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Preparation and biodegradability of chitin derivatives having mercapto groups

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Two kinds of chitin derivatives having mercapto groups have been prepared, and the properties, including solubility and susceptibility to lysozyme, are discussed. Chitosan was first modified by *N*-acylation with *S*-protected mercaptoacetic acid, followed by selective acetylation of the remaining amino groups and deprotection to give derivatives having mercaptoacetyl groups at the C-2 amino groups. In an alternative preparation of mercapto-chitins, tosyl-chitin was treated with potassium thioacetate and then with alkali to afford derivatives having mercapto groups at the C-6 positions. The derivatives showed much enhanced swelling and solubility in organic solvents. Biodegradability of the mercapto derivatives was evaluated with lysozyme, and they turned out to be more susceptible to enzyme hydrolysis than the original chitin.

INTRODUCTION

Chitin, a naturally occurring abundant polysaccharide, is expected to be useful as a versatile multi-functional polymeric material, since it has three kinds of functional groups at the C-2, C-3, and C-6 positions. Controlled chemical modification of chitin would be possible, making use of the difference in reactivity among the functional groups, which would open a way to full development of its high potential in both basic and applications research. Although a wide variety of modification reactions has been reported thus far (Muzzarelli, 1977; Kurita, 1986), difficulties have generally been encountered in preparing derivatives without structural ambiguity owing primarily to its intractable nature. Regioselective substitution at desired positions is especially difficult, and this in turn makes quantitative discussion difficult on the relationship between the structure and properties.

We have been engaged in the development of facile modification reactions under mild conditions to enable regioselective modification leading to sophisticated molecular design (Kurita *et al.*, 1991*a*; Nishimura *et al.*, 1991). In an extension of studies on chemical modification to prepare derivatives with well-defined structures, our attention has been focused on the effective incorporation of mercapto groups, functional groups playing important roles in biomolecules, into chitin and on the evaluation of the resulting mercapto-chitins as a novel type of biologically active polymeric materials. This paper deals with the preparation of mercapto-chitins and characteristic properties including biodegradability.

EXPERIMENTAL

General

Chitin isolated from shrimp shells was a gift from Katokichi Co. Ltd, Kagawa, Japan and was a white powdery material. It was treated with 1 mol/litre aqueous sodium hydroxide at 100°C for 8 h and washed with deionized water. The degree of deacetylation was 0·15. Chitosan was prepared by deacetylating chitin with 40% aqueous sodium hydroxide at 120°C for 5 h under nitrogen. The degree of deacetylation was 0·90. Tosyl-chitin with a degree of substitution of 0·87 was prepared as reported previously (Kurita *et al.*, 1991*b*, 1992). Commercially available mercaptoacetic

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acid and benzyloxycarbonyl chloride (*Z*-chloride) were of 90% purity and used without further purification. Solvents were purified in the usual manner and stored over molecular sieves of 3 Å mesh. Solvents used for treating mercapto-chitins were purged with nitrogen for 1 h just before use to prevent oxidation of the mercapto groups.

IR and UV spectra were taken with IR-700 and Ubest-30 spectrophotometers (Japan Spectroscopic Co. Ltd, Tokyo, Japan), respectively. ^{1}H NMR spectra were recorded on a JNM-GX270 (JEOL Ltd, Tokyo, Japan) at 270 MHz, tetramethylsilane being used as the internal reference. X-Ray diffraction diagrams were obtained by the powder method using nickel-filtered CuKα radiation with a RAD-IA diffractometer (Rigaku Corporation, Tokyo, Japan). The degree of deacetylation was determined by conductometric titration with a conductivity meter CM-40S (TOA Electronics Ltd, Tokyo, Japan).

