Chitosanase from the Plant Pathogenic Fungus, *Fusarium solani*

Makoto SHIMOSAKA, Masahiro NOGAWA, and Mitsuo OKAZAKI
Gene Research Center and Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Tokida 3-15-1, Ueda, Nagano 386 (JAPAN) FAX: +81 268 21 5331
Email: mashimo@giptc.shinshu-u.ac.jp

Abstract

The plant pathogenic fungus, *Fusarium solani* f. sp. *phaseoli* SUF386 secreted a chitosanase in the culture using either glucose or *N*-acetylglucosamine as a carbon source. Chitosan, a substrate for chitosanase, inhibited cell growth significantly when added exogenously. Chitosanase purified from the culture filtrate had a molecular mass of 30 kDa, and catalyzed an endo-type cleavage of chitopentaose, chitosan (0% and 30% acetylation), and glycol chitosan. Based on partial amino acid sequences of the purified chitosanase, two degenerate oligonucleotides were synthesized and used as PCR primers to amplify and isolate the genomic copy of the chitosanase gene (*csn*). *F. solani csn* has an open reading frame encoding a polypeptide of 304 amino acid residues and containing a putative 19-amino acid residue signal sequence. Comparison with the cDNA revealed the presence of three introns in the coding region. Southern blot analysis indicated that *csn* is present as a single copy in the genome of *F. solani* SUF386.

Keywords: chitosan, chitosanase, *Fusarium solani*, gene cloning, plant pathogenic fungus, nucleotide sequence

Materials and methods

Strain. *F. solani* f. sp. *phaseoli* SUF386 (=*Hypomyces solani* f. sp. *phaseoli* IFO9974) was used as a source of enzyme and as a sorce of DNA for gene cloning.

Chitosanase purification. Cells were grown in 4-liters of Czapek-Dox medium containing Glucose (2%) as a carbon source and

supplemented with 0.1% each of yeast extract, casamino acids, and peptone at 26 °C for 7 days with vigorous shaking. Ammonium sulfate was added to the culture filtrate to give 80% saturation. The resulting precipitate was dissolved in 40 ml of 25 mM sodium acetate buffer, pH5.6 (buffer A), and then the sample was desalted by passage through a Sephadex G-25 column. The sample was put on a hydroxyapatite column (2.1 x 23 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.0. Proteins with chitosanase activity were eluted from the column by washing with 20-500 mM potassium phosphate buffer. After desalted by a Sephadex G-25 column, the sample was applied on a CM-Sepharose CL-6B column (2.1 x 17 cm) equilibrated with 20 mM sodium acetate buffer, pH4.5. Proteins were eluted with a linear gradient of the same buffer containing 0-1.0 M potassium chloride. Fractions with chitosanase activity were pooled and put on a Sephadex G-100 column equilibrated with buffer A. Active enzyme was eluted with buffer A, and used as the purified enzyme.

Chitosanase assay. Reducing sugars liberated during the hydrolysis of glycol chitosan or chitosan (30% acetylation) were measured. One unit (U) of activity was defined as the amount of the enzyme liberating 1 μmol of the reducing sugar from the substrate per minute using glucosamine as standard.

Primer designs, RT-PCR, and cloning of chitosanase gene. The determined nucleotide sequences of N-terminal and internal regions of the purified chitosanase were used to design two degenerate primers, A and B. Using total RNA prepared from *F. solani* SUF386, first-strand cDNA was synthesized with primer B. The resulting cDNA was used as a template to amplify DNA fragments using a set of primers (A and B) and *Taq* DNA polymerase. The genomic DNA library of *F. solani* SUF386 (1 x 10^5 clones, average insert sizes of 15 kbp, lambda DASH II vector) were screened for plaque hybridization using RT-PCR amplified cDNA as a probe.

