FACILE PREPARATION OF TRITYLATED AND TRIMETHYLSILYLATED DERIVATIVES STARTING FROM β-CHITIN

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Abstract

Chemical reactivity of β-chitin isolated from squid pens has been studied in tritylation and trimethylsilylation to elucidate the possibility of facile modifications in simple manners. When β-chitin was treated with trityl chloride in pyridine in the presence of 4-dimethylaminopyridine, the primary hydroxy groups at C-6 were tritylated easily. Trimethylsilylation of β-chitin also proceeded smoothly with hexamethyldisilazane and trimethylsilyl chloride in pyridine. Under appropriate conditions, full substitution was achieved in either case. The reactions of α-chitin were, however, much more sluggish. The resulting highly substituted products were readily soluble in common organic solvents.

Keywords: β-chitin, α-chitin, chitosan, chemical modification, tritylation, trimethylsilylation, 4-dimethylaminopyridine, solubility

Introduction

Since α-chitin is easily available in quantity from crab and shrimp shells, extensive studies have been carried out on this ordinary chitin. Modification reactions of α-chitin is, however, rather sluggish in most cases under heterogeneous conditions owing to the insoluble nature, and the derivatives are often structurally ambiguous. It is hence considered significant to prepare derivatives with well-defined structures by controlled modifications.

Although only limited attention has been paid to β-chitin, it is another interesting form of chitin with properties considerably different from those of α-chitin. β-Chitin is much less abundant, but it may be obtained from various species, squid pens being the most promising source. β-Chitin is characterized by weak intermolecular forces because of the parallel arrangement of the molecules [1] and may have higher potential as a starting material than the ordinary α-chitin for efficient chemical modifications under mild conditions.

β-Chitin swells in some organic solvents and has proved to exhibit marked reactivity compare to α-chitin in some reactions including N-deacetylation [2], acetylation, tosylation [3], and acetolysis [4]. It is noteworthy that even the derived chitosan showed higher reactivity in N-phthaloylation than chitosan derived from α-chitin [5]. Tritylation was also suggested to proceed smoothly by a preliminary study [3]. Here

we report efficient tritylation and trimethylsilylation reactions of β-chitin as compared with those of α-chitin.

Materials and methods

Chitins

α-Chitin and β-chitin were isolated from shrimp shells and squid pens, respectively, according to the procedure reported previously [2].

Tritylation

Chitin was dispersed in pyridine, and trityl chloride and 4-dimethylaminopyridine (DMAP) were added. The mixture was heated at 90 °C, concentrated, and poured into ethanol to isolate the substituted product.

Trimethylsilylation

A dispersion of chitin in pyridine was treated with a mixture of hexamethyldisilazane and trimethylsilyl chloride. The silylated chitin was isolated by pouring the mixture into methanol or by extracting with acetone.

Results and discussion

Tritylation

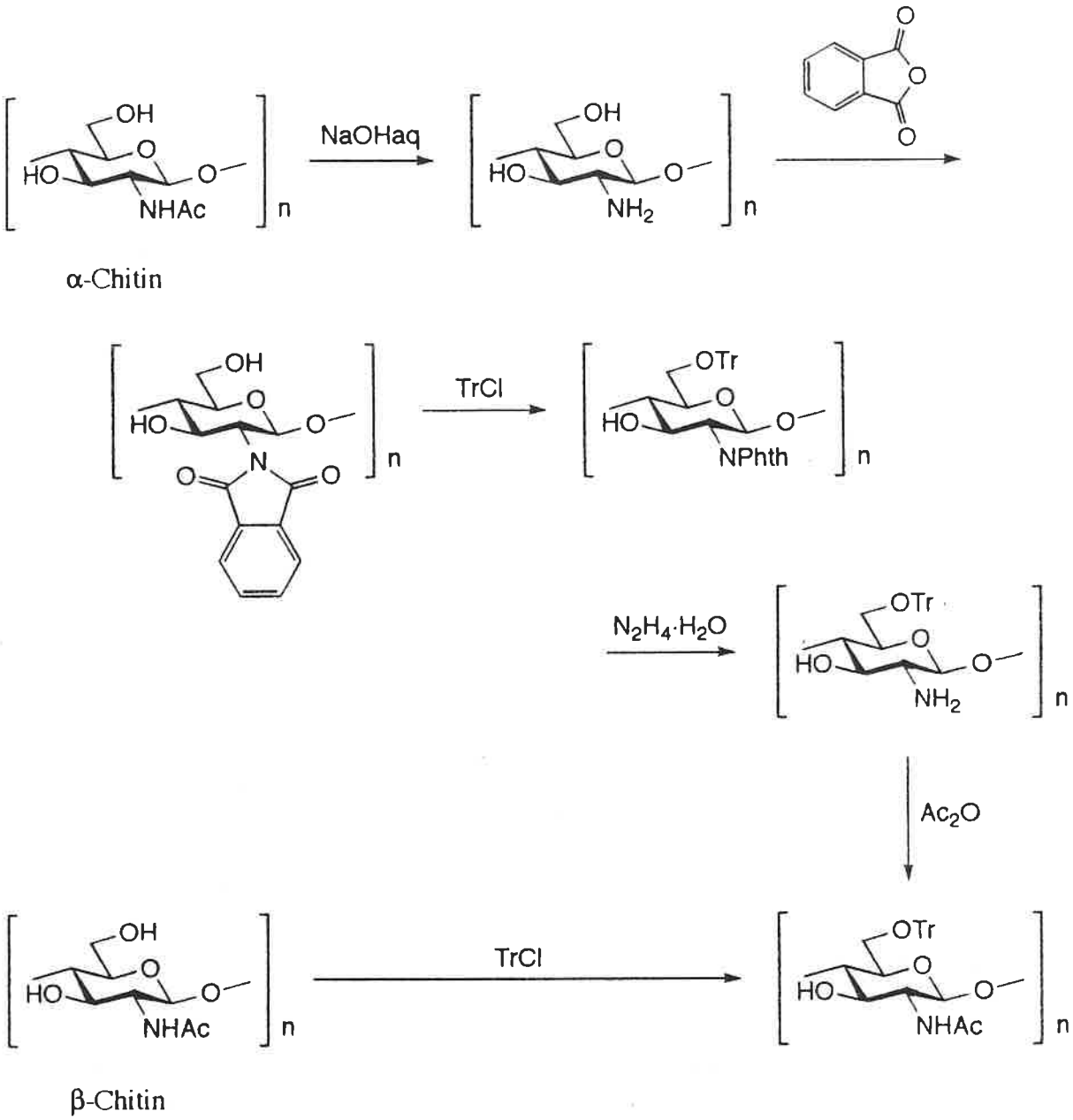
The trityl group is so bulky that substitution occurs only at the primary hydroxy group and not at the secondary hydroxy group. Tritylation would hence be a powerful tool for selective protection of the primary hydroxy groups of chitin if complete substitution is possible. However, α-chitin is resistive to direct tritylation, and tritylated chitin could be prepared through multi-step reactions based on *N*-phthaloyl-chitosan [6] as shown in Scheme 1. β-Chitin was expected to show higher reactivity in tritylation, and direct one-step tritylation was examined (Scheme 1).

Direct tritylation was carried out in pyridine with ten equivalents of trityl chloride at 90 °C, but no appreciable substitution was observed. DMAP sometimes works as a catalyst in acylation, and actually it was confirmed to be effective for tritylation of chitin. The degree of substitution (ds) was dependent on the amount of DMAP, and as summarized in Table 1, full substitution could be attained with an appropriate amount of DMAP. Tritylation of α-chitin was also attempted under similar conditions, but the reaction was very sluggish leading to only a low ds around 0.1.

Table 1. Tritylation of chitins[a]

chitin	DMAP/ pyranose[b]	time (h)	ds[c]
β-chitin	0	24	0
β-chitin	1.7	24	0.61
β-chitin	6.0	72	1.02
α-chitin	1.7	24	0.10

[a] With 10 equiv of trityl chloride in pyridine at 90 °C.
[b] Mole ratio.
[c] Determined from the C/N ratio of elemental analysis.



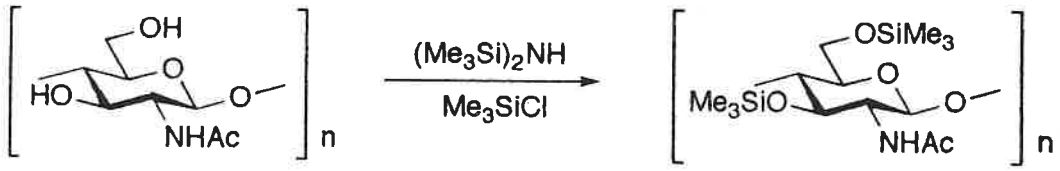
Scheme 1.

The fully tritylated product was soluble in polar organic solvents such as pyridine and dimethyl sulfoxide and would be useful as an organosoluble precursor for regioselective modification reactions.

Trimethylsilylation

In the course of our studies on regioselective modifications based on soluble precursors, trimethylsilylation of 3-*O*-acetyl-2-*N*-phthaloyl-chitosan was found to increase the solubility markedly, and the silylated derivative became soluble even in low-boiling solvents such as dichloromethane, dichloroethane, and THF [7]. Silylated derivatives of chitin are thus considered of interest in view of both the solubility and reactivity. If direct trimethylsilylation of chitin becomes possible, therefore, it would be useful as a novel type of modification reaction. High reactivity of β-chitin suggested the possibility of

direct trimethylsilylation, and the reaction was studied in comparison with that of α-chitin (Scheme 2).



Scheme 2.

β-Chitin was treated with ten equivalents of hexamethyldisilazane and trimethylsilyl chloride in pyridine. The reaction proceeded slowly at room temperature, but facilely at 70 °C as listed in Table 2. The silylated products were isolated by pouring the reaction mixture into methanol. Those with high ds values were, however, soluble in acetone and isolated with acetone.

Table 2. Trimethylsilylation of chitins[a]

chitin	temp (°C)	time (h)	ds[b]
β-chitin	r. t.	24	0.16[c]
β-chitin	r. t.	48	0.32[c]
β-chitin	70	24	2.05[d]
α-chitin	70	24	0.99[d]

[a] With 10 equiv of hexamethyldisilazane and trimethylsilyl chloride in pyridine.
[b] Determined from the C/N ratio of elemental analysis.
[c] Isolated with methanol.
[d] Isolated with acetone.

The silylated products showed improved affinity for solvents and were readily soluble in acetone when the ds values were high. Films could thus be prepared by solution casting. Subsequent treatment of the silylated chitin films with aqueous acetic acid resulted in desilylation to afford chitin films.

Conclusion

Tritylation and trimethylsilylation have proved to proceed efficiently with β-chitin under appropriate mild conditions to give fully substituted derivatives, in sharp contrast to the quite sluggish reactions with the ordinary α-chitin under similar conditions. These results indicate the high potential of β-chitin as a starting material for controlled modification reactions. The resulting organosoluble products would be useful for further controlled modification reactions.

Acknowledgment

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Preparation of Chitin Derivatives via Ring-Opening Reaction with Cyclic Acid Anhydrides

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Abstract

Water-soluble chitin derivatives were prepared via ring-opening reactions with various cyclic acid anhydrides in lithium chloride/N,N-dimethylacetamide system. Some cyclic acid anhydrides such as succinic, maleic, glutaric, and phthalic anhydrides gave successfully water-soluble chitin derivatives.

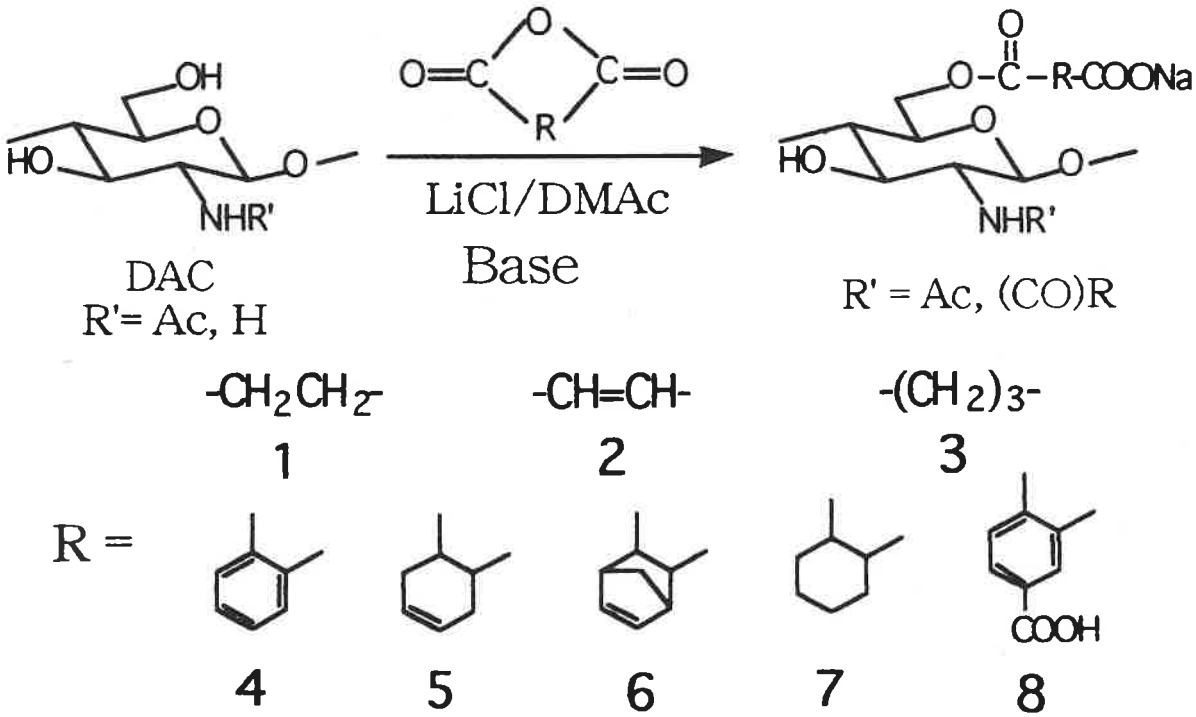
Keywords: Lichium chloride, Dimethylacetamide, Cyclic acid anhydride, Esterification, Water soluble

Introduction

Chitin, a natural mucopolysaccharide composed of N-acetylglucosamine units, is one of the most abundant polymers in nature. At present its industrial utilization, however, is undeveloped compared to the structurally similar cellulose. Commonly, slightly N-deacetylated chitins (below 10% of deacetylation) have been also regarded as "chitin". Chitosan (above 90% of deacetylation) and partially deacetylated chitin (DAC) have various degree of deacetylation (DDA). Chemical modification of chitin is difficult in general because of its strong hydrogen-bonded network structure. The polar aprotic solvent composed of lithium chloride (LiCl) and N,N-dimethylacetamide (DMAc) allows a range of organic reactions including typical alcohol modification reactions such as esterification and carbamate formation. A binary solvent system, LiCl/DMAc, dissolves chitin or cellulose. Chemical modifications of cellulose in the

LiCl/DMAc system have been studied by numerous workers [1-3]. Only a few example was reported on the chemical modification of chitin in the LiCl/DMAc system such as chlorination, acylation and so on [4-6]. We would now like to study the synthesis of novel chitin derivatives soluble in water or organic solvents under the homogeneous conditions.

Herein we report the preparation and characterization of water-soluble chitin derivatives via ring-opening reactions with cyclic acid anhydrides (Scheme 1).



Scheme 1.