(*S*-Benzyloxycarbonyl)mercaptoacetic acid

To a solution of 10·2 g (0·10 mol based on 90% purity) of mercaptoacetic acid in 96 ml of 2 mol/litre aqueous sodium hydroxide were added 9·5 g (0·033 mol based on 90% purity) of *Z*-chloride and 12 ml of 2 mol/litre aqueous sodium hydroxide at 0°C with stirring. The addition of the same amounts of *Z*-chloride and sodium hydroxide was repeated two more times. The slightly cloudy mixture was stirred for 1 h at 0°C and washed with ether. The aqueous layer was separated and acidified with dilute hydrochloric acid to pH 2 to precipitate the product. It was collected by filtration, dried, and recrystallized from a mixture of benzene and hexane to give 11·5 g (52%) of *Z*-mercaptoacetic acid as white plates, m.p. 84–85°C. IR (KBr): ν 1725 and 1698 (C=O), 1157 (C–O), and 742 and 695 cm^{-1} (phenyl). ^{1}H NMR (chloroform-*d*): δ 3·72 (s, 2H, SCH_2), 5·27 (s, 2H, Ph-CH_2), 7·37 (s, 5H, phenyl), and 9·50 ppm (s, 1H, COOH).

Anal. Calcd for $C_{10}H_{10}O_4S$: C, 53·09; H, 4·46. Found: C, 52·82; H, 4·44.

***N*-(*Z*-Mercaptoacetyl)-chitin**

Chitosan, 5·0 g, was dissolved in 150 ml of 10% aqueous acetic acid, and the solution was diluted with 150 ml of methanol. To the solution was added a solution of 21·0 g (0·093 mol; 3 molar equivalents to the free amino groups of chitosan) of *Z*-mercaptoacetic acid and 19·2 g (0·093 mol) of dicyclohexylcarbodiimide (DCC) in 500 ml of tetrahydrofuran (THF) with stirring at room temperature. The white cloudy mixture was stirred for 16 h at room temperature and poured into 500 ml of 5% aqueous sodium hydrogen carbonate. After 3 h, the precipitate was filtered and washed with deionized water until neutral. It was further washed with ethanol, then acetone, in a Soxhlet extractor for 6 h each, and dried. The yield of a white powdery material was 8·4 g.

The remaining amino groups were then acetylated as follows. Part of the acylated product obtained above, 7·5 g, was swollen in 150 ml of methanol, and 7·0 ml (7·6 g; 10 molar equivalents to the pyranose units) of acetic anhydride was added. After stirring for 16 h at room temperature, the mixture was poured into 500 ml of ice water. The precipitate was filtered, washed consecutively with water, methanol, and ether, and dried to give 6·4 g of *N*-(*Z*-mercaptoacetyl)-chitin as a white powdery material. The degree of substitution was 0·30 as determined by elemental analysis. IR (KBr): ν 3424 (O–H), 1708 (SC=O), 1658 (amide I), 1555 (amide II), 1151 (C–O), and 746 and 697 cm^{-1} (phenyl).

Anal. Calcd for $(C_8H_{13}NO_5)_{0.7}(C_{16}H_{20}NO_7S)_{0.3}\cdot 1{\cdot}4H_2O$: C, 44·84; H, 6·48; N, 5·03. Found: C, 44·71; H, 6·12; N, 5·04.

Z-Mercaptoacetylation depended upon the efficiency of stirring as in the tosylation (Kurita *et al.*, 1992), and thus smaller scale reactions starting from 0·5 g of chitosan with efficient stirring generally resulted in higher substitution degrees (see Table 1).

***N*-Mercaptoacetyl-chitin**

To 6·0 g of *N*-(*Z*-mercaptoacetyl)-chitin swollen in 120 ml of methanol was added 3·0 g of sodium methoxide. The mixture was stirred under nitrogen for 16 h at room temperature and centrifuged. The product was collected by filtration, washed with methanol and then ether, and dried to give 6·4 g of *N*-mercaptoacetyl-chitin as a white powder. The substitution degree of free mercapto groups was roughly estimated to be 0·3 by Ellman's method (Ellman, 1959), where the reaction with Ellman's reagent had to be carried out in a heterogeneous swollen state owing to insolubility of the product in water. IR (KBr): ν 3432 (O–H), 1652 (amide I), 1554 (amide II), and 1150–1000 cm^{-1} (pyranose).

Anal. Calcd for $(C_8H_{13}NO_5)_{0.7}(C_8H_{13}NO_5S)_{0.3}\cdot 5{\cdot}0H_2O$: C, 31·72; H, 7·65; N, 4·62; S, 3·18. Found: C, 31·54; H, 6·42; N, 5·02; S, 2·90.