Results and discussion

Effect of carbon sources on chitosanase production by F. solani SUF386. The chitosanase activity in the culture fluid was assayed when the strain SUF386 was grown in Czapek-Dox (CD) synthetic medium containing various carbon sources (Fig. 1). The strain secreted a large amount of chitosanase (2.8 mU/ml) in CD containing *N*-acetylglucosamine (GlcNAc) as a carbon source, while the activity remained low (0.58 mU/ml) in CD containing glucose (Glc). It is noteworthy that the strain did not grow at all in CD containing 0.25% chitosan (30% acetylation) as a sole carbon source. This growth cessation was due to growth inhibition caused by exogenously added chitosan, since the strain could not grow at all even in CD containing both Glc and chitosan. In contrast to other microbial chitosanases which were induced by chitosan, chitosanase of *F. solani* SUF386 seemed to be produced constitutively [1].
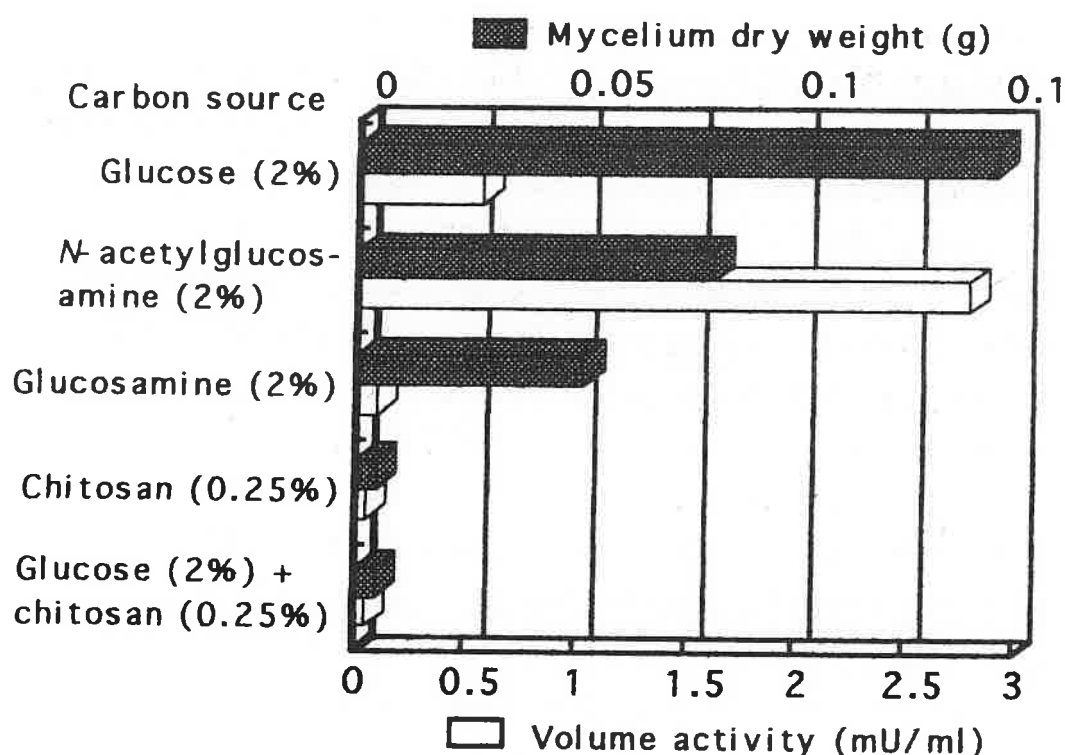

Fig. 1 Effect of carbon sources on chitosanase production

Cells were grown in 50 ml of Czapek-Dox synthetic medium at 26 °C for 7 days. Initial inoculum corresponded to 0.01 g (dry weight) cells.

Purification and characterization of chitosanase. Chitosanase was purified to homogeneity from the culture fluid of the strain SUF386 with a 36-fold purification and 10% recovery. At all chromatography steps, chitosanase activity was eluted in a single peak, indicating that the strain secreted a single kind of chitosanase. The molecular mass of the purified chitosanase was estimated to be 30 kDa by SDS-polyacrylamide gel electrophoresis. The enzyme showed the maximum activity at pH5.6 and 40 C. Substrate specificity of the chitosanase was examined. The enzyme hydrolyzed glycol chitosan and chitosan (30% acetylation) effectively. The enzyme degraded chitosan (0% acetylation) to a 60% level as compared to chitosan (30% acetylation). Glycol chitin and carboxymethyl cellulose were not hydrolyzed at a significant level. The chitosanase exhibited a rapid reduction in the viscosity of chitosan solution, suggesting an endo-type cleavage reaction. The enzyme could hydrolyze chitopentaose to chitobiose and chitotriose, but not hydrolyze chitobiose, chitotriose, and chitotetraose.