Materials and methods

Partially deacetylated chitin (DDA=20%: DAC-20) from shrimp shell was purchased from Wako Pure Chemical Industries Co., Ltd. IR spectra were recorded as KBr pellets on a Shimadzu FT-IR 4200 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in D_2O on JEOL JMN-GX270 spectrometer, sodium 3-(trimethylsilyl)propanesulfonate as an internal standard. Molecular weight was determined by GPC with pullulan as standard on Shimadzu LC-6A apparatus (column, Asahipak GS-220H, -310H, and -510H; eluent, 0.1 M phosphate buffer (pH=7.4); 1.0 ml/min; 50℃).

A typical procedure for the succinylation of DAC was

described as follows. To a solution of LiCl in DMAc (5% w/v: 200 ml) was added DAC (2.0 g), and the mixture was stirred at room temperature for 3 h to give a clear solution. Succinic anhydride (10 g, 100 mmol) was added to 200 ml of a 1.0 %(w/v) solution of DAC (2.0 g) in 5%(w/v) LiCl/DMAc. The mixture became colored to deep purple by the addition of triethylamine (Et_3N, 10.2 g), and then turned to gel within 30 min. After 24 h stirring, the reaction mixture (containing gel) was poured into MeOH (400 ml). The precipitate was filtered, and the polymer was dissolved in 200 ml of water. The product was precipitated by addition of 3 M HCl to adjust the pH 1-2. The precipitate was filtered, washed with MeOH, and dried to give a protonated product (H type). To obtain a salt formed product (Na type), the product was dispersed in 200 ml of H_2O, and adequate amount of Na_2HPO_4 was added to achieve pH 8-10 to give clear solution. The solution was dialyzed and lyophilized. ^1H NMR (270 MHz, D_2O): δ 1.94 and 2.00 (br s, 2.4 H, NHCO$\underline{CH_3}$, 2.46 (br s, 2.5 H, O(C=O)$\underline{CH_2}$), 2.62 (br s, 2.5 H, $\underline{CH_2}$COONa), 3.6-4.2 (br m, 5.8 H, H-2, H-3, H-4, H-5, and H-6 of monosaccharide residue (MR)). ^{13}C NMR (68 MHz, D_2O): δ 24.9 (NHCO$\underline{C}H_3$), 32.9 (O(C=O)$\underline{C}H_2$), 34.5 ($\underline{C}H_2$COONa), 57.6 (C-2 of MR), 65.4 (C-6 of MR), 74-75 (C-3 and C-5 of MR), 82.7 (C-4 of MR), 104-105 (C-1 of MR), 177.2 (NH$\underline{C}$OCH$_3$), 178.0 (O$\underline{C}$=O), 183.3 ($\underline{C}$OONa). Anal. Calcd for $C_{12.8}H_{18.0}O_{8.7}N$: C, 47.29; H, 5.54; N, 4.31. Found: C, 47.15; H, 5.76; N, 4.00.

Other DAC derivatives were also prepared with the same procedure as above.

Results and Discussion

At first of the reaction, we used pyridine as base. The reaction, however, did not proceeded successfully even at high temperature (entry 2 in Table 1). So we tested the effect of pKa value of base on the substitution of succinyl group to DAC. As shown in Table 1, the reaction proceeded successfully by use of strong bases such as DMAP or Et_3N. The reaction temperature did not affect the DS value by use of DMAP (entry 3 and 4). In the absence of base, the reaction did not proceeded (entry 1). From the ^{13}C NMR analysis of the product (1), almost all of signals at C-6 position of monosaccharide residue in 1 was sifted from 62.7 ppm to

65.4 ppm. This result means that the substitution site is mainly at C-6 position of hydroxyl groups in DAC molecule. The amino group in DAC-20 would be fully substituted with succinyl groups though the shift of signals at C-2 position of MR was not observed (because of the small amount of amino group (20%) aginst total monosaccharide residue) .

Table 2 shows the preparation of various DAC derivatives via ring opening reaction with some cyclic acid anhydrides. The reaction proceeded successfully under the reaction

Table 1. Effect of pKa of Base on the Succinylation of DAC[a)]

Entry	DAC-20	Base[b)]		Temp	Yield[c)]	DS
	mg		pKa	℃	%	
1	200	None		110	95	0.1
2	200	Pyridine	5.3	110	90	0.2
3	200	DMAP	9.7	110	97	0.6
4	600	DMAP	9.7	25	90	0.6
5	200	Et_3N	11.0	110	88	0.4

a) Solvent, 5%(w/v) LiCl in DMAc(10 ml/100 mg of DAC); succinic anhydride, 10 equiv. per MR; time, 2 h.
b) Base, 10 equiv. per MR; DMAP: 4-dimethylaminopyridine.
c) Yield(%) = $\frac{\text{(Weight of product) x (MW of MR) x 100}}{\text{(Weight of DAC) x [(MW of MR) + 100 x DS]}}$

Table 2. Preparation of Various DAC Derivatives[a)]

Entry	Anhydride[b)]		Base		Yield	DS[c)]	Mn[d)]
		equiv		equiv	%		(x10^4)
1	Succinic	10	Et_3N	10	84	1.3	17
2	Maleic	10	Et_3N	10	72	1.0	N.D.
3	Glutaric	6	DBU	5	100	0.8	N.D.
4[e)]	Phtharic	10	DMAP	10	99	1.3	11
5	THP	6	DMAP	5	71	1.4	11
6	Norb	10	Et_3N	10	73	1.0	19
7	Cycl	6	Et_3N	5	68	1.2	5
8	Trimellitic	6	Et_3N	5	73	1.0	11

a)DAC (DDA=20%), 0.8-2.0 g; solvent, 5%(w/v) LiCl in DMAc (100 ml/1 g of DAC); time, 24 h; temp, 25℃.
b) THP, tetrahydrophthalic anhydride;
Norb, *cis*-5-norbornene-*endo*-2,3-dicarboxylic anhydride;
Cycl, *cis*-1,2-cyclohexanedicarboxylic anhydride.
c) Determined by ^1H NMR; DDA of the products were ca. 20%.
d) N.D., not determined. e) Time, 2 h; temp, 80℃.

conditions tested in Table 2, though the reactivity of cyclic acid anhydrides is slightly different. Glutalic anhydride did not react to DAC molecule by use of DMAP and Et_3N, and only proceeded by use of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base (entry 3). These results suggests that more strong base is required for the reaction. Though the detail reactivity of each cyclic acid anhydride will be required for studying the reaction mechanism, water-soluble DAC derivatives were obtained successfully under the conditions listed in Table 2.

Table 3. Solubility Data of DAC Derivatives[a)]

Structure	DS	Mn ($x10^4$)	Type	Solvent			
				H_2O	DMSO	$CHCl_3$	EtOH
1	1.3	17	H	−	−	−	−
			Na	+	−	−	−
2	1.3	12	Na	+	−	−	−
3	1.1	5	Na	±	−	−	−
4	1.3	11	H	−	+	−	−
			Na	+	−	−	−
5	1.4	11	Na	+	−	−	−
6	1.3	19	Na	±	−	−	−
7	1.2	5	Na	+	−	−	−
8	1.1	11	H	−	−	−	−
			Na	+	−	−	−

a) Concentration of DAC derivatives (DDA=ca. 20%), 2 mg/ml; time, 1 day; +, soluble; ±, partially soluble; −, insoluble.

Table 4. Effect of pH on the Solubility of DAC Derivatives[a)]

DAC derivative			pH						
Structure	DS	Mn($x10^4$)	1	3	5	7	9	11	13
1	0.6	17							
1	1.3	17							
4	1.3	11							

a) DAC derivatives were dissolved in H_2O (2 mg/ml) and the pH was adjusted by 0.1 M HCl and 0.1 M NaOH. White bar, soluble pH; black bar, insoluble pH; shaded bar, ester linkage was hydrolyzed.

Table 3 shows the solubility of various DAC derivatives. Almost all of DAC derivatives (**1**-**8**, H and Na type) did not dissolve in organic solvent such as dimethylsulfoxide (DMSO), N,N-dimethylacetamide (DMAc), pyridine, $CHCl_3$, EtOH, and acetone. Only H type of **4** dissolved in DMSO that would be caused by the hydrophobic properties of phenyl groups. The insolubility of the H type of **8** in DMSO would be caused by the two hydrophilic hydroxyl groups. Na form of DAC derivatives dissolved or partially dissolved (**3** and **6**) in H_2O though H form of the derivatives did not dissolved. The effect of pH on the solubility of **1** and **4** was also tested (Table 4). SuccinylDAC (**1**) was dissolved at the pH range from 4.5 to 11.0. At the pH below 4.5, the water insoluble materials was precipitated which was redissolved by the addition of 0.1 M NaOH to pH 7. At the pH above 11, however, water insoluble materials did not redissolved by addition of 0.1 M HCl to pH 7. In the case of phthalorylDAC (**4**), the similar phenomenon was also observed and the soluble pH range was from 3 to 11. These results suggest that the hydrolysis of ester linkage was occurred at the pH above 11. The insolubility of **1** and **4** at the pH below 4.5 or 3 would be caused by the change of counter cation from sodium carboxylate (-COONa) to carboxyl groups (-COOH).

In conclusion, water-soluble DAC derivatives could be prepared via the ring-opening reaction with various cyclic acid anhydrides. These derivatives would be expected for the medical use as new type of anionic polysaccharide.

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PREPARATION OF *N*-ACETYLCHITOOLIGOSACCHARIDES FROM CHITOSAN BY ENZYMATIC HYDROLYSIS FOLLOWED BY *N*-ACETYLATION

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Abstract

The purpose of this study is to develop the method for preparing *N*-acetylchitooligosaccharides [(GlcNAc)n] with high degree of polymerization by enzymatic and chemical reactions. Chitosans with various degrees of *N*-acetylation (DA) were hydrolyzed by enzymes and hydrolyzates were acetylated with acetic anhydride. (GlcNAc)n (n=1~7) were detected in the reaction mixtures by HPLC. The yield of hexaose was more than 20% when using lipase, cellulase, and hemicellulase preparations. The mixture (51mg) of pentamer (13%), hexamer (72%), and heptamer (15%) was obtained by selective precipitation from aqueous solution by adding methanol, starting from chitosan (200mg) with DA of 22%. The hydrolysis of chitosans with DA of 10~30% by these enzymes followed by *N*-acetylation is suitable to prepare higher (GlcNAc)n

Keywords: Chitosan, *N*-acetylchitooligosaccharide, hemicellulase, cellulase, lipase, *N*-acetylation, HPLC

Introduction

Chitooligosaccharides [(GlcN)n], *N*-acetylchitooligosaccharides [(GlcNAc)n], and heterooligosaccharides composed of GlcN and GlcNAc are of special interest in agriculture and medicine. They have activities as elicitors, antibacterial agents, immunoenhancers, and lysozyme inducers [1-4]. Higher *N*-acetylchitooligosaccharides such as hexaose and heptaose show high activity at a very low concentration.

The development of the methods of preparing these (GlcNAc)n is attracting growing interest. These have been prepared by chemical, enzymatic and fermentation methods. The processes are hydrolysis of chitin and chitosan and also synthesis

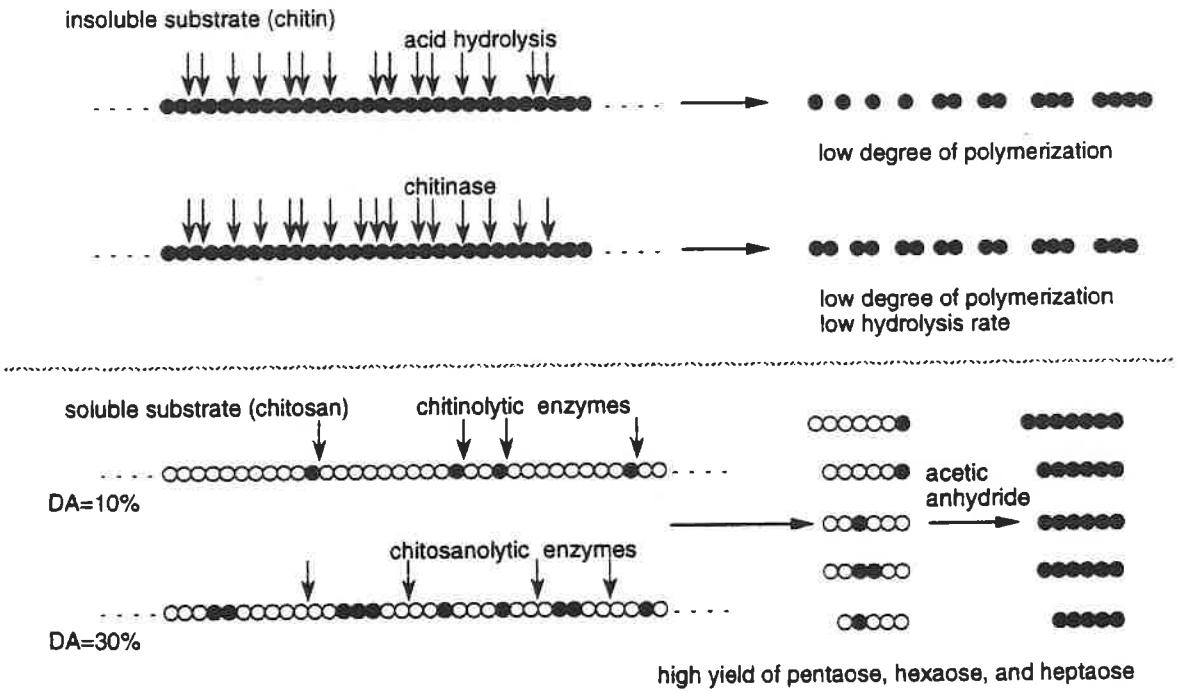
from lower oligosaccharides. Barker et al. reported that monomer to heptamer were obtained by hydrolysis of chitosan with hydrochloric acid, *N*-acetylation, and chromatography [5]. Sakai reported that hexaose was obtained with yield of about 5% by hydrolysis of chitin with hydrochloric acid [6]. Fluorohydrolysis of chitin in anhydrous hydrogen fluoride was also tried [7]. One special procedure is chemical synthesis of hexaose from biose [8].

Effective procedure for preparing higher oligosaccharides from lower ones is a transglycosylation reaction using chitinase and lysozyme [9-11].

Chitin and chitosan are hydrolyzed by chitinase, chitosanase, and lysozyme as well as other enzyme preparations. But there is almost no report on the method for preparing higher oligosaccharides by hydrolysis with these enzymes [12].

In the case of acid hydrolysis, chitin is randomly cut in the solid state. The products have low degree of polymerization. In the case of enzymatic method, chitin is randomly cut by chitinase in the solid state. Soluble higher (GlcNAc)n produced are immediately hydrolyzed by enzyme in the solution. A main product is biose. These methods are not good for preparation of higher (GlcNAc)n (top of Scheme 1).

In the case of degradation of chitin by chitinase, hydrolyzed sites can not be regulated. If chitosan is used as a substrate in a



Scheme 1 Methods for preparation of *N*-acetylchitooligosaccharides

homogeneous state, hydrolyzed sites could be regulated. So higher oligosaccharides will be obtained as speculatively shown in Scheme 1 because chitosan has GlcNAc residues partially and chitinase recognizes GlcNAc residues. In scheme 1 the closed circles are GlcNAc residues and the open circles are GlcN units. Hydrolyzates are heterooligosaccharides composed of GlcN and GlcNAc. These are converted to (GlcNAc)n by *N*-acetylation with acetic anhydride. This speculation has been applied onto hydrolyses of chitosan using chitinase as well as lysozyme [13, 14]. With this concept, we tried development of the methods of preparing higher (GlcNAc)n using hemicellulase, cellulase, and lipase.