6-Deoxy-6-acetylthio-chitin

To a solution of 5·5 g of tosyl-chitin with a degree of tosylation of 0·87 in 200 ml of dimethyl sulfoxide (DMSO) was added 5·0 g of potassium thioacetate (0·044 mol; 3 molar equivalents to the pyranose units). The solution was stirred under nitrogen at 80°C for 16 h, and the resulting brown cloudy mixture was poured into 500 ml of methanol. The precipitate was collected by centrifugation, washed thoroughly with methanol and then ether, and dried to give 3·1 g of a pale tan solid. IR (KBr): ν 3406 (O–H), 1700 (SC=O),

Table 1. Preparation of *N*-(*Z*-mercaptoacetyl)-chitins[a]

Molar ratio[b] (mol/mol)	Degree of substitution	Calculated			Found		
		C	H	N	C	H	N
1	0·01	43·19	6·85	6·23[c]	43·08	6·70	6·23
2	0·31	46·72	6·27	5·20[d]	46·73	6·11	5·21
3	0·51	47·90	6·05	4·62[e]	47·96	5·76	4·64
4	0·37	47·10	6·20	5·01[f]	47·16	6·01	5·03
5	0·20	44·73	6·54	5·39[g]	44·68	6·03	5·63
10	0·15	44·95	6·55	5·65[h]	44·94	6·50	5·39

[a]Starting from 0·5 g of chitosan.
[b]*Z*-Mercaptoacetic acid/amino group.
[c]$(C_8H_{13}NO_5)_{0.99}(C_{16}H_{20}NO_7S)_{0.01}\cdot 1{\cdot}1H_2O$.
[d]$(C_8H_{13}NO_5)_{0.69}(C_{16}H_{20}NO_7S)_{0.31}\cdot 0{\cdot}8H_2O$.
[e]$(C_8H_{13}NO_5)_{0.49}(C_{16}H_{20}NO_7S)_{0.51}\cdot 0{\cdot}8H_2O$.
[f]$(C_8H_{13}NO_5)_{0.63}(C_{16}H_{20}NO_7S)_{0.37}\cdot 0{\cdot}8H_2O$.
[g]$(C_8H_{13}NO_5)_{0.80}(C_{16}H_{20}NO_7S)_{0.20}\cdot 1{\cdot}2H_2O$.
[h]$(C_8H_{13}NO_5)_{0.85}(C_{16}H_{20}NO_7S)_{0.15}\cdot 1{\cdot}0H_2O$.

1667 (amide I), 1538 (amide II), and 1150–1000 cm^{-1} (pyranose).

Anal. Calcd for $(C_8H_{13}NO_5)_{0.13}(C_{10}H_{15}NO_5S)_{0.87}\cdot 0{\cdot}8H_2O$: C, 43·63; H, 6·14; N, 5·22. Found: C, 43·93; H, 5·48; N, 4·47.

6-Deoxy-6-mercapto-chitin

6-Deoxy-6-acetylthio-chitin obtained above, 3·0 g, was swollen in 65 ml of methanol, and 1·5 g of sodium methoxide was added. The mixture was stirred under nitrogen at room temperature overnight. The resulting swollen product was isolated by centrifugation, washed consecutively with deionized water, 0·1 mol/litre acetate buffer of pH 4·8, and methanol, and dried to give 2·8 g of 6-deoxy-6-mercapto-chitin as a pale tan solid. The degree of substitution was roughly estimated by Ellman's method (Ellman, 1959) in a heterogeneous swollen state to be 0·9. IR (KBr): ν 3418 (O–H), 1659 (amide I), 1532 (amide II), and 1150–1000 cm^{-1} (pyranose).

Anal. Calcd for $(C_8H_{13}NO_5)_{0.13}(C_8H_{13}NO_4S)_{0.87}\cdot 3{\cdot}5H_2O$: C, 34·29; H, 7·19; N, 5·00; S, 9·95. Found: C, 33·94; H, 6·04; N, 4·74; S, 9·89.

Hydrolysis by lysozyme

Susceptibility of mercapto-chitins to lysozyme was evaluated by determining an increase in the amount of reducing ends with ferricyanide (Imoto & Yagishita, 1971) as follows.