Primer designs, RT-PCR, and cloning of chitosanase gene. Based on the determined amino acid sequences, two degenerate primers (A and B) were synthesized (Fig. 2). A reverse transcription-
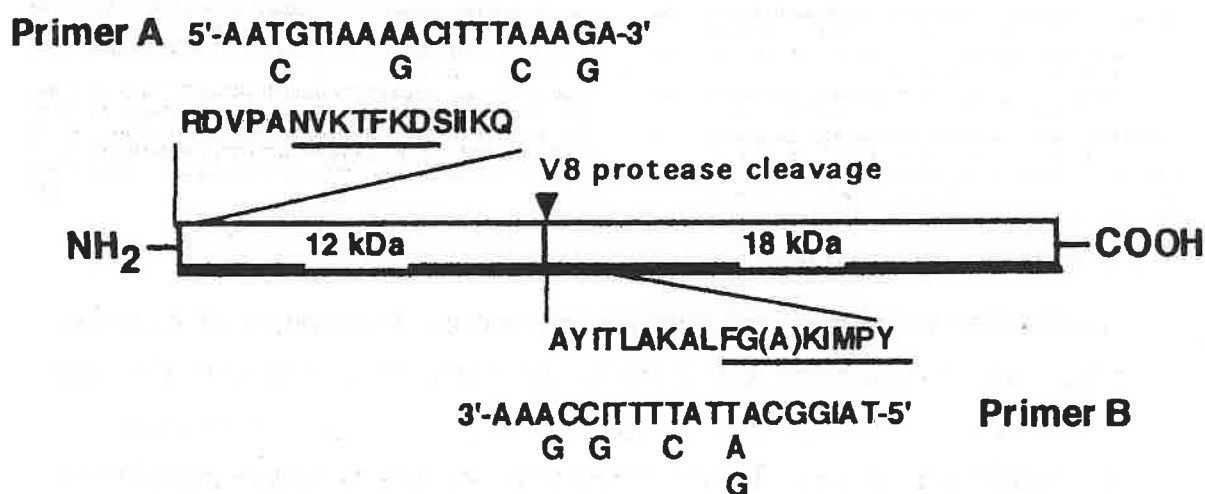

Fig. 2. Amino acid sequence of the purified chitosanase and primer design. A set of primer was designed based on the underlined amino acid sequences. The letter I in the primer sequence denotes inosine.

mediated PCR (RT-PCR) was performed for first-strand cDNAs that were synthesized from SUF386 total RNA using primer B. As a result, a single specific product 500 bp in size was obtained. This 500-bp amplified fragment was used as a hybridization probe for genomic DNA library screening, and the nucleotide sequences of the isolated genomic DNA fragments were determined.