Materials and methods

Chitosans were obtained from Katakura Chikkarin Co., Japan. Partially *N*-acetylated chitosans (PNACs) were prepared according to the method of Hirano et al. [15]. Lipase, cellulase, and hemicellulase were obtained from Amano Pharmaceutical Co., Japan and used without further purification. Authentic (GlcNAc)n(n=2~6) were purchased from Seikagaku Kogyo Co., Japan. The chitinolytic activity was assayed according to the method described in the literature [16]. Oligosaccharides in enzymatic reaction mixtures were analyzed by high performance liquid chromatography (HPLC) according to the method described in previons papers [13, 14]. Briefly, chitosan (10mg) was dissolved in acetate buffer (1.6mL) and the solution was mixed with an enzyme solution (0.4mL). The mixture was incubated at 37℃ for several days, diluted with methanol, reacted with acetic anhydride, and stirred for one hour. The solution was diluted with water and acetonitrile and supernatant was analyzed by HPLC. In this work the yield is defined as percentage weight ratio of each oligosaccharide to chitosan.

Results and discussion

In prerious papers [13, 14, 17, 18], we studied the hydrolysis behavior of PNACs using lysozyme and chitinase and tried to prepare higher (GlcNAc)n . But in the case of lysozyme yields of higher oligosaccharides were low: 16, 10, and 18% for biose, triose, and tetraose, respectively. The yields of biose and tetraose in the lysozyme method are higher than those in the case of hydrochloric acid hydrolysis [6].

In the case of chitinase hydrolysis tri-, tetra-, penta-, and hexa-saccharides of GlcNAc were obtained with yields of 25.3, 25.5,

19.6, and 12.3%, respectively. Chitinase hydrolysis was advantageous over the lysozyme process and hydrochloric acid hydrolysis.

Commercially available chitinase is purified to some extent. Then the cost is high although amount of chitinase used is small. In order to reduce the production cost, we tried utilization of low-cost enzyme preparations such as lipase, cellulase, and hemicellulase because it has been reported recently that these enzyme preparations were active on chitosan [19, 20].

The hydrolyzates produced from PNACs by hemicellulase (*Aspergillus niger*) were analyzed by HPLC. The yield of hexaose was about 25% when using chitosans with DA from 9% to 22%. Next this hemicellulase preparation was characterized. The effect of pH on enzyme activity against PNAC with 59% DA was determined (pH4.0~5.5). The optimal pH range was 4.0~4.5. The enzyme retained 55% of the maximum activity even at pH5.5 and 50% of the initial activity after 19 days incubation at PH4.5. The relationship between susceptibility of PNACs to the enzyme and DA (5~56%) was examined. The susceptibility increased with increrased DA (Fig. 1). Then the chitinolytic activity of this hemicellulase preparation is higher than the chitosanolytic activity.

In the case of lipase (*Aspergillus niger*), a similar relationship between DA and yields of (GlcNAc)n was observed as in the case of hemicellulase. The yield of hexaose was about 20% when using chitosans with DA of 14~22% and decreased with increased DA. In the case of cellulase (*Aspergillus niger*), the yield of hexaose was about 25% when using chitosans with DA of 14~22%. The susceptibility of chitosans with various DA to these preparations were similar to that to hemicellulase preparation (Fig. 1). The chitinolytic activity is higher.

For the preparative-scale production, chitosan (200mg) was hydrolyzed by hemicellulase (200mg) and acetylated with acetic anhydride. Higher (GlcNAc)n were separated as described in Fig.2. They were selectively precipitated from an aqueous solution of (GlcNAc)n mixtures by adding methanol, because the solubility of (GlcNAc)n in water-methanol mixture decreases with increased n. The solubility was examined as follows: methanol (5mL) was added to an aqueous solution (2mL) of pentaose (2.34mg),and hexaose (4.64mg). 76% of pentaose remained in the solution while only 18% of hexaose remaind. In addition, 96% of hexaose were precipitated from the solution when more methanol (2mL) was added. Addition of three or four fold of methanol made it possible to separate hexaose from lower oligosaccharides. In the case of

Fig.2, the mixture of pentaose, hexaose, and heptaose was obtained as lyophilized powder with yield of 51mg which contained 36.7mg of hexaose. This yield was lower than that expected from the analytical-scale experiments but this method is simple and does not include a chromatographic step.

The yield of hexaose was higher than that when using chitinase. But the yields of triose and tetraose were lower than those when using chitinase. It is difficult to explain these defferences. The main activity of the hemicellulase preparation against chitosan was chitinolytic activity but it was found that this preparation contained the hydrolase activities against chitohexaose and *N,N*-diacetylchitobiose. The chitinase preparation didn't have the activity against them. It might be considered that the defference of the yields is caused by the composition of chitinolytic, chitosanolytic, and exo-glucosidase activities and the action modes of these activities. But these activities have not been studied quantitatively. It largely depends upon future systematic studies.

Conclusion

Higher (GlcNAc)n such as hexaose and heptaose were

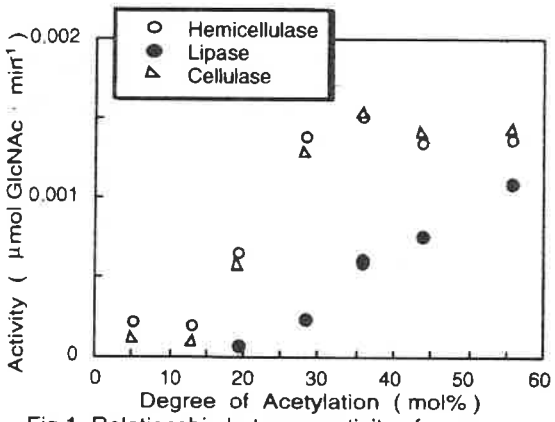


Fig.1 Relationship between activity of enzyme preparations and DA of chitosans

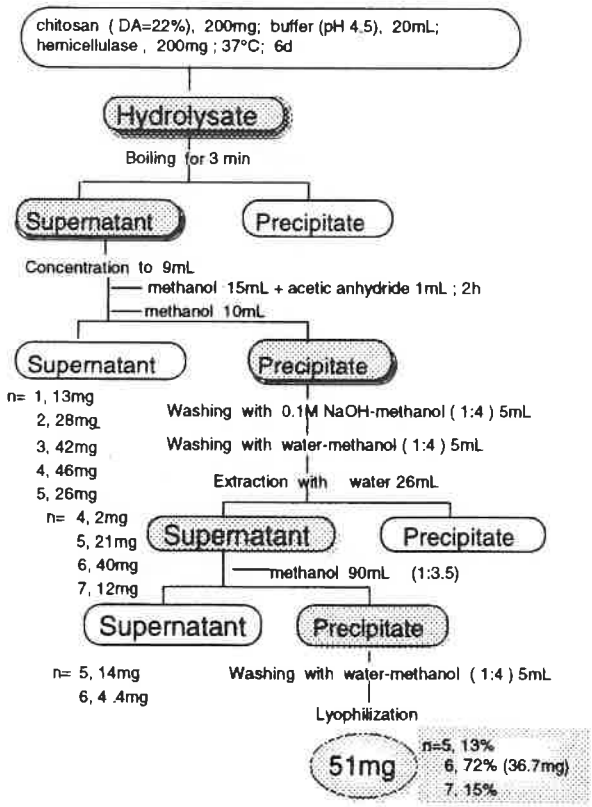


Fig.2 Process of preparation and separation of (GlcNAc)n

prepared by hemicellulase. The yield of hexaose was about 18% when using hemicellulase preparation. Hemicellulase is more suitable for the preparation of hexaose and heptaose.

Acknowledgements

We thank Katakura Chikkarin Co. for chitosan samples, Amano Pharmaceutical Co. for enzyme preparations, Ms. K. Iida and Ms. H. Morino for their skillful technical assistance.

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Effect of Sonication on the Acid Degradation of Chitin and Chitosan

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Abstract

Chitin and chitosan are hydrolyzed by hydrochloric acid and by formic acid employing sonolysis concomitantly, and the produced oligosaccharides in the degraded fluid are analyzed with high performance liquid chromatography (HPLC). Effect of sonication on the produced amount of oligosaccharides obtained by the degradation is investigated in relation with ultrasound frequency. The degree of deacetylation of $(GlcNAc)_n$ of the produced oligosaccharides is also investigated. The positive effects of sonication on production of these oligomers are observed most strongly for 45 kHz ultrasonic irradiation, which is in accordance with OH radical forming ability in Fricke solution.

Keywords: Chitin; Chitosan; Hydrolysis; Sonication

1. Introduction

The degradation of polymers by ultrasound has been investigated for more than fifty years. During the sonolysis of aqueous solutions of polymers, polymer chains are ruptured by the action of hydrodynamic shear forces near the cavitation bubbles [1]. Polymers are also attacked by active chemical species and are thermally decomposed in some cases [1]. In the previous paper [2], we reported the effect of ultrasound on degradation of chitin to N-acetyl chitooligomers during acid hydrolysis by hydrochloric acid. In the present experiment, formic acid will also be used during hydrolysis, and effects of ultrasound frequency on the degradation of chitin and chitosan will be investigated.

In case of the degradation of chitin, it is necessary to be careful to avoid deacetylation because chitin has an acetylamino group. Therefore, the combination of the acid degradation and sonolysis, which is able to degrade polymers without dependence on temperature of the bulk

solution, is considered to be an effective method. Composition ratios and absolute amounts of N-acetyl chito and chitosan oligosaccharides of the products are examined to make clear whether the acid-sonolysis method is adequate to prepare the oligosaccharides, especially higher components of them.

2. Materials and methods

Samples: Chitin (Chitin-1) flake and a series of $(GlcNAc)_n$ (n=2-7) and a series of $(GlcN)_n$ (n=2-7) of the purest grade were kindly donated by Yaizu Suisan Kagaku Industry Co., Ltd. Chitosan powder(Chitosan-1, 10cp/0.5%, degree of deacetylation; 94%) was also kindly donated by Hokkaido Soda Co. Ltd. A series of samples having different degrees of deacetylation and viscosity-[Chitosan 10B, 9B, 8B, 7B and Chitin EX] was purchased from Katokiti Co., ltd.

Ultrasonic apparatus: ①Branson B-220H (frequency 45 kHz, output power 60 W, volume of bath 3 l), ②Branson 1210J (47 kHz, 60 W), ③Branson 2210J (47 kHz, 70 W) and Honda W-113 (28 kHz, 45 kHz, 100 kHz) were used. When Branson 2210J was used, frequency was denoted as 47' kHz.

Experiment 1: Chitin-1 and chitosan-1 (3 g) were dispersed and dissolved in 20 ml of chilled concd HCl at 5 ℃ or lower in a 100 ml flask, equipped with a cooling device, and the flask was clamped in the center of the sonication baths of ①, ② and ③. The temperature of the bath water was controlled at about 37 ℃ for the irradiation of 0-2 h. Then, the degraded fluid was dispersed into 80 ml of chilled water at 5℃ or lower, mixed for 30 min with stirring, and allowed standing in a refrigirator over night, followed by centrifugation. The filtrate was neutralized with the addition of sodium hydroxide solution (2.5 mol dm^{-3}) under well stirring with a magnetic stirrer and made to be 250 ml, followed by separation of precipitates. The supernatant was demineralized with an electric dialyzer (Micro Acilyzer G 1, AsahiKasei Kogyo Co., Ltd.) using an Aciplex cartrige AC-220-10 and analyzed with HPLC.

Experiment 2: Chitin-1, Chitin EX, Chitosan-1, Chitosan 10B, 9B, 8B and 7B (0.2 g, 1 g, 3 g) were degraded in 20 ml of concd HCl and various concentration of formic acid in a 50 ml Pyrex test tube [23 mm diameter × 250 mm high] for 0-2 h at 37℃ under ultrasonic irradiation with 28 kHz, 45 kHz and 100 kHz (100 W, Honda W-113). The liquid inside the flask was maintained at the same level as that of the water in the bath, in order to obtain sufficient reproducibility in the standing wave field. During the sonication the sam-

ple solution was bubbled with an air pump. Then, the same procedure as in Experiment 1 was applied for the degraded fluid, and the final neutralized fluid was less than 150 ml. 10 ml of the filtrate was demineralized and analyzed with HPLC.

HPLC analysis: An aliquot of demineralized liquid was used for analysis by HPLC. Sample injection volume was 25 μl passed through a 0.22 μm membrane filter (Millex-GS). The HPLC system consisted of a Hitachi 683-30 chromatograph, a LSI RI-98 RI detector (Labosystem Co., Ltd.), and a D-2500 chromato-integrator (Hitachi Ltd.). Sugars were separated on a Asahipak NH2p-50 column (Asahipak NH2p-50 4.6ϕ X 250 mm, guard column ;Asahipak NH2p-50G 4.6ϕ X 10 mm, Asahi Kasei) using acetonitrile and water mixtures (65:35) as the mobil phase, at a flow rate of 0.8 ml/min. The peaks eluted in the first 20 min were analyzed on the basis of the retention time of each standard substance of $(GlcNAc)_n$ or $(GlcN)_n$ and the mixture of standard substances of $(GlcNAc)_n$. The concentration of each saccharide was estimated from the peak area on the chromatogram.

Estimation of relative absorbed energy by solvent: To estimate relative absorbed energy by the solvent during sonolysis, relative OH radical formation in Fricke solution was measured. Fricke solution prepared by the standard method [3] was irradiated in a flask or in a test tube as in the case of Experiment 1 or Experiment 2.

Analysis of the degradation products: Demineralized liquid was condensed and dried using a freeze dryer (Yamato Model DC-31) and then in vacuo until its weight became constant in a desiccator. The yield(%) of the hydrolysis products was calculated against the initial amount of sample. Degree of deacetylation was examined with colloid titration.

3.Results and Discussion

Condition of sonication for degradation by HCl: Components of oligosaccharides detected in 20 min by HPLC in Experiment 1 are shown in Fig.1, which indicates that sonication with ①45 kHz is the most effective, and sonication with 47 kHz or 47'kHz comes in the order of effectiveness. Fig. 2 also shows that ultrasonic irradiation enhances degradation of chitin and chitosan. The amounts of produced chitin oligosaccharides increased with time under these conditions, and about 70% of chitin-1 dissolved in concd HCl was degraded to N-acetyl chitooligosaccharides in 120 min. However, the higher oligomers ($n \geqq 6$) are produced in 100 min most efficiently [2]. On the other hand, the absolute

amounts of produced oligomers from chitosan-1 are small. In the solution being left after demineralization relatively large amounts of higher oligomers were precipitated.

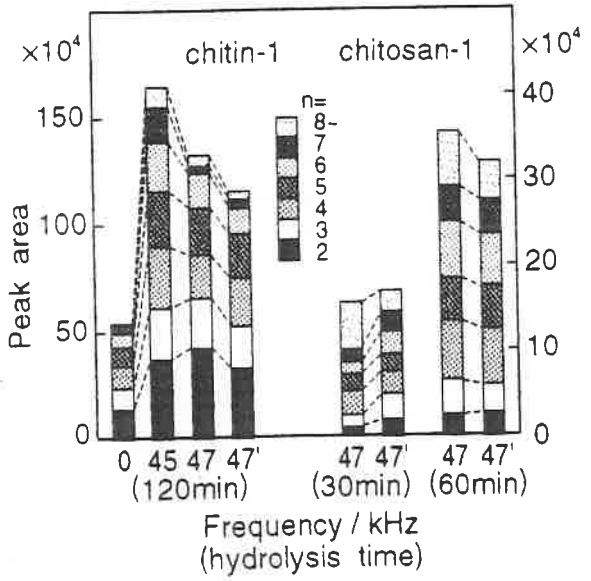

Fig. 1 Components of $(GlcNAc)_n$ and $(Glc)_n$ (n=2 ~8 ~) in the degradation fluid obtained from 3g of chitin-1 and chitosan-1 in 20ml of concd HCl employing sonolysis under 0, 45 kHz, 47 kHz and 47' kHz frequencies for chitin-1 and 47kHz and 47' kHz for chitosan-1.