Lysozyme (from egg white; Seikagaku Kogyo Co. Ltd, Tokyo, Japan), 15 mg, was dissolved in 500 ml of 0·1 mol/litre acetate buffer of pH 4·5. A ferricyanide solution was prepared by dissolving 0·5 g of potassium ferricyanide in 1000 ml of 0·5 mol/litre sodium carbonate.

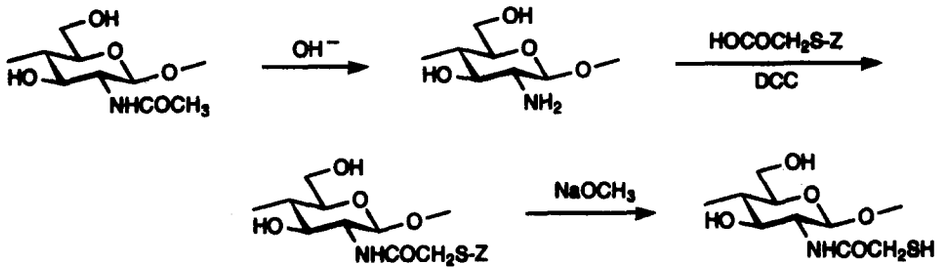
To a dispersion of 25 mg of chitin or a chitin derivative, pulverized to 100-mesh pass, in 50 ml of 0·1 mol/litre acetate buffer of pH 4·5, 25 ml of the lysozyme solution was added, and the mixture was shaken at 36°C. After a given time, 3 ml of supernatant of the mixture was taken in a test tube, and 4 ml of the ferricyanide solution was added. The mouth of the tube was covered with aluminium foil, and the solution was heated in boiling water for 15 min. It was cooled with water for 5 min, and the absorbance at 420 nm was measured. Control reactions were carried out in the absence of lysozyme, and the mercapto groups were confirmed not to interfere with the determination with ferricyanide under these conditions.

RESULTS AND DISCUSSION

Mercapto groups may possibly be introduced into chitin by several methods, but in view of preparing derivatives with well-defined structures, modification reactions should be carried out under mild conditions in homogeneous solution to enable regioselective modification. Chitosan and tosyl-chitin were expected to have high potentials as starting materials for this purpose because of the solubility and presence of reactive functional groups. Two approaches were thus examined.

Introduction of mercapto groups at the C-2 positions

Acetylation of the amino groups of chitosan or partially deacetylated chitin proceeds efficiently with DCC in aqueous organic solvents, and with a water-soluble carbodiimide (1-ethyl-3-(3-dimethylamino-



Scheme 1.

propyl)carbodiimide) in water, practically no reaction occurs (Kurita *et al.*, 1977). The reaction was therefore examined primarily with DCC under various conditions.

Direct mercaptoacetylation of chitosan with mercaptoacetic acid was unsuccessful, and hence the mercapto group was protected with the benzyloxycarbonyl group (*Z*-group). Addition of *Z*-mercaptoacetic acid and DCC in THF to a chitosan solution in a mixture of aqueous acetic acid and methanol, which is a good solvent for chitosan, generally caused the formation of a cloudy mixture, implying the reaction to proceed in homogeneous or almost homogeneous solution (Scheme 1). DCC was again confirmed to be much superior to the water-soluble carbodiimide in the *Z*-mercaptoacetylation. The remaining free amino groups were then fully acetylated with acetic anhydride in methanol to give acyl derivatives with well-defined structures, although *N*-acetylation with acetic acid as a solvent component would have taken place to some extent in addition to the expected *Z*-mercaptoacetylation.

The acylation was examined with various amounts of *Z*-mercaptoacetic acid to elucidate the effects on the acylation. As summarized in Table 1, the degree of acylation increased with the amount of the acylating agent and reached 0·51 with a three-fold excess amount. With larger amounts of acylating agent, the acylation degree decreased considerably. This is reasonably interpreted in terms of the solvent compositions; with a small amount of acylating agent up to three-fold excess, the reaction mixture remains almost homogeneous, while with a large amount, chitosan precipitates out as a result of the increased amount of THF. The reaction was also attempted in DMSO, but no appreciable extent of substitution occurred even at elevated temperatures on account of the reaction under heterogeneous conditions.