The determined sequence contains a single open reading frame (ORF) (Fig. 3). The amino acid (aa) sequence deduced from this ORF includes the both aa sequences determined from the purified protein. Judging from the location (at the 20th residue) of the arginine residue corresponding to the N-terminus of the purified chitosanase, the preceding 19 amino acids are likely to be a signal sequence required for secretion of mature chitosanase. Comparison of the genomic DNA sequence to the cDNA sequence revealed the existence of three introns. Primer extension analysis revealed two distinct transcription start points in the upstream region of the ORF. Upstream of each of the start points, a TATA box-like sequence was found (Fig. 3).

```
CCATCTTGGCGTAAGCCGGCCAACCCAGCCGCACTCTTGTTCCGTCCTGTCAGATTGGAC   60
GAAGCGGTGGGCGGGCATCCGCATAAACGTGAATGGATCGCCGTCCGGGGGCGGACAGGA  120
ATTGGATGGATGCGACGTAAGGCTGAGCTGAAACATACAGGATGGATAAATGGGCGGCTT  180
TTAATAAAGCTGCGTGCGCCCCGATGATCCGTTCTTGACATTAAATAGAGCTGGAGAGCC  240
TCGTTTGACTTGAAATCAACCAAGCCCTTCTTCCTCGGTCAATATGCCTTCTCTTCGAAA  300
                                          M  P  S  L  R  N
CACCCTGCTGGCGTCCCTCCTCGCCGCGTCCGTCTCTGGACGCGACGTGCCGGCCAATGT  360
 T  L  L  A  S  L  L  A  A  S  V  S  G  R  D  V  P  A  N  V
CAAGACCTTCAAGGACAGCATCATCAAGCAGGGCTCTTGCAAGAGCACTCTAGCCACCGG  420
 K  T  F  K  D  S  I  I  K  Q  G  S  C  K  S  T  L  A  T  G
CTTCTTTAGCAGCGACGGCGACTCTGGCAgtatgtacctcctctcaagacacacaatctc  480
 F  F  S  S  D  G  D  S  G  T
acactcaccaacatctccagCCTACTCGTACTGCGGCGACCACGTCAAGGACTACAACGTC  540
            Y  S  Y  C  G  D  H  V  K  D  Y  N  V
ATCTACCTCCAGGGCAAGAACCGGCAAGCTCGTCAACATGGACATTGACTGCGACGGCGTC  600
 I  Y  L  Q  G  K  N  G  K  L  V  N  M  D  I  D  C  D  G  V
CAGGGCAGCCCCGCCGACGACGGCCGCTGCGGCTCCTCGGGCGACACCCAGTCCATCACG  660
 Q  G  S  P  A  D  D  G  R  C  G  S  S  G  D  T  Q  S  I  T
TCGTTCCAGTGGGTCCTCGAGTCGTACGGCACCTCGCAAAAGGACCTCGACGCAAACATC  720
 S  F  Q  W  V  L  E  S  Y  G  T  S  Q  K  D  L  D  A  N  I
CACCCCTACATCGTCTTTGGCAACGAGGGCACAAAGAAGGGGTGGAAGACGTTTGACCCG  780
 H  P  Y  I  V  F  G  N  E  G  T  K  K  G  W  K  T  F  D  P
GAGAAGCACGGCATCAAGCCTTTGAGCGTCATGGCTGTTGTGTGCGGCAACAAGATGgtg  840
 E  K  H  G  I  K  P  L  S  V  M  A  V  V  C  G  N  K  M
ggctcttctgtctctgtgaggcaaaaagccaatggcttacagtggtggtctagTTCTACG  900
                                                        F  Y  G
```

```
GCATCTGGGGTGACGAAAACGGCGACGACGGCGACCAGCCCATGGTCGGCGAGGCGTCCA  960
 I  W  G  D  E  N  G  D  D  G  D  Q  P  M  V  G  E  A  S  I
TCTCGGCTCGCCACCGCCTGCTTCGGCAAGTCGATGAACGGAAACTTTGGCCACAGCGACG 1020
 S  L  A  T  A  C  F  G  K  S  M  N  G  N  F  G  H  S  D  D
ACGACGTCCTCTACATCGCCTTTCCCGGCGCCGACGCCGTCCCGGGAGCAAAGGGCGCCA 1080
 D  V  L  Y  I  A  F  P  G  A  D  A  V  P  G  A  K  G  A  K
AGTGGAACGCAAAGAACTTTGACGAGTTCCAGACCAGCATCACCAGTCTCGGCGACAAGC 1140
 W  N  A  K  N  F  D  E  F  Q  T  S  I  T  S  L  G  D  K  L
TGATCAAGCGCATCGGAGGTACCAACAACGGCGGAGGAGAGCACTGGCGGTGGAAGCGGAA 1200
 I  K  R  I  G  G  T  N  N  G  G  G  D  T  G  G  G  S  G  N
ACACTTGCTCTTGGCCCGGACACTGCCAAGatacattggtcctgtcgtgcatgacctgtc 1260
 T  C  S  W  P  G  H  C  Q  G
tcgccgttaacatcatcatgtagGTGCCGCTTGCAAGACTGGCGATGACTGCTCCGACGA 1320
                        A  A  C  K  T  G  D  D  C  S  D  D
CCTCATCTGCACAAAGGGCAAGTGCTCGCCTCCATGAGGGTCGTCTGGAGCAACCAAGAC 1380
 L  I  C  T  K  G  K  C  S  P  P  *
CAAGACGGCATGCAAGGCCAAGAAATCTTGAGCCATGTTAGGTAGTCTTGGAGGATGGTT 1440
GTGCCCAGAGAAGCGCTGTTTGTCTACGTGTTTGCGCAATAAACACATGTAGAAACAACT 1500
TTGCATATATATCTTTCCATGTTCTTGTATATAAATCTCAACATGGGAAATTCATAGATATT 1560
CTTGGCGGATCTCTTTTTCGTCGTCCCAGGCTATGTCTTTTTTTCTTCAGTTACATGATC 1620
AGCAACCTATGCTCCAACCATAATGATCCTTCCATCCGTCACGGGAGCAATGCACCGCGT 1680
CCAGTCGAC                                                    1689
```

Fig. 3. Nucleotide sequence and deduced amino acid sequence of *F. solani* chitosanase. Solid underline in the amino acid sequence indicates the signal sequence. Wavy underlines indicate amino acid sequences determined using the protein sequencer. Three introns are shown by lower case letters. TATA box-like sequences, a CT-rich region, and polyadenylation signal sequences are underlined. Two transcription starting points and polyadenylation sites are double-underlined.