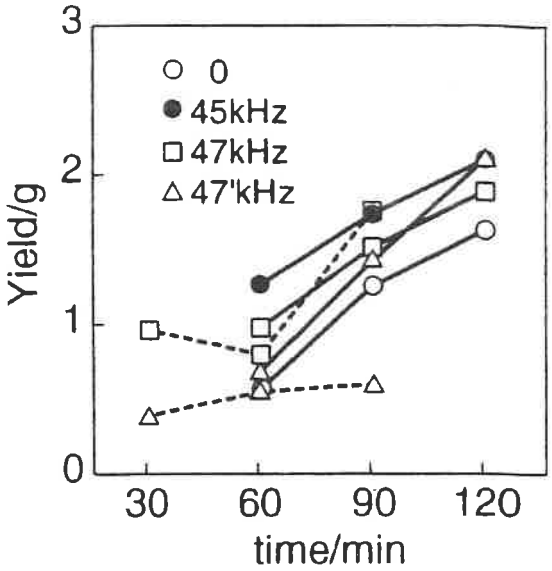

Fig. 2 Effect of ultrasound irradiation on the yield of oligomers obtained from 3 g of chitin-1 (—) and chitosan-1 (···) in Experiment 1.

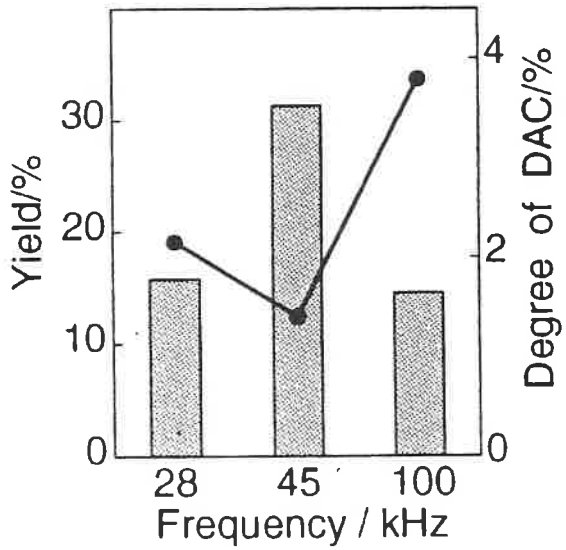

Fig. 3 Effects of ultrasound irradiation (60 min) on the yield of N-acetyl chito-oligosaccharides obtained from 3g of chitin -1 in Experiment 2 and the degree of deacetylation of the obtained product.

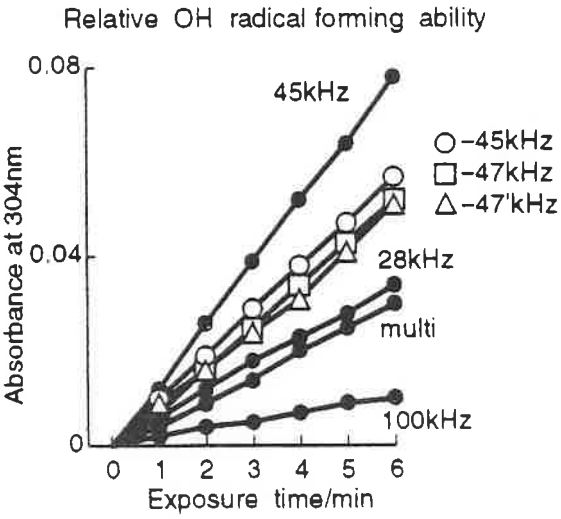

Fig. 4 Production of OH radical in Fricke solution exposed to 28 kHz, 45 kHz, 100 kHz and multi freq. (28 kHz, 45 kHz, 100 kHz each 1 sec in tern, consecutively) ultrasound of 100 W in a 50 ml test tube (all denoted by ●), and ①-45 kHz (○), ②-47kHz(□) and ③-47' kHz(△) ultrasound in a 100 ml flask.

Table 1 Degradation of chitin and chitosan

solvent	sample 1g	Freq. /kHz	time /min	yield/g colloid	yield/g oligomers	HPLC peak area×10⁴ (n: main oligomer)[a]
formic 85% acid	chitosan-1	0	60	1.07	0.30	13.8(n=7)
		28	60	0.69	0.38	16.3(n=4)
	chitin-1	0	60	1.18	—	8.2
		multi	30	1.09	—	15.1
		multi	60	0.87	1.09	34.3
49%	Chitosan 10B	28	120	3.88	0.04	60.8(n=5)
		45	120	3.97	0.07	58.3(n=2)
		0	120	2.41	0.04	22.3(n=8 ~)
	(0.2g)	28	120	2.09	0.08	18.3(n=7)
75%		45	120	—	1.78	—
98%		45	120	—	1.61	—
49%	Chitin EX	0	120	1.28	0.14	23.8
		45	120	1.59	0.09	29.1(n=8 ~)
concd HCl	Chitosan 9B	45	120	1.97	0.42	26.4(n=7)
	Chitosan 7B	45	120	0(2<)[b]	1.93(0.05)	—
	Chitin EX	0	120	2.23	0.23	—
		45	120	1.74	0.41	24.2(n=8 ~)

a) n: number of $(GlcNAc)_n$ or $(Glc)_n$ produced mainly.
b) being left before demineralization

Effect of sonolysis: Fig. 3 shows the results of Experiment 2 carried out with 20 ml of concd HCl under sonication with 28 kHz, 45 kHz and 100 kHz. For concd HCl, 45 kHz ultrasound was most effective on yields of degradation products, and 28 kHz and 100 kHz came in the order of effectiveness. The yield(%) of saccharides in the soluble fraction hydrated under 45 kHz irradiation (Fig. 3), produced in 60 min by acid-sonolysis was about four times higher as compared with that obtained by HCl degradation only. The degrees of deacetylation of produced oligomers are very low as shown in Fig. 3. Under 45 kHz ultrasound irradiation, the degree of deacetylation is minimun. In Experiment 2, 10 ml of the neutralized solution after irradiation by ultrasound of 45 kHz, 28 kHz and 100 kHz was condensed to 5.9 ml, to 6.3 ml and to 6.9 ml during the demineralization. It suggests that sonication with 45 kHz ultrasound would maximally accerelate dehydration of water surrounding saccharide chains. For formic acid, as shown in Table 1, the amounts of products of N-acetyl chitooligomers and chitosan oligomers were very changeable with sample, solvent and other conditions, because of changes of solubility of produced oligomers during preparation, especially for higher oligomers. Some of the data listed in Table 1 indicate that a degradation reversal phenomenon, that is, hydration is clearly observed for products left standing in the aqueous solution for long time.

4.Conclusions

Sonication around the frequency of 45 kHz enhances effectively the degradation of chitin and chitosan by concd HCl and formic acid. It is in accordance with OH radical forming ability in Fricke solution (Fig. 4), which is proportional to the absorbed energy (dose) by the solvent according to ref.3. That the ultrasonic waves of 28 kHz were less effective (Fig. 3) might be ascribed to the less effective energy absorption by the solvent. The yield of oligosaccharides for 100 kHz irradiation is a little lower than 28 kHz, but the yield is higher than that expected from the OH radical yield in Fricke solution shown in Fig. 4. Therefore, whether the increase in the yield shown in Fig. 3 is frequency dependent or 'dose 'dependent remains open to question. Investigation on this point is now in progress.

According to Riesz et al.[4], around the collapsing cavitation bubbles, hydrophobic molecules would accumulate preferentially. For saccaride chain, scavenging of hydrogen atoms and hydroxyl radicals would take place. The process will enhance the yield of oligosaccharides and the monomer. The presence of Cl atoms and Cl_2 molecules would accelerate exclusion of water layers around the saccharide chain. Without dehydration around the chain, degradation of the saccharide chain seems to be difficult. Under the dehydrated condition, chain scission will easily occur by the attack of active chemical species.

The combination of acid degradation and sonolysis does not require longer than two hours to get N-acetyl chito and chitosan oligosaccharides. For the preparation of higher oligomers such as pentamer and hexamer, the separation [5] should be carried out without delay.

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GRGDS-GRAFTED CHITOSAN FOR BIOMIMETIC COATING

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Abstract

We have covalently grafted on chitosan an oligopeptide containing the RGDS sequence specific to endothelial cell attachment, via a water-soluble agent, the 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride. Immobilization of GRGDS was confirmed by ATR-FTIR analysis, and the yield of the grafting reaction, calculated from UV spectroscopy measurements, reached approximately 90%.

Keywords : chitosan, peptide, GRGDS, ATR-FTIR, UV spectroscopy, endothelial cells, carbodiimide, covalent grafting.

Introduction

Surface modification is the most common technique to improve biomaterial haemocompatibility. One of the approaches consists of the preparation of biomimetic surfaces. As the first step of such procedure, we propose the grafting on the chitosan of a biologically active oligopeptide containing the RGDS sequence, namely Arginine-Glycine-Aspartic acid-Serine, specific to endothelial cell attachment. The RGDS sequence is an active cell-binding site present in adhesive proteins like fibrinogen, fibronectin, vitronectin and von Willebrand factor. This modified chitosan could be used to coat vascular implants and therefore to induce the formation of a new endothelium at the biomaterial surface.

The GRGDS peptides were introduced as lateral substitutes by chemical modification of the chitosan. This polysaccharide was chosen because its pendant $-NH_2$ groups constitute reactive sites for the grafting, in using a coupling reagent, the 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), in mild conditions. Furthermore, its hydrophilic behavior is expected to protect the immobilized peptides in maintaining aqueous environment around them.

Experimental

Materials

The chitosan (Fluka) was purified by precipitation in butanol at 4°C and dried under vacuum at 40°C. Its molar mass, measured by size exclusion chromatography, is 520 000 $g.mol^{-1}$. The EDC (SIGMA) was used as received. The GRGDS peptides (Bachem) were stored at 4°C before use.

Synthesis

The chitosan was solubilized in 0.5% acetic acid solution. The pH of the solution was adjusted to 4.5 with a phosphate buffered saline solution (PBS). Then, the peptides and the

EDC in PBS were added. EDC was introduced with an excess of 3, proportionally to the expected substituted $-NH_2$ groups of the chitosan. The excess of EDC is necessary to prevent the formation of peptide coupling. The reaction was carried out at 4°C during 3 hours. The modified chitosan was recuperated in an ultracentrifugation cell with a cut-off of 30 000 $g.mol^{-1}$, which separated it from non-grafted products and salts. It was dried under vacuum during 48 hours.

Measurements

The spectrometer FTIR was a Perkin Elmer Paragon 1000 equipped with the Golden Gate Single Reflection Diamond ATR. Pure and dried products were compressed against the crystal with a calibrated force to obtain comparable and reproducible spectra.

UV Spectra have been recorded with an UVIKON apparatus. All the samples were diluted in 0.5% acetic acid solution used as reference. This technique has allowed us to determine the yield of the substitution.

Results and discussion

The microstructure of the chitosan was identified by ATR/FTIR (figure 1). The spectrum of purified chitosan shows characteristic peaks of amide I (-C=O) absorption at 1652 cm^{-1}, amide II (-NH-) at 1586 cm^{-1}, amide III at 1313 cm^{-1}, and CH stretching band at 2874 cm^{-1}. Other peaks at lower wave numbers near 1150 and 1050 cm^{-1} are respectively attributed to C-N and C-O ether groups. The content of units bearing NH_2 groups was calculated according to Miya [1], from the ratio of the absorption bands of (-CH) and amide I groups. The deacetylation degree of the chitosan was 91%.

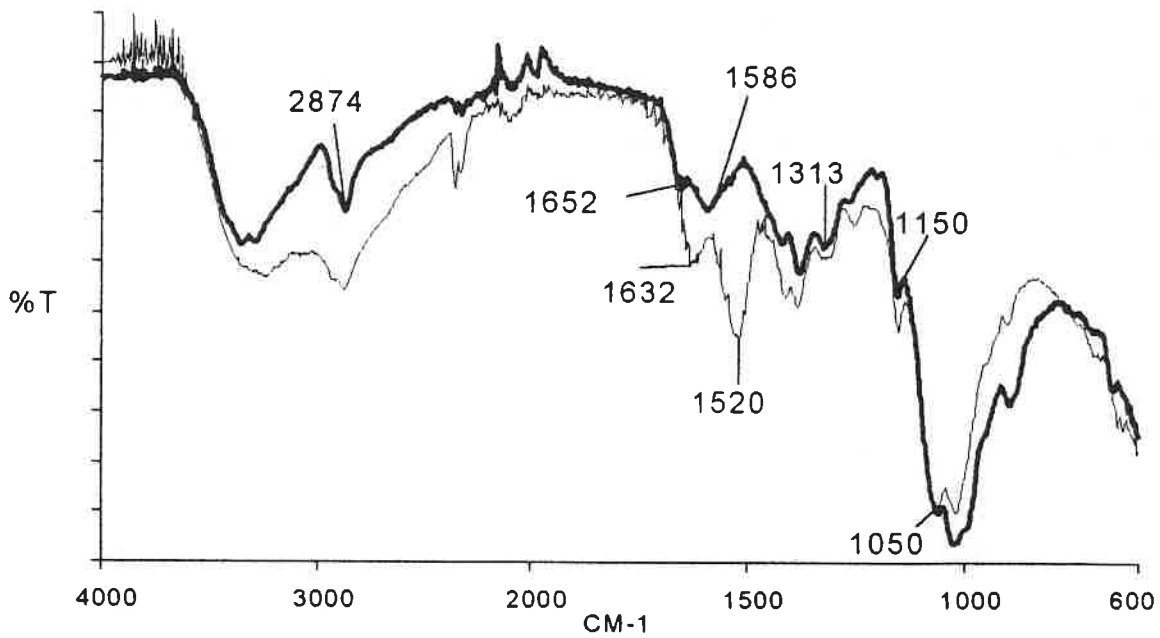


Figure 1 : ATR/FTIR spectra of chitosan (▬) and GRGDS-grafted chitosan (–).

The purified chitosan exhibited an UV band absorbance at 219nm, which followed the Beer-Lambert's law with a molar extinction coefficient ξ_{219} of 0.1374 $l.g^{-1}.cm^{-1}$.

The amide I band of the IR spectrum of GRGDS peptides has contribution at 1638 cm^{-1}, and the amide II band at 1522 cm^{-1}. The amide III region is located between 1137 and 1264 cm^{-1} (figure 2).
The UV spectrum of the GRGDS showed a maximum located at 221 nm. This absorption band yielded to the Beer-Lambert's law. The molar extinction coefficient of the peptide was ξ_{221} = 1.8358 $l.g^{-1}.cm^{-1}$.