The products, *N*-(*Z*-mercaptoacetyl)-chitins, were obtained as white powdery materials. The structure of the acylated products was confirmed by elemental analysis and IR spectroscopy. The IR spectra showed a band at 1708 cm^{-1} due to the carbonyl of the *Z*-groups, amide I and II bands at 1658 and 1555 cm^{-1}, and bands at 746 and 697 cm^{-1} due to the phenyl of the *Z*-groups. Figure 1 illustrates the IR spectrum of a typical

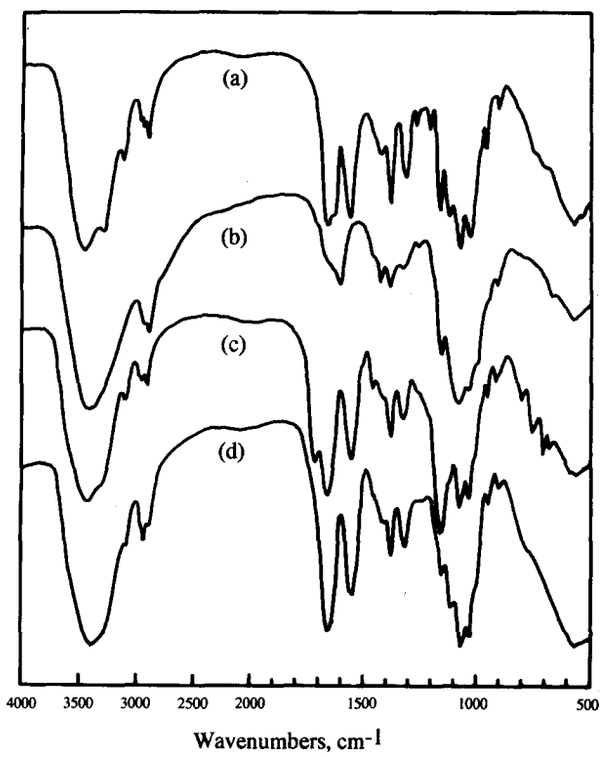
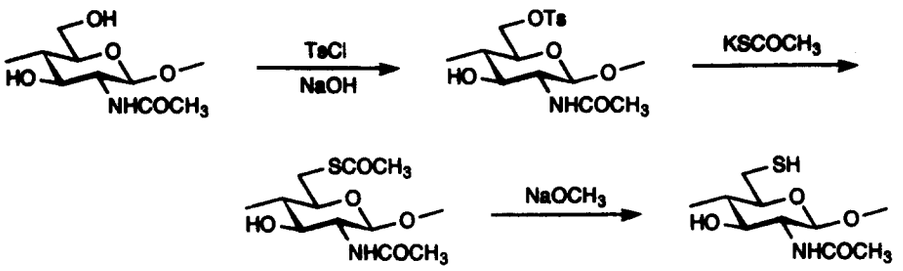


Fig. 1. IR spectra of (a) chitin (degree of deacetylation: 0·15), (b) chitosan (degree of deacetylation: 0·90), (c) *N*-(*Z*-mercaptoacetyl)-chitin (substitution degree: 0·30), and (d) *N*-mercaptoacetyl-chitin (substitution degree: 0·30) (KBr method).

example of *N*-(*Z*-mercaptoacetyl)-chitin along with those of chitin and chitosan.

The acylated products obtained above were then deprotected with sodium methoxide in methanol. The reaction proceeded smoothly in a swollen state, and *N*-mercaptoacetyl-chitins were isolated as white powdery materials. Figure 1 includes the IR spectrum of the deprotected product (*N*-mercaptoacetyl-chitin — see Fig. 1(d)), which resembles that of chitin. Complete removal of the protecting groups was evident from the disappearance of the characteristic bands due to *Z*-groups in the spectrum, elemental analysis, and determination of free mercapto groups by Ellman's method.



Scheme 2.

