sample No. **2-12** and **3-11** in Table 4).

The factors in the water-solubility of chitin/chitosan–PEG hybrid are as follows:

(A) advantageous factors for the water-solubility

1. the increase of hydrophilicity by PEG introduction
2. the disturbance of hydrogen bond between chitosan molecules by PEG introduction to amino group of chitosan

(B) disadvantageous factors for the water-solubility

1. the increase of total molecular weight of chitosan–PEG hybrid which results in high viscosity of the solution and the poor solubility
2. the decrease of hydrophilic amino group of chitosan
3. the increase of the hydrogen bond between *N*-acetyl-D-glucosamine units of hybrids

The factors (A)-1 and (A)-2 were supported by the following phenomena. The higher molecular weight PEG is able to give a higher hydrophilicity to chitosan. In the case of the solubility in PBS, all soluble chitosan–PEG hybrids (which had DA = 0.14) had the weight ratio of PEG in hybrids of more than 0.59, and chitosan–PEG hybrids which had weight ratio of PEG in hybrids from 0.43 to 0.52 were partial soluble (Table 4). It is easily supposed that the weight ratio of PEG in chitosan–PEG hybrid, factor (A)-1, would be a most important factor which controls the water-solubility of hybrid. This means that the high molecular weight PEG is able to give the water-solubility to chitosan with lower DS of PEG than the low molecular weight PEG.

On the other hand, DS value concerning factor (A)-2 seems to be not so important. However, in the case of chitosan–PEG hybrids which were insoluble in PBS, when pH of chitosan–PEG hybrids solution in aqueous 1% acetic acid solution were changed by adding aqueous 1 M and 0.1 M NaOH solution, these hybrids were soluble until pH = 8 (the cases of sample Nos **2-8-2** and **2-10-1** in Table 4), nevertheless chitosan as the source of hybrids precipitated at pH = 6–7 (in Table 4). Additionally, sample No. **2-8-1** was soluble until pH = 10, but a little precipitate appeared during standing at pH = 11. Sample No. **2-9** was partially insoluble in aqueous 1% acetic acid, but the precipitation did not occur by the increase of pH until 8. A little precipitation like a cloud was observed at pH = 10. These results are explained by the fact that PEG side chain disturbs the hydrogen bond between amino groups of chitosan and results in the delay of the precipitation. This result seems to support the effect of factor (A)-2. Sample No. **3-10**, which has a higher degree of acetylation, was insoluble in aqueous 1% acetic acid and formed a gel. Moreover, the solubility did not change and the precipitate like sample No. **2-9** did not appear. This result is explained by the fact that the solubility was not affected by the change of pH because sample No. **3-10** has little amino group.

The acetylation of chitosan–PEG hybrid would not increase the solubility of hybrid in all buffers (Table 4). The conversion from amino group to acetylamide group decreased hydrophilicity, factor (B)-2, and increased the hydrogen bond between *N*-acetyl-D-glucosamine units of hybrids, factor (B)-3 (Gardner et al., 1975; Minke et al., 1978). In PBS which was the poorest solvent for these hybrids, the hybrids were divided into three groups (Fig. 4). The first group was the soluble group (sample No. **3-4**, **3-6**, and **3-8** in Table 4). This group had high DS, high weight ratio of PEG in hybrid, and middle DA. The second group was the insoluble group (sample No. **3-1**, **3-2**, and **3-3** in Table 4) which had low DS, high molecular weight of PEG, high weight ratio of PEG, and low DA. The third group was the insoluble group (sample No. **3-7**, **3-9**, and **3-10** in Table 4) which had middle DS, high weight ratio of PEG, and high DA. Comparing these three groups, DA and DS are important to control the water-solubility in addition to the weight ratio of PEG in hybrid. So, the water-solubility of chitin–PEG hybrid was effectively influenced by DS, DA, the molecular weight of PEG (which gave enough hydrophilicity with low DS), and the weight ratio of PEG in hybrid.

4. Conclusions

Chitosan–PEG hybrid, partially acetylated chitosan–PEG hybrid, and chitin–PEG hybrid were prepared. Solubility in water was dependent on the molecular weight of PEG, the weight ratio of PEG in hybrid, DS, and DA. The modification with high molecular weight PEG improved the water-solubility of chitosan keeping the main skeleton intact. Additionally, water-soluble chitin–PEG hybrid was obtained by the acetylation of chitosan–PEG hybrid.

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