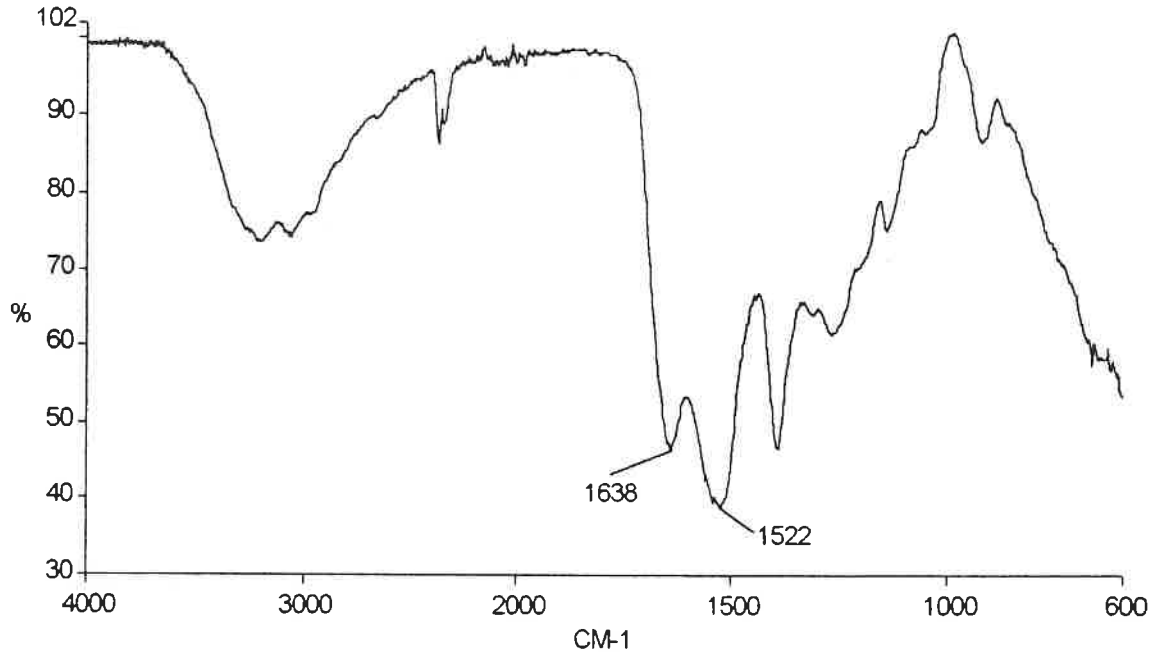
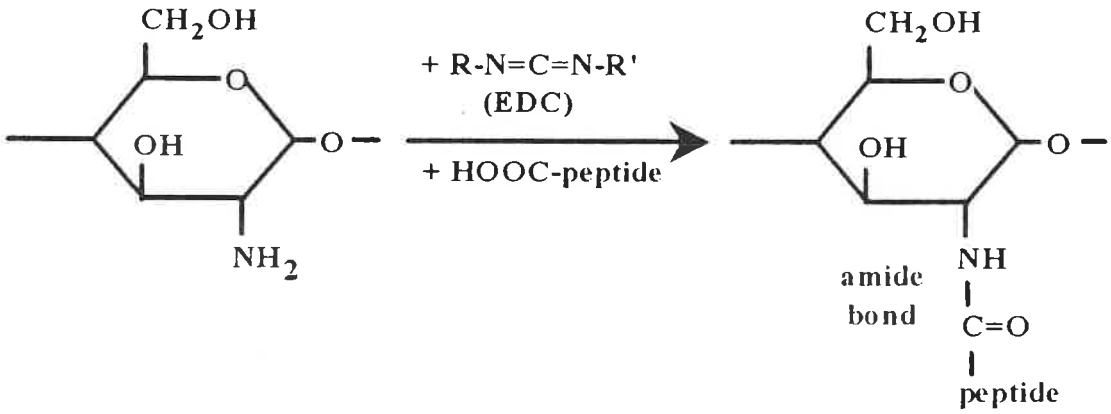


Figure 2 : ATR/FTIR spectrum of GRGDS peptides.

The synthesis of GRGDS-grafted chitosan was carried out in one step (scheme 1). In the literature [2], the EDC firstly reacts with the ionized carboxyl groups to give a compound o-acylisourea. This intermediate product can react again with the carboxyl groups of peptides to produce a carboxylic anhydride. An amide bond is formed by the reaction of the amino groups of the chitosan with the carboxylic anhydride and the o-acylisourea. Thus, a covalent grafting between the peptides and the chitosan is obtained.



Scheme 1 : Synthesis of GRGDS-grafted chitosan
(R : CH_3-CH_2, R' : $(CH_2)_3NH(CH_3)_2^+$, Cl^-).

Grafting of GRGDS was confirmed by ATR/FTIR measurements. The spectrum of the modified chitosan for an expected degree of $-NH_2$ substitution of 10% is presented in figure 1. The amide I and amide II absorption bands of the peptides grafted on chitosan have respectively contributions at 1632 and 1520 cm^{-1}.

In order to verify that the EDC is not consumed by the reaction between chitosan and acetic acid in which the chitosan is solubilized, we have applied the same protocol of synthesis but without the peptides. The ATR/FTIR spectrum of the recuperated product is presented in figure 3. The intensity of the (-C=O) absorption band located at 1644 cm^{-1} is higher than that of the chitosan. The degree of deacetylation of this polymer, calculated from the spectrum, is equal to 82.5%. According to the mechanism of reaction proposed by Nakajima et al. [2], the reaction of the EDC and chitosan in the acetic acid leads to a reacetylation of the chitosan, and consumed about 8.5% of NH_2. However, as the amide peaks are predominant in the GRGDS-grafted chitosan spectrum (figure 1), the reaction between the carboxylic groups of the peptides and the chitosan seems to be favored, and thus, this has led us to neglect the reaction with the acetic acid in the yield calculation.

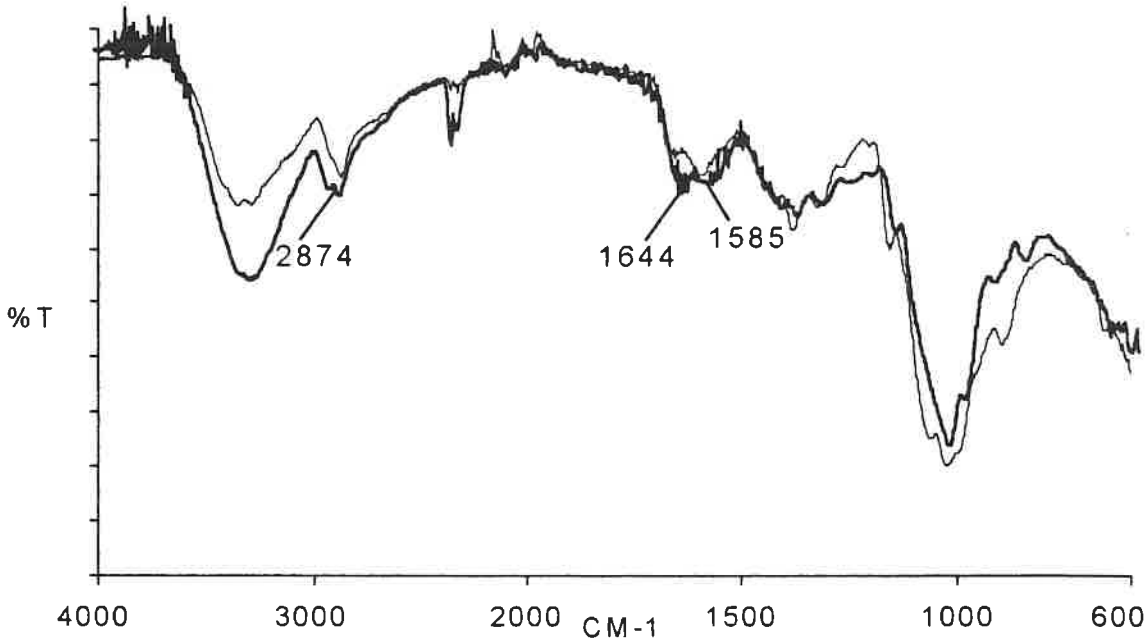


Figure 3 : ATR/FTIR spectra of chitosan (–) and product of reaction between chitosan, EDC and acetic acid (▬).

The chitosan was grafted with different concentrations of GRGDS peptides from 5, 10 to 15% of substituted $-NH_2$ groups. As expected, the intensity of the IR bands of amide bonds in the modified chitosan increases with the content of grafted GRGDS (figure 4).

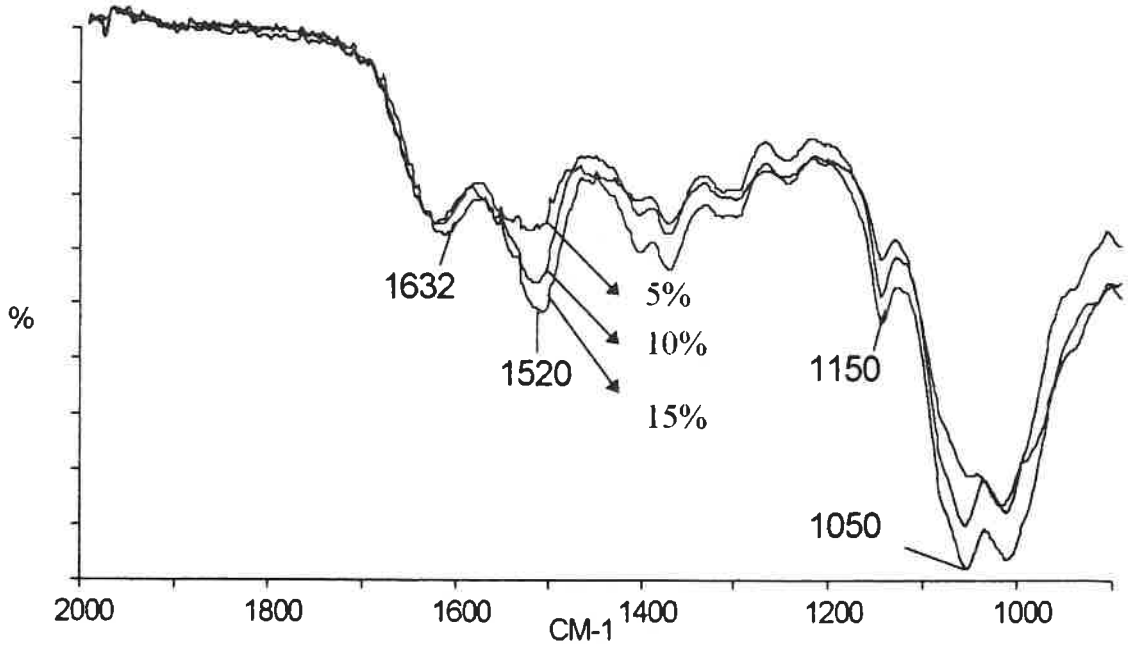


Figure 4 : ATR/FTIR spectra of GRGDS-grafted chitosans with different degrees of substitution.

The analysis of the UV spectra of modified chitosans (figure 5) has allowed us to calculate the yields of the reaction.

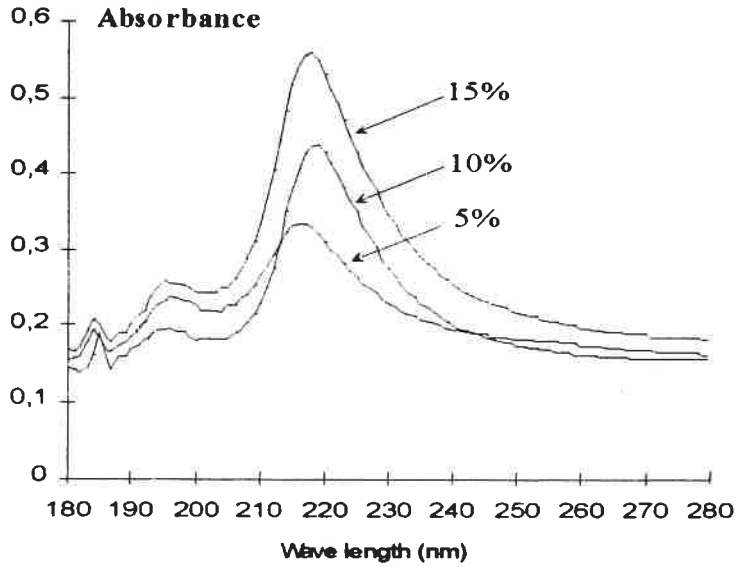


Figure 5 : UV spectra of GRGDS-grafted chitosans with different degrees of substitution.

In substracting the theoretical chitosan absorbance to grafted-chitosan absorbance, the concentration of peptides in the synthetized polymer can be deduced with the Beer-Lambert's laws established for the native products. Whatever the GRGDS concentrations, yields were higher than 80%. Results are summarized in table 1. The peak in the UV spectra of chitosan bearing 8.7 and 12.7% of GRGDS tends to shift to 221 nm and to become sharper, close to the characteristic peptide absorbance (figure 5).

Expected degree of NH_2 substitution (%)	5	10	15
yield of the reaction (%)	86	87	84
Experimental degree of NH_2 substitution (%)	4.3	8.7	12.7

Table 1 : Yields of grafting of GRGDS onto $-NH_2$ groups of chitosan.

Conclusion

Conditions were found for the preparation of chitosan bearing GRGDS peptides as lateral groups through a covalent amide bond. The yield of the synthesis reached approximately 90%. The rate of grafted peptide has no influence on the reaction yield. A side reaction has been shown. The reaction of EDC with the acetic acid led to the reacetylation of the chitosan.

This modified chitosan could be used to coat implants in contact with blood. Its biological effectiveness would have to be assessed by endothelial cell culture.

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Acknowledgments

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N,N,N-TRIMETHYL CHITOSAN CHLORIDE (TMC) OF HIGH DEGREE OF SUBSTITUTION AS A POTENTIAL ABSORPTION ENHANCER FOR HYDROPHILIC DRUGS

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Abstract

Quaternary ammonium salt derivatives of chitosan, having degrees of substitution up to 70%, have been synthesized by varying the steps and the duration of the reaction. These cationic polymers have been prepared by reductive methylation of a 93% deacetylated chitosan by methyl iodide at 60^0C and the counterion I^- was exchanged with Cl^- in an aqueous solution of 10% NaCl.The characterization of these compounds has been done by ^{1}H-NMR spectroscopy .

We found out that a second reaction step is necessary to obtain high degrees of quaternization.

Derivatives having degrees of substitution of approximately 20 and 60% have been chosen to be tested for their ability in opening the tight junctions of intestinal Caco-2 cell monolayers, thus decreasing the transepithelial electrical resistance (TEER) and increasing the transport of a hydrophilic marker compound (^{14}C-mannitol) at an intestinal pH value of 7.2.

The results are showing that only the highly substituted TMC (degree of substitution 60%) is able to decrease the TEER and to increase the transport of the model compound.

Keywords: Chitin, chitosan, N,N,N-trimethyl chitosan chloride, Caco-2 monolayers, TEER, permeability enhancer.

Materials and methods

TMC: Chitosan (93% deacetylated) was a gift from Pronova Biopolymer A.S. (Drammen, Norway) methyl iodide and 1-methyl-2-pyrrolidinone were obtained from Acros (Geel, Belgium).

One step synthesis: To prepare TMC, a mixture of chitosan, sodium iodide, methyl iodide was stirred in a basic 1-methyl-2-pyrrolidinone solution for one hour at 60^0 [1]. The product was precipitated using ethanol and subsequently isolated by centrifugation. After washing with ethanol and centrifugation, the material was dissolved in water, to which 1M HCl in ethanol was added carefully, thus exchanging the iodide for chloride. Centrifugation and washing with ethanol yielded a white, water soluble powder, which was dried in vacuo at 40°C.

Two step synthesis : The intermediate product N-trimethyl chitosan iodide obtained after the reaction mentioned above (one step synthesis) was allowed to react with methyl iodide under the same conditions for 30 min. An additional amount of methyl iodide and NaOH-pellets were added and the stirring was continued for 1 hour.