Introduction of mercapto groups at the C-6 positions

In an alternative approach to incorporating mercapto groups into chitin, tosyl-chitin was chosen as a soluble and reactive precursor. Tosyl-chitin was subjected to the reaction with thioacetate followed by *S*-deacetylation to generate free mercapto groups (Scheme 2).

Tosyl-chitin was prepared by the interfacial solution method, where tosylation was supposed to take place preferentially at the C-6 positions (Kurita *et al.*, 1991*b*, 1992). The reaction of tosyl-chitin with thioacetate proceeded in homogeneous solution in DMSO. At 60°C, however, the IR spectra of the products showed weak bands at 1598, 1176, and 813 cm^{-1} characteristic of tosyl groups, indicating incomplete substitution. Although the reaction was rapid at 100°C, the mixture assumed a dark brown color, and heating at 80°C proved appropriate judging from complete substitution and only slight discoloration. Figure 2 supports complete displacement of tosyl groups. The products, 6-deoxy-6-acetylthio-chitins, were obtained as pale tan solids.

The acetylthio derivatives obtained here were then treated with methoxide in methanol to remove *S*-acetyl groups. The deprotection proceeded in a swollen state in methanol, and the resulting 6-deoxy-6-mercapto-chitins were obtained as pale tan powdery materials. The IR spectra of the mercapto-chitins had no band due to *S*-acetyl groups and are quite similar to that of chitin as shown in Fig. 2. Formation of the mercapto derivatives was confirmed by elemental analysis and direct determination of mercapto groups by Ellman's method.

Properties of the derivatives

Qualitative solubility of the derivatives in excess solvents showed much improved swelling and solubility in organic solvents, unlike the insoluble nature of starting chitin and chitosan. The mercapto derivatives swelled to considerable extents in low boiling solvents such as THF and methanol as well as in common polar solvents. Moreover, *N*-(*Z*-mercaptoacetyl)-chitin was soluble in *N,N*-dimethylacetamide containing lithium chloride. The results are listed in Table 2.

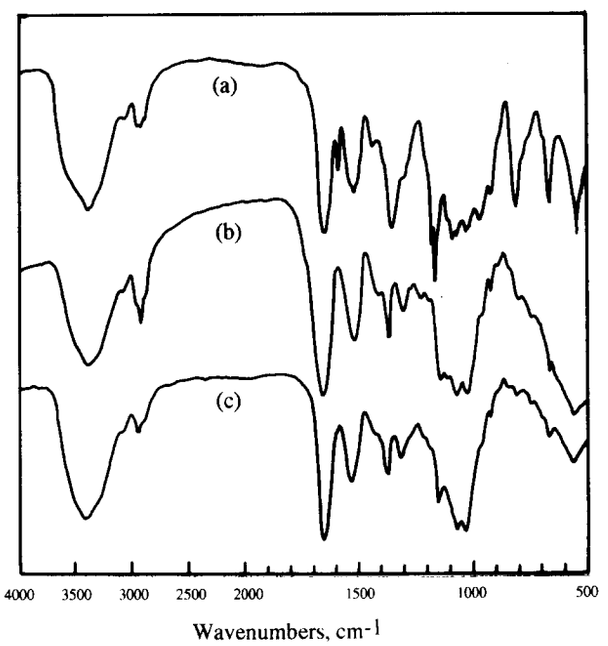


Fig. 2. IR spectra of (a) tosyl-chitin (substitution degree: 0·87), (b) 6-deoxy-6-acetylthio-chitin (substitution degree: 0·87), and (c) 6-deoxy-6-mercapto-chitin (substitution degree: 0·87) (KBr method).

All four kinds of derivatives prepared here, *N*-(*Z*-mercaptoacetyl)-chitin, *N*-mercaptoacetyl-chitin, 6-deoxy-6-acetylthio-chitin, and 6-deoxy-6-mercapto-chitin, were found to be amorphous from X-ray diffraction diagrams even at low substitution degrees, as in other cases of introduction of appropriate substituents into chitin and chitosan (Kurita *et al.*, 1988, 1990).

Hydrolysis of mercapto-chitins by lysozyme

Chitin is hydrolyzed by lysozyme, and destruction of the crystalline structure enhances susceptibility to the enzyme (Nishi *et al.*, 1983). Substitution, however, sometimes lowers the hydrolysis rate as in the carboxymethylation at the C-3 positions (Tokura *et al.*, 1990). The two kinds of mercapto derivatives were thus evaluated as substrates for lysozyme.

Table 2. Solubility data of chitin and the derivatives

	Solubility[a]						
	DMAc/LiCl[b]	DMAc	DMF[c]	DMSO	THF	MeOH	H_2O
2-ZMA-chitin[d]	+	±	±	±	−	−	−
2-SH-chitin[e]	±	±	±	±	±	±	±
6-SAc-chitin[f]	±	±	±	±	−	−	−
6-SH-chitin[g]	±	±	±	±	±	±	±

[a] +, soluble; ±, partially soluble or swelled; −, insoluble.
[b] *N,N*-Dimethylacetamide containing 5% lithium chloride.
[c] *N,N*-Dimethylformamide.
[d] *N*-(*Z*-Mercaptoacetyl)-chitin (degree of substitution: 0·30).
[e] *N*-Mercaptoacetyl-chitin (degree of substitution: 0·30).
[f] 6-Deoxy-6-acetylthio-chitin (degree of substitution: 0·87).
[g] 6-Deoxy-6-mercapto-chitin (degree of substitution: 0·87).

Hydrolysis was carried out under heterogeneous suspension conditions in acetate buffer of pH 4·5 at 36°C and followed by determining the amount of resulting reducing ends with ferricyanide. The decrease in absorbance at 420 nm due to ferricyanide was plotted against time as a measure of the extent of hydrolysis. As shown in Fig. 3, both the mercapto derivatives were hydrolyzed efficiently to higher extents than chitin itself. This is ascribable to the disordered or loose arrangement of chitin molecules caused by the introduction of substituents. Higher accessibility to *N*-mercaptoacetyl-chitin than 6-deoxy-6-mercapto-chitin may be associated with the improved disorderedness; the former has mercapto groups additionally to chitin backbones, while the latter has them in place of hydroxyl groups. Hydrolysis almost leveled off after about 24 h, probably because of the limited accessible portion under heterogeneous conditions. The decrease in absorbance of 0·12 in the case of *N*-mercaptoacetyl-chitin corresponds to the formation of 5·1 μmol of reducing end in the mixture, as calibrated with *N*-acetylglucosamine of known concentration, from 25 mg (117 μmol of repeating units) of the chitin derivative, indicating that about 4% of the glycosidic linkages were hydrolyzed.

CONCLUSIONS

Efficient procedures for introducing mercapto groups into chitin at the C-2 and C-6 positions have been established. The modification reactions could be conducted in solution under mild conditions starting from chitosan and tosyl-chitin. The resulting mercapto derivatives showed characteristic properties such as high susceptibility to lysozyme, high swelling, and enhanced solubility, and are hence expected to be useful as novel functional materials in various fields, including bioseparation, adsorption, immobilization and biodegradation.

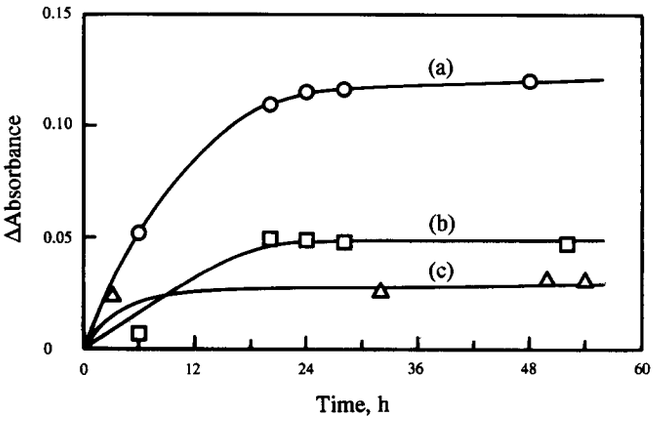


Fig. 3. Hydrolysis of (a) *N*-mercaptoacetyl-chitin (substitution degree: 0·30), (b) 6-deoxy-6-mercapto-chitin (substitution degree: 0·87), and (c) chitin with lysozyme in acetate buffer of pH 4·5 at 36°C.

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