The product was dissolved in a NaCl aqueous solution, instead of HCl, to exchange the iodide with chloride. The polymer was precipitated with ethanol, isolated by centrifugation and thoroughly washed with ethanol. In vacuo drying yielded a white, water-soluble powder. ^{1}H-NMR-spectra were measured in D_2O at 80°C, using a 300 or 600 MHz spectrometer (Bruker, Switzerland). No attempts were made to remove the residual water from the NMR- sample, because at 80°C this peak does not interfere with the spectrum of the polymer

Caco-2 cells: Caco-2 cell cultures of passages 82 and 94 were seeded in Costar Transwell plates (Costar, The Netherlands). DMEM (Dulbecco's Modified Eagle's Medium, pH=7.4) supplemented with foetal calf serum was used as culture medium. The cells were kept at 37^{0}C in an atmosphere of 95% air and 5% CO_2, the medium was changed every second day until cells were 22-24 days old. Caco-2 cell confluent monolayers seeded on 24- and 6- well plates were used to measure the TEER by a Millicell ERS meter (Millipore Corp. USA) and the transport of ^{14}C-mannitol (MW 182 57mCi/mmol Amersham Life Sciences, UK) respectively. The radioactivity of the obtained samples was determined after adding 3 ml of scintillation cocktail (Packard Instruments Co., USA). Solutions of chitosan HCL (Pronova, Norway), TMC Cl^--after the one step synthesis and TMC Cl^--after the two step synthesis in DMEM serum free buffered with HEPES were applied apically at different concentrations and pH values for the TEER experiments. For the transport experiment the above preparations contained also the hydrophilic marker. After finishing the experiments the cell monolayers were checked with the trypan blue exclusion technique [2].

Results and discussion

Chitosan is a biocompatible and biodegradable polymer which shows several valuable properties in the biomedical field. It can be used as a bacteriostatic, homeostatic, anticholesterenemic or anticoagulant agent, as a wound dressing, and as a pharmaceutical additive in drug delivery systems[3]. Recently, it has been refered that this polymer enhances the absorption of peptide and protein drug across nasal[4] and intestinal epithelia[5]. Chitosan glutamate does not only enhance the in vitro transport of small hydrophilic compounds like ^{14}C -mannitol [2], but also the transport of high molecular weight peptide drugs like 9-desglycinamide, 8-arginine vasopressin. Chitosan hydrochloride has been validated as an intestinal absorption enhancer in vivo of the peptide drug buserelin when it was coadministered in rats[6]. However, at intestinal pH values (7.2) chitosan is deprotonated and insoluble (apparent pK_a 5.6).

TMC has already been reported as a permanently quaternized chitosan derivative for its superior solubility in a wider pH range in comparison with chitosan [1,7]. This property makes TMC a promising candidate for an absorption enhancer[8]. The degree of substitution was expected to play an important role on the ability of this polymer to open the tight junctions of intestinal epithelia and to enhance the transport of hydrophilic compounds.

The ^{1}H-NMR spectrum of the obtained TMC after one step synthesis is depicted in Fig. 1. It clearly shows a peak at 3.1 ppm assigned to $N(CH_3)_3^+$, together with a smaller peak at 3.4 ppm assigned to $N(CH_3)_2$, according to a previously reported peak assignment[1]. We calculated the degree of quaternization of this product to be 35%.

To investigate the effect of the degree of quaternization, we attempted to synthesize TMC with preferably higher substitution degrees.

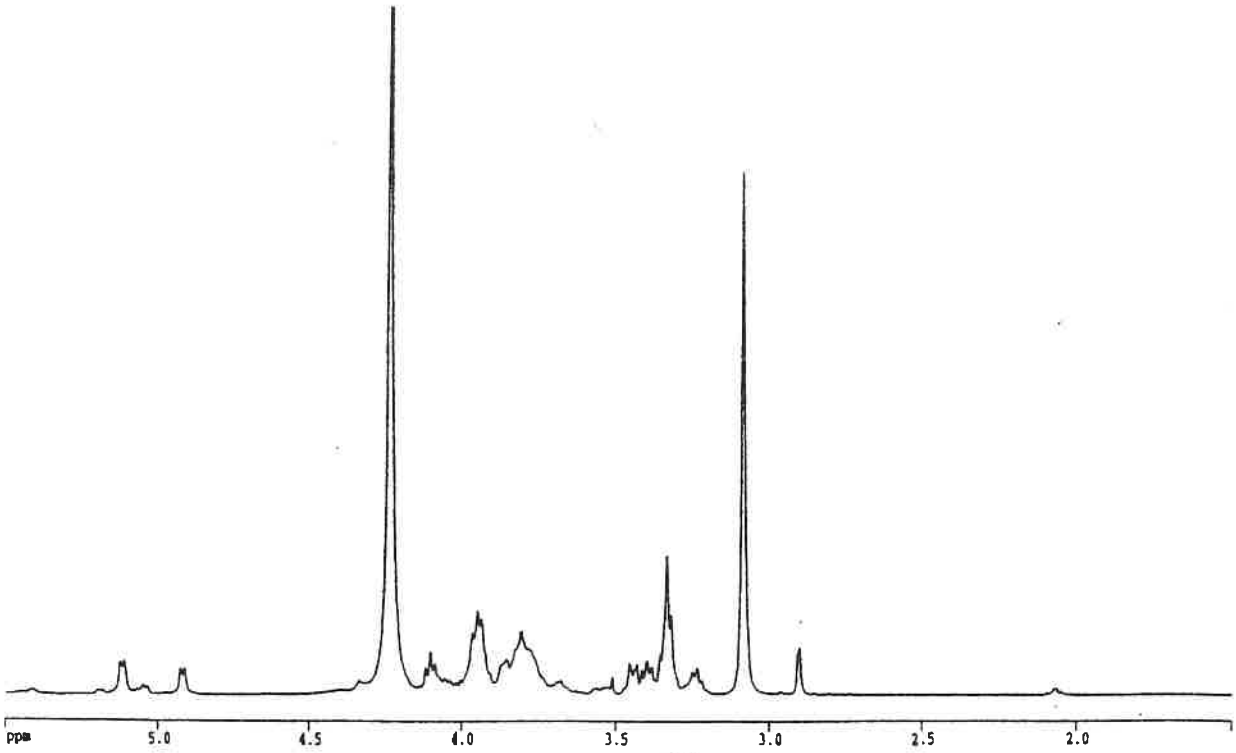

Fig. 1. ^{1}H-NMR spectrum of N-trimethyl chitosan chloride after one step synthesis
Reported peak assignement:-$N^{+}(CH_3)_3 \rightarrow$ 3.1ppm; D.S.= 35%.
Proposed peak assignement:-$N^{+}(CH_3)_3 \rightarrow$ 3.4ppm; D.S.= 15%
D.S.= Degree of Substitution

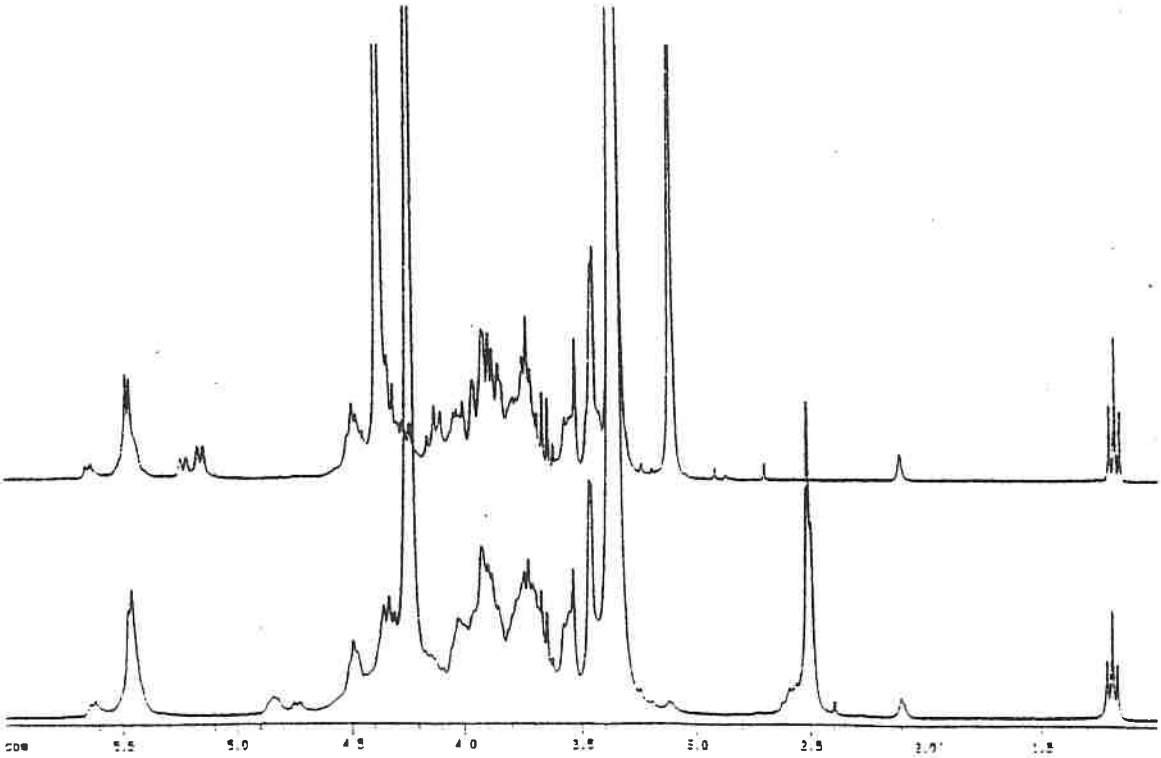

Fig. 2. ^{1}H-NMR spectra of N-trimethyl chitosan chloride after a two step synthesis. Up:the peak assigned to the quaternized group has disappeared, the peak assigned to the tertiary amino group has increased substantially and a new peak appeared at 2.4 ppm.Down :upon addition of DCl (one drop) the peak which had appeared at 2.5 ppm shifted after acidification to the supposed quaternized position 3.1 ppm.

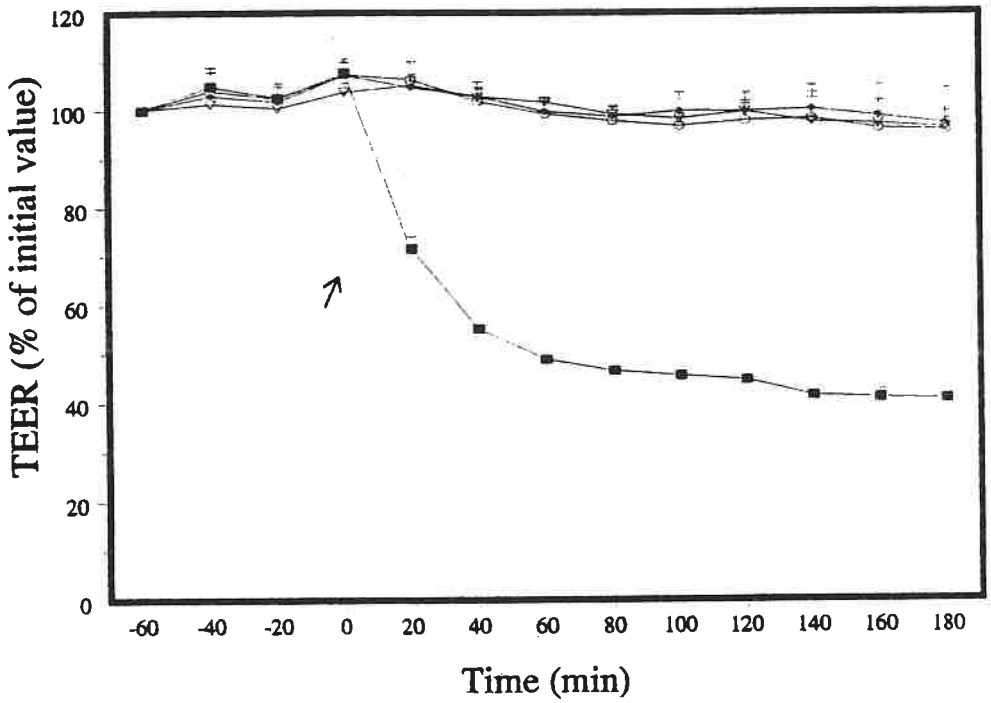

Fig. 3. Effect on TEER of Caco-2 cell monolayers treated with 0.5% (w/v) (■) TMC60 ,(▽) TMC20 and (✧)ChitosanHCl compared to the control (O) at pH=7.2; at 120 min the cells were washed and returned into pure culture medium. (mean ± SD; n=3).

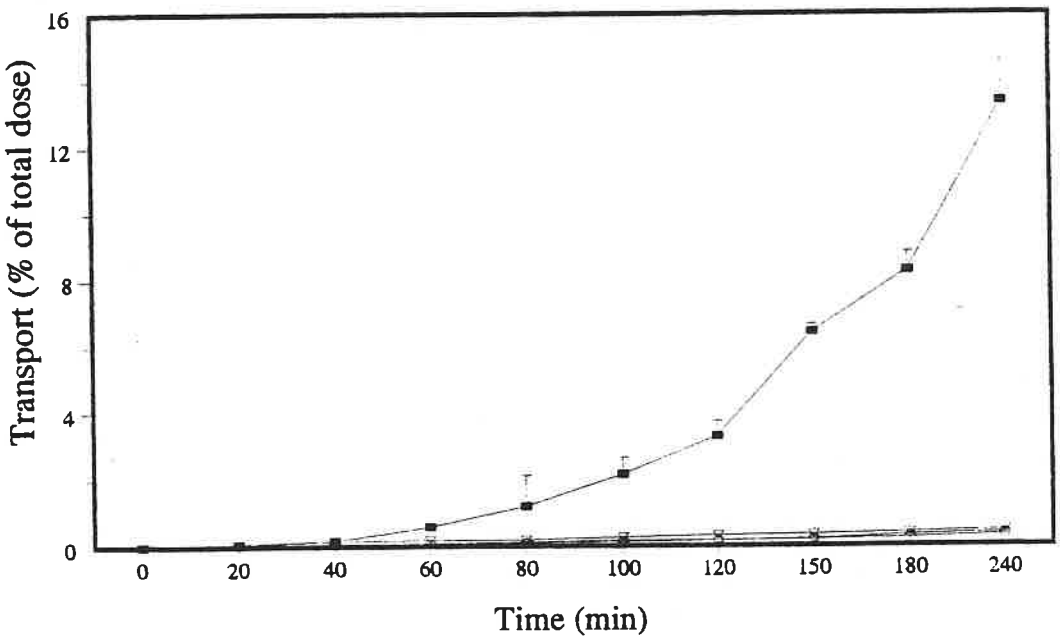

Fig 4. Transport of ^{14}C-mannitol through Caco-2 cell monolayers incubated with 0.5% (w/v) (■) TMC60, (▽) TMC20 and (✧) ChitosanHCl compared to the control (O) at pH=7.2 (mean ± SD; n=3).

A ^{1}H-NMR spectrum of the product obtained via the two step synthesis showed unexpected results when compared to the intermediate material. The peak assigned by Le Dung et al. (1994) to the quaternized amino group has disappeared, the peak assigned to the tertiary amino group has increased substantially, and a new peak appeared at 2.5 ppm (Fig. 2). Upon addition of a drop of DCl, this new peak shifted to the quaternized position (Fig. 2). It is very unlikely that the NMR-signal of a quaternized amino group will shift upon acidification of the solution. Therefore, the signal at 2.5 ppm in the neutral solution must be from the dimethylated amino group, which, upon acidification, will shift to a lower field. This leaves the peak at 3.4 ppm, which must be assigned to the quaternary amino group.
These results indicate that the product after the one step synthesis[1] is mainly dimethylated chitosan with only 10-15% degree of quaternization while using the two step method, degrees of quaternization of at least 60% can be obtained when the degree of trimethylation is calculated according to the peak assignement described in the above paragraph. This also explains our observation that a poorly water soluble polymer was obtained after the first step, when the iodide was exchanged for chloride using NaCl instead of hydrochloric acid. Upon acidification, this material rapidly dissolved in water.
Further evidence was obtained when the two compounds were tested for their ability of decreasing the TEER and therefore opening the tight junctions of Caco-2 intestinal epithelia and enhancing the permeability of a mainly paracellularly transported hydrophilic marker like ^{14}C-mannitol.
The product after the one step synthesis showed almost no difference with the control when these two polymers were tested for their ability of decreasing the TEER, while the product obtained after the two step synthesis decreased the resistance significantly (Fig. 3). The decrease of the resistance is a strong indication that the tight junctions are opened. The same effect was observed when these two compounds were compared for their efficiency to enhance the paracellular transport of the hydrophilic marker compound ^{14}C-mannitol. (Fig 4).

Conclusions

A two step reaction in the synthesis of TMC is necessary to obtain high degrees of quaternization. The degree of quaternization is a critical value for the efficiency of TMC as transport enhancer through Caco-2 monolayers. TMC is a chitosan derivative that shows better enhancing permeability properties than chitosan at pH values higher than 7.0. It is expected that the highly substituted TMC will be able to increase the absorption of peptide or peptidomimetic drugs in vivo as well.

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PREPARATION AND PROPERTIES OF CHITIN AND CHITOSAN DERIVATIVES

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Abstract

Chitosan was modified with polyethyleneglycol(PEG)-aldehyde in the various reaction conditions. Then modified chitosan (chitosan-PEG hybrid) was converted to modified chitin (chitin-PEG hybrid) by the acetylation with acetic anhydride (Scheme 1).

The water-solubility of these hybrids was investigated in buffers (0.01 M PBS pH 7.2 and 0.2 M Na_2CO_3 buffer pH 10). Some of these derivatives were soluble in these buffers. Moreover, the solubility in organic solvents was investigated, and these hybrids exhibited the affinity to organic solvents.

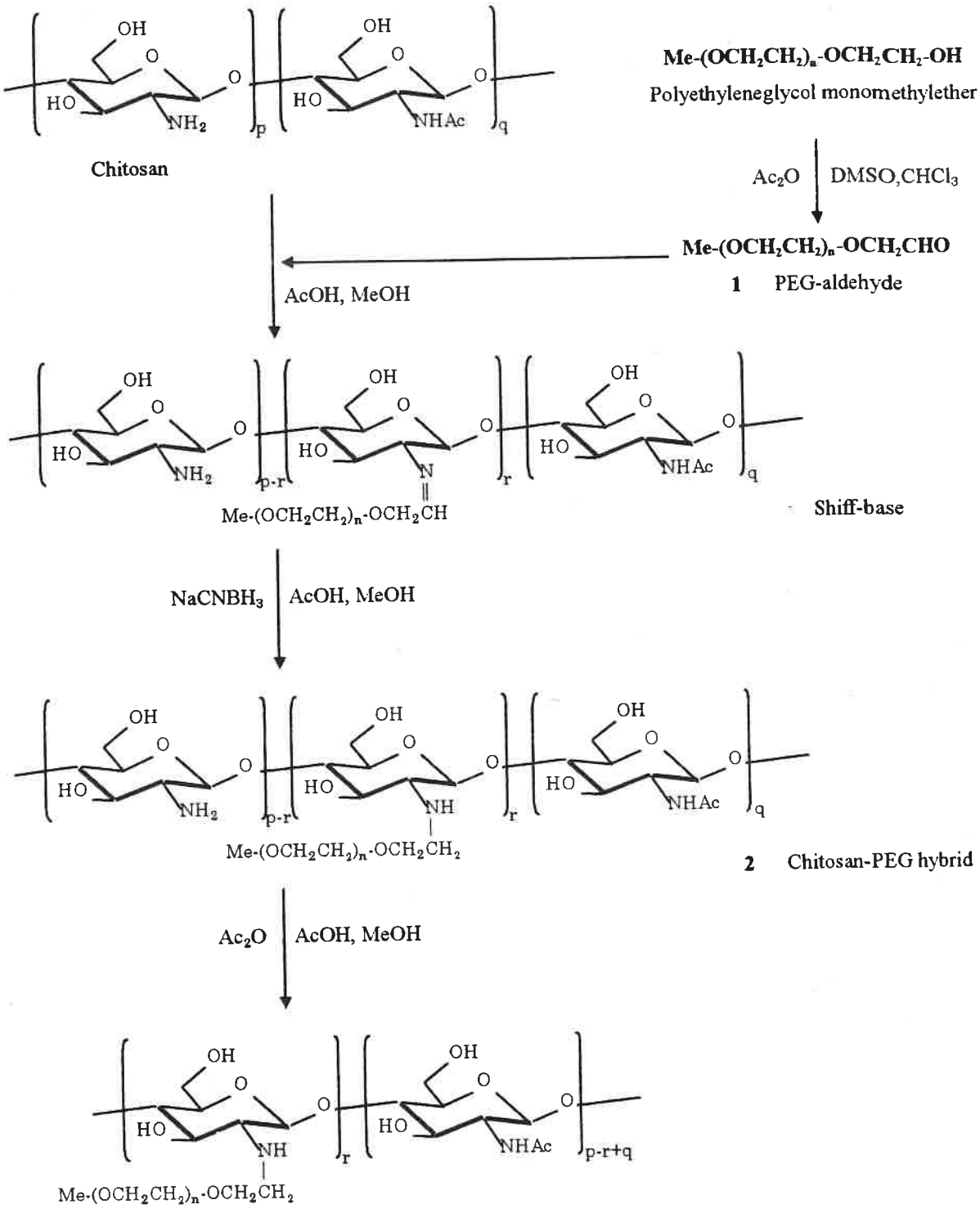
Keywords: Chitin, chitosan, polyethyleneglycol, derivative, solubility.

Materials and methods

Chitosan was obtained from Kyowa Technos Co. The molecular weight (Mn, determined by GPC) was 28000 and the degree of deacetylation was 86% (determined by ^{1}H-NMR). Polyethylenglycol monomethylether (PEG, Mn=2000) were obtained from Aldrich Chemical Co. Other reagents were all chemical grade and used without further purification.

PEG-aldehyde was prepared by the oxidation reaction of PEG with DMSO/acetic anhydride[1]. Acetic anhydride was added to PEG solution in anhydrous dimethylsulfoxide containing 6% chloroform under an Ar atmosphere and the mixture was stirred for 9 h at room temperature. Then the reaction mixture was poured into diethylether. The precipitate was reprecipitated two times from chloroform solution with diethylether. After drying, white powder was obtained. The degree of the conversion (DC) from hydroxy group to aldehyde group (0.53 to 0.65) was estimated by Schales' method with the calibration curve

of glutaraldehyde[2].



Scheme 1. Preparation of chitin/chitosan-PEG hybrid

The preparation of chitosan-PEG hybrid was performed by Harris's method[1]. Chitosan was dissolved in a 2:1 mixture of 2% aqueous acetic acid and methanol. An aqueous solution of PEG-aldehyde was added to chitosan solution and the mixture was stirred. After pH

adjustment to 6.5 with 1 M aqueous NaOH, an aqueous solution of $NaCNBH_3$ was added to the reaction mixture dropwise for 20 min and the solution was stirred for 30 h at room temperature. After dialysis against 0.05 M aqueous NaOH and water, the solution was centrifuged. The precipitate and the supernatant were freeze-dried and washed with acetone in order to except for unreacted PEG. After drying, white powders from supernatant and precipitate were obtained as water soluble and insoluble chitosan-PEG hybrid, respectively.

Chitosan-PEG hybrid was converted to chitin-PEG hybrid by the acetylation with acetic anhydride in acetic acid and methanol solvent[3]. Acetic anhydride was added to chitosan-PEG hybrid solution in a 1:4 mixture of 1% aqueous acetic acid and methanol. After stirring for 2-12 h at room temperature, the pH was adjusted to 12.0 with 1M aqueous NaOH. The reaction mixture was evaporated and dialyzed against water. After freeze-dry, white powder was obtained as chitin-PEG hybrid.

The solubility of chitin and chitosan-PEG hybrid was evaluated in buffers and in each organic solvents. The samples were soaked in each buffer at 5 mg/ml and organic solvent at 1 mg/ml, and were observed the solubility after standing for 4 days.

Results and discussion

According to Harris's method[1)], the chitosan products modified with PEG were insoluble in water, and the degree of substitution (DS) of PEG against monosaccharide residue was not over 0.1. We considered the precipitation of chitosan in the reduction process of schiff-base by $NaCNBH_3$ would suppress the smooth reduction of the schiff-base (Scheme 1). Then we added two steps, the neutralization before the reduction of schiff-base and the addition of aqueous $NaCNBH_3$ solution by dropwise, to avoid the precipitation of chitosan caused by sudden pH change. These results are summarized in Table 1. Additionally, the free PEG in the reaction mixture was hardly separated from chitosan-PEG hybrid and couldn't be purified by the dialysis and the precipitation with the addition of organic solvent. But free PEG was well removed from the reaction mixture by washing with acetone after freeze-dry.

The bond between chitosan and PEG could be confirmed by the peak of -NH-$\underline{CH_2}$- at 2.7ppm and 49.4ppm on ^{1}H- and ^{13}C-NMR spectra, respectively, as reported by Loubaki *et al.*[4] and Holme *et al.*[5].

Table 1. Preparation of chitosan-PEG hybrid and its water-solubility

Run No.	Reaction Molar Ratio		Chitosan-PEG hybrid			Solubility[d)]	
	-CHO of PEG /-NH_2 of chitosan	$NaCNBH_3$ /-CHO of PEG	Yield[a)]/%	DS[b)]	Weight ratio PEG/hybrid[c)]	0.01M PBS (pH7.2)	0.2M Na_2CO_3 buffer (pH10)
2-1	0.96	3	93	0.37	0.82	+++	+++
2-2	0.48	5	107	0.16	0.66	+++	+++
2-3	0.29	10	81	0.16	0.66	+++	+++
2-4 [e)]	0.19	10	60	0.12	0.59	+++	+++
			28	0.05	0.37	-	-
2-5 [e)]	0.10	10	27	0.09	0.52	++	+++
			70	0.05	0.37	-	±

a) Yield is indicated with the amount of recovered chitosan in chitosan-PEG hybrid calculated by DS.
b) DS means the degree of substitution of PEG to monosaccharide residue of chitosan determined by ^{1}H-NMR.
c) The weight ratio means the weight ratio of PEG in chitosan-PEG hybrid calculated by DS and Mn of PEG (Mn=2000).
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 , ++ : partial soluble , +++ : soluble
e) Two fractions of chitosan-PEG hybrid were obtained. Upper line indicates water-soluble fraction and lower line indicates water-insoluble fraction respectively.

DS value was calculated with relative peak intensities of the methylene protons of PEG and H-1 proton of monosaccharide residue.

$$\text{Corrected peak intensity of PEG methylene} = \frac{\text{whole intensity of 2.9-3.8 ppm}}{\text{H-1 intensity at 4.5-5.0 ppm}} - 6$$

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

DS value was dependent on the reaction molar ratio of PEG-aldehyde to chitosan. The hybrids which DS were over 0.12 dissolved in 0.01 M PBS (pH 7.2) and 0.2 M Na_2CO_3 buffer (pH 10) as shown Table 1.

The results of the N-acetylation reaction of water-soluble chitosan-PEG hybrids and these water-solubility are shown in Table 2. The acetylation decreased water-solubility of hybrids, but some hybrids kept the water-solubility in PBS and Na_2CO_3 buffer. Especially,

run **3-1** and **3-2** had little D-glucosamine units, and mainly consisted of N-acetyl-D-glucosamine and N-PEG-D-glucosamine units. They were defined as water-soluble chitin-PEG hybrid in Scheme 1. So the water-solubility of these hybrids was dependent on DS and DA value.

Table 2. Preparation of chitin-PEG hybrid and its water-solubility

Run No.	Chitosan-PEG hybrid	Reaction Molar Ratio	Acetylated chitosan-PEG hybrid		Solubility[d]	
	DS[a] of PEG	$Ac_2O/-NH_2$ of hybrid	Yield[b]/%	DA[c]	0.01M PBS (pH7.2)	0.2M Na_2CO_3 buffer (pH10)
3-1	0.37	3.9	92	0.64	+++	+++
3-2	0.37	10.0	98	0.65	+++	+++
3-3	0.16	2.2	97	0.70	+++	+++
3-4	0.16	5.1	100	0.65	+++	+++
3-5	0.16	3.1	101	0.79	±	±
3-6	0.12	3.0	97	0.72	+	±

a) DS means the degree of substitution of PEG to monosaccharide residue of chitosan determined by ^{1}H-NMR.
b) Yield is caluculated from the weight of chitosan in acetylated chitosan-PEG hybrid.
c) DA means the degree of N-acetylation determined by ^{1}H-NMR.
The reason why the sum of DS and DA was over 1.0 was the error for the evaluation by ^{1}H-NMR.
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 , ++ : partial soluble , +++ : soluble

The solubility in various organic solvents are shown in Table 3. Both chitosan-PEG hybrid and chitin-PEG hybrid had the high affinity to organic solvents in comparison with chitosan[6].

These results mean that chitin/chitosan-PEG hybrid might be applicable to the biomedical fields and be useful for the chemical modification reaction in various reaction conditions.

Conclusion

Chitosan-PEG hybrid and chitin-PEG hybrid were prepared. The modification with high molecular weight PEG improved the solubility of chitin and chitosan in neutral and alkaline pH buffers keeping the chitin and chitosan skeleton. The solubility in the buffers was dependent on DS and DA value. Furthermore, the modification with PEG improved

the affinity to organic solvents.

Table 3. Solubility of chitin/chitosan-PEG hybrid in various organic solvents

Run No.	Chitosan-PEG hybrid			Solubility[d]						
	DS[a]	DA[b]	Weight ratio[c] PEG/hybrid	MeOH	$CHCl_3$	AcCN	DMSO	DMF	n-Hexane	Et_2O
Chitosan	0	0.14	0	−	−	−	−	−	−	−
2-1	0.37	0.14	0.82	±	±	+++	+	+	−	−
3-2	0.37	0.65	0.82	−	±	−	++	±	−	−

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 1H-NMR.

c) The weight ratio means the weight ratio of PEG in chitin/chitosan-PEG hybrid calsulated using DS and DA values.

d) The solubility was evaluated at the concentration 1 mg/ml in each solvent, and the solubility was checked after standing for 4 days. - : precipitate , ± : swelling , + : gel or suspension , ++ : partial soluble , +++ : soluble. AcCN : acetonitrile, DMSO : dimethylsulfoxide, DMF : dimethylformamide, Et2O : diethylether

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PREPARATION AND PROPERTIES OF CHITOSAN-G-PEG*

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Abstract

Chitosan-g-PEG was prepared by schiff's base formation between monomethoxypoly (ethylene glycol)-aldehyde (mPEG-CHO) and chitosan and reduction with $NaCNBH_3$. The degree of substitution of PEG in chitosan (D.S) was investigated by IR, NMR and elemental analysis. Water swelling rate, mechanical property and antithrombogenicity were measured. The water swelling rate of chitosan-g-PEG increased with the increasing of D.S. Chitosan-g-PEG showed higher break elongation than that of chitosan, with only a little loss in tensile strength. It also exhibited more effective antithrombogenicity than chitosan.

Keywords: Chitosan; Poly (ethylene glycol); Graft reaction; Antithrombogenicity; Swelling rate; Mechanical properties.

Materials and methods

1. Preparation of aldehyde-terminated poly (ethylene glycol) (mPEG-CHO)

Monomethoxy poly (ethylene glycol) (mPEG) (Aldrich, M_w 5000, 2000) was used. The terminal hydroxyl group of mPEG was oxidized to an aldehyde (mPEG-CHO) based on the method given by Harris[(1)] and modified by Gerard R[(2)] .

2. Preparation of PEG-grafted chitosan (chitosan-g-PEG)

Chitosan-g-PEG was prepared following a method reported by Harris[(1)] with some modifications. Chitosan (0.164g, 1mmol, Katakura Chikkarin, Japan, acetylated degree 5%) was dissolved in 20 ml NaAc-HAc buffer (pH=2.7, 3.4, 4.1, 4.9, 5.3). Then predetermined amount of mPEG-CHO and $NaCNBH_3$ (10 ml) were added in chitosan solution with vigorous stirring. The reaction system was left stirring at room temperature for 48hrs. Then NaOH solution was poured in to adjust pH to 7 or higher, the products were isolated by centrifugalization or dialysis methods according to whether they precipitated in NaOH solution or not.

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3. Film formation

Sample was dissolved in 2% HAc solution, then was casted onto a glass plane which was formerly treated with trimethyl chloride silicon. After being dried at 37°C for a certain time , the film was washed by (1) a 1:1 mixture of NaOH solution (20%) and methanol , (2) methanol , then desiccated for characterization.

4. Instruments

FT-IR. spectroscopy: Nicolet 5SX spectrometer was used. Resolution was $2cm^{-1}$, scanning number was 32.

N.M.R Spectroscopy: ^1H NMR measurement was performed on a Jeol NMR spectrometer (500 MHz). The sample was dissolved in 2% formic acid D_2O solvent in 5-mm tube. ^1H chemical shifts were expressed in ppm downfield from the signal for sodium 3-(trimethyl, silyl) propane sulfonate.

Elemental Analysis: Elemental analyses were performed with a MOD-1106 type apparatus.

Stress-strain measurement: Samples stripe of 3×50mm was used to measure stress-strain curve with the equipment of Instron Universal Tensile Tester, Model 1122, at relative moisture , the load being 20N and draw speed being 20mm/min.

5. Swelling studies

Dry sample was weighted and dipped in deionized water at 37°C for 24hrs. Then it was wiped by dry tissues to remove surface water and weighted. The swelling ratio was calculated as follows:

$$S(\%)=(W_w-W_d) / W_d \times 100\%$$

W_w----wet sample weight, W_d----dry sample weight

6. Antithrombogenicity test

In vitro antithrombogenicity was measured using the method given by Imai and Nose[3]. Sample films of 20×20mm were placed on the bottom of beakers at 37°C and putted on the surface with citrated blood (0.25ml) from rabbit, then recalcificated with 0.02ml of 0.2M calcium chloride to initiate blood clotting , and keep at 37°C. After the appropriate time, distilled water (50 ml) was poured into the beakers and shaken for 10 minutes to hemolize erythrocytes which was not entrapped in thrombus. The concentration of the free hemoglobin in the resulting solution was colorimetrically measured at 540nm. The antithrombogenicity of sample tested was expressed by % recovery of blood when the absorbency of the blank solution in which 0.25 ml citrated blood was hemolized with 50ml distilled water, is 100%.

Results And Discussion

1. Graft reaction of mPEG and chitosan

To graft PEG onto chitosan consists of two steps. The first is the

condensation of aldehyde groups in PEG and amine groups in chitosan to form a Schiff's base of N-substituted imine. The second step is a hydrogenation of the Schiff's base by $NaCNBH_3$ to form a N-substituted amine.

Step 1: chitosan-NH_2 + mPEG-CHO→ chitosan-N=C-mPEG

Step 2: chitosan-N=C-mPEG+ $NaCNBH_3$ →chitosan-$NHCH_2$-mPEG

The reactions are both pH sensitive[4]. Fig.1shows the influence of pH (from 2.73 to 5.3) of the system on the grafting efficiency of PEG. As can be seen in Fig.1, the optimum pH of the reaction is about 4.9.

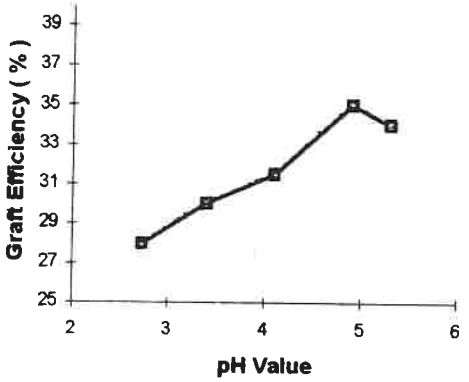


Fig.1 The relationship between pH and grafting efficiency of PEG

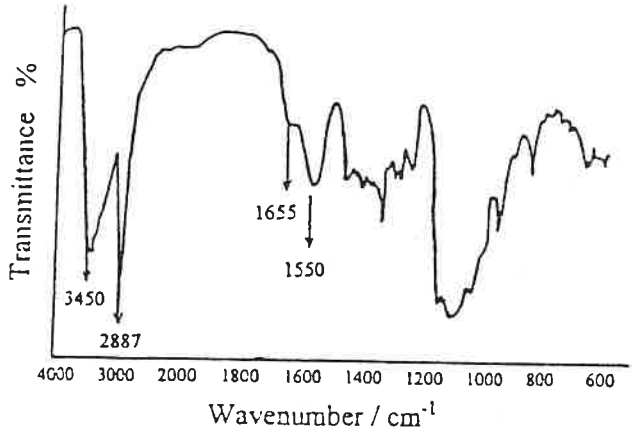
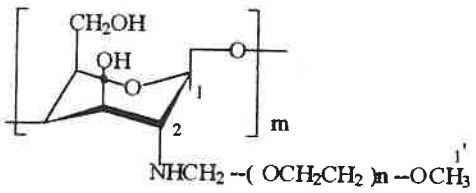


Fig. 2 The IR spectrm of chitosan-g-PEG

2. The characterization of D.S (Degree of Substitution of PEG)

The structure of the products can be clearly identified in IR spectrum (in Fig. 2). Three characteristic bands, hydroxyl vibration at 3450 cm^{-1}, amide-I at 1655cm^{-1} and amide II at 1550cm^{-1} represent chitosan[5] . The grafted PEG was confirmed by the typical intensive band resulting from $-CH_2-$ groups in PEG at 2887cm^{-1} .



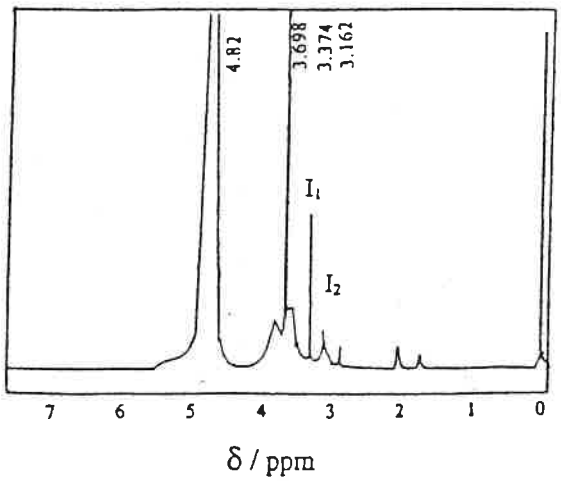


Fig.3 The ^{1}H-NMR spectrum of chitosan-g-PEG

A ^{1}H-NMR spectrum of chitosan-g-PEG is shown in Fig.3. Resonance line appearing at 2.06 ppm was assigned to CH_3 of N-acetyl residue. Resonances at 3.162 ppm and 4.82 ppm were assigned to the protons of C_2 (I_2) and C_1 respectively. Resonance in the range of 3.5-3.8 was attributed to the protons at C_3, C_4, C_5, C_6 and methylene of PEG. Resonance line at 3.374 ppm was assigned to the end group of methyene protons in PEG ($I_{1'}$)[6, 7].

The resonance absorption of methylene proton in PEG and C_1 ,C_3, C_4, C_5, C_6 protons in chitosan all appeared at from 3.4 to 4.4 ppm and were difficult to be separated. However, the absorption at 3.374 ppm can represent the PEG content and the absorption at 3.162 ppm can represent the chitosan content, thus the D.S of mPEG can be calculated by the following equation.

$$DS=I_{1'}/3I_2$$

DS is mole content of mPEG for each glucosamine unit in chitosan
$I_{1'}$ is the absorption intensity of methylene protons in mPEG
I_2 is the absorption intensity of protons of C_2 in chitosan

Elemental analysis was also used to determinate the D.S of PEG by measuring the content of C, H, N and calculating the ratio of C/N of the sample.

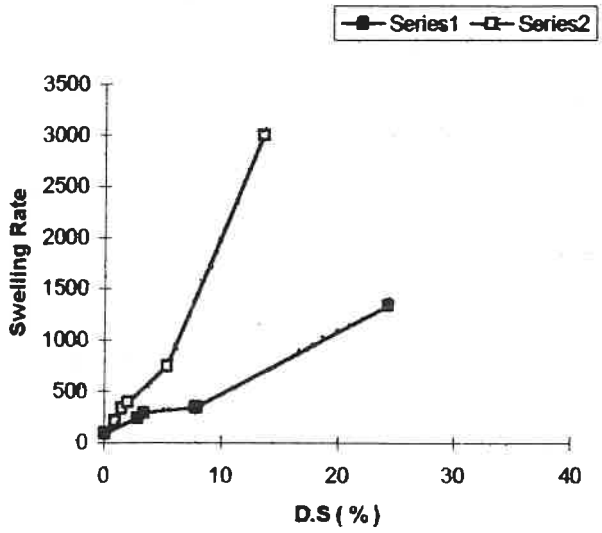
Table 1 listed the D.S of chitosan-g-PEG measured by NMR and elemental analysis method.

Table 1 Characterization of D.S of chitosan-g-PEG

code	M_w of mPEG	NH_2/CHO molar ratio	D.S	
			^{1}H-NMR	Elem. Analy.
2000-1	2000	5/1	4.6	5.3
2000-2	2000	5/2	8.1	7.9
2000-3	2000	5/3	24.7	24.3
2000-4	2000	5/4	27.8	32.2
5000-1	5000	10/1		5.4
5000-2	5000	10/2		13.6
5000-3	5000	10/3		16
5000-4	5000	10/4		28.9

3. Swelling test

By measuring the weight of samples before and after soaking them in 37°C water for 24hrs, the swelling rate of each sample was calculated and shown in Fig.4. The swelling rate of chitosan-g-PEG increased as the content of PEG in sample increased. Because sample bearing side chain of PEG 5000 had higher molecular weight of the grafted PEG, it displayed higher water absorbability when the value of D.S was same as that with side chain of PEG 2000.



1. PEG: 2000, 2. PEG: 5000

Fig. 4 The swelling rate of chitosan-g-PEG

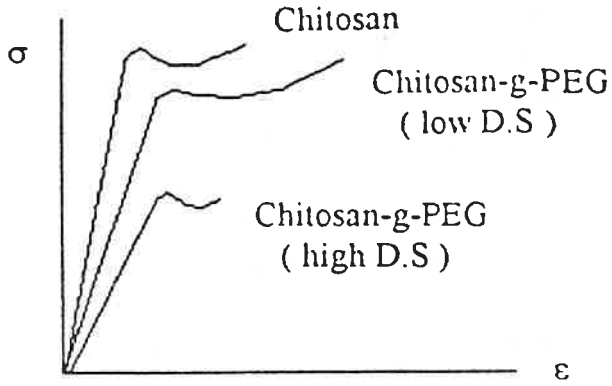
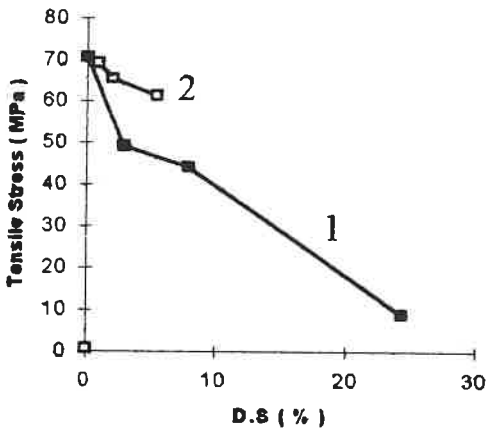


Fig. 5 Three types of stress-strain curve

4. Mechanical properties

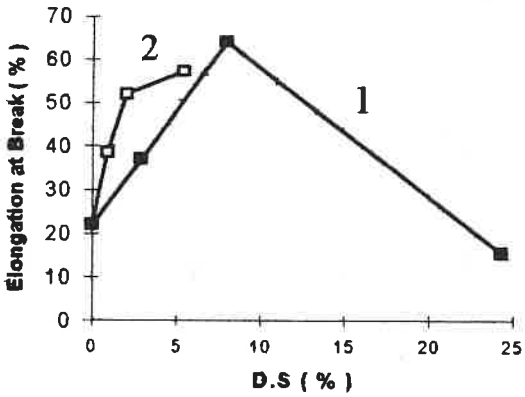
Three types of stress-strain curve are shown in Fig.5. Chitosan (1) gave the highest yield stress and breaking strength, but its elongation at break was relatively low. Chitosan-g -PEG with low D.S of PEG (2) gave the best mechanical properties, though its yield stress and breaking strength decreased slightly. It was more elastic and tougher than chitosan and had much longer elongation at break. As for chitosan-g-PEG with high D.S of PEG (3), the mechanical properties were poor.

Fig. 6 and Fig. 7 show the relationships between tensile strength, break elongation and D.S respectively. It is clear that with the increase of D.S of PEG , the tensile strength at break decreased, and the elongation at break increased. Chitosan-g-PEG with side chain of PEG 5000 was better than that of PEG 2000 when mechanical properties were compared, for the former showed less loss in tensile strength and more increase in elongation than the latter.



1. PEG: 2000; 2. PEG: 5000

Fig. 6 Relationship between tensile strength and D.S



1. PEG: 2000; 2. PEG: 5000

Fig. 7 Relationship between break elongation and D.S

5. Antithrombogenicity of chitosan-g-PEG

By Imai and Nose's method antithrombogenicity of chitosan-g-PEG was expressed by % recovery of blood after blood coagulating on sample surface for 30 and 60 minutes separately. The results are given in table 2.

Table 2 Antithrombogenicity of chitosan-g-PEG

Sample	D.S%	% Recovery of blood	
		30min	60min
Chitosan	0	25	24
Chitosan-g-PEG2000	7.9	56	35
Chitosan-g-PEG2000	24.3	93	80
Chitosan-g-PEG5000	2	39	32
Chitosan-g-PEG5000	16	78	78

Table 2 shows the relative concentration of uncoagulated blood after blood coagulating on sample surface for 30 or 60 minutes. All PEG grafted samples showed improved antithrombogenicity than the original chitosan. Further observation revealed that the antithrombogenicity of PEG grafted samples also depended on the amount of grafted PEG chain, with the increasing of D.S thromboresistance ability are also increased for both PEG2000 and PEG5000.

References

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