Southern blot analysis of genomic DNA. Genomic DNA of *F. solani* SUF386 was digested completely with various restriction endonucleases, and Southern blot analysis was carried out using *csn* cDNA as a probe. Only one band was detected for almost all digestions, indicating that SUF386 possesses only a single copy of *csn* [2].

Conclusion

(1) Computer analysis of the deduced amino acid sequence of *F. solani* chitosanase (*csn*) revealed no significant identity to any protein sequences in the available protein database. *F. solani csn* would have an evolutional origin distinct from that of bacterial counterparts [3]. This nucleotide sequence of *csn* is the first one reported for a fungal chitosanase. It must await the determination of *csn* sequence from other fungal strains to investigate the relationships of fungal chitosanases to bacterial chitosanases and other microbial 1,4-glycosidases (e.g. chitinase, lysozyme, cellulase).

(2) The physiological role of chitosanase in *Fusarium* remains to be elucidated. The severe inhibition of cell growth caused by exogenously added chitosan makes it difficult to postulate that chitosanase plays a role in utilization of chitosan as a nutrient. One plausible explanation is that chitosanase might degrade chitosan contained in cell walls of *Fusarium*. If this is the case, the chitosanase would play a role in cell division or autolysis. Disruption of chitosanase gene is now underway to clarify changes in cellular morphology of *F. solani*.

References

[1] M. Shimosaka, M. Nogawa, Y. Ohno, and M. Okazaki, *Biosci. Biotech. Biochem.*, 57, 231-235 (1993).
[2] M. Shimosaka, M. Kumehara, X.-Y. Zhang, M. Nogawa, and M. Okazaki, *J. Ferment. Bioeng.*, 82, 426-431 (1996).
[3] M. Shimosaka, M. Nogawa, X.-Y. Wang, M. Kumehara, and M. Okazaki, *Appl. Environ. Microbiol.*, 61, 438-442 (1995).

DEACETYLATION OF CHITIN-OLIGOSACCHARIDES BY CHITIN DEACETYLASE FROM *COLLETOTRICHUM LINDEMUTHIANUM*

Ken TOKUYASU, Hiroshi ONO, Mayumi OHNISHI-KAMEYAMA, Kiyoshi HAYASHI, Shioka HAMAMATSU, and Yutaka MORI

National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Kannondai, Tsukuba, Ibaraki 305, JAPAN, Fax:+81-298-38-7996 E-mail: tokuyasu@nfri.affrc.go.jp

Abstract

Chitin deacetylase purified from a Deuteromycete, *Colletotrichum lindemuthianum* ATCC 56676 could hydrolyse chitin-oligosaccharides (2mer-6mer) into deacetylated products. According to the data from HPLC and FAB-MS analyses, chitin-oligosaccharides whose degrees of polymerization are more than three could be converted into fully-deacetylated corresponding chitosan-oligosaccharides.

N,N'-diacetylchitobiose, however, was predominantly converted into a partially deacetylated product, 2-acetylamino-4-0-(2-amino-2-deoxy-ß-D-glucopyranosyl)-2-deoxy-D-glucose. The potential of this enzyme for producing fully or partially deacetylated chitin-oligosaccharides is discussed.

Keywords: Chitin deacetylase; *Colletotrichum lindemuthianum*; Chitin-oligosaccharides; Chitosan-oligosaccharides; Enzymatic deacetylation

Materials and methods

Materials.- Chitin-oligosaccharides were purchased from Seikagaku Kogyo Co. Japan and chitosan-oligosaccharides were kindly provided by Prof. Usui of Shizuoka University, Japan. All other chemicals were of reagent grade.

Microorganism.- *Colletotrichum lindemuthianum* (ATCC56676) was obtained from the American Type Culture Collection.

Purification of chitin deacetylase.- Chtin deacetylase (E.C.3.5.1.41) secreted in the culture media was purified by the method of Tokuyasu *et al.*[1] and the enzyme activity was measured

according to the spectrophotometric method of Dische and Borenfreund[2]. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of GlcN residue (with free amino group) per minute when incubated with glycol chitin as the substrate.

Deacetylation of chitin-oligosaccharides.- The reaction mixture (400 μl) was composed of each chitin-oligosaccharide as the substrate (0.2%), 10 mM sodium tetraborate/ HCl (pH 8.5) and purified enzyme solution [0.1 U for $(GlcNAc)_{4-6}$, 0.4 U for $(GlcNAc)_3$ and $(GlcNAc)_2$]. The deacetylation reaction was performed at 45 °C and monitored by HPLC.

Monitoring of the deacetylation reaction by HPLC.- Monitoring of the reaction was carried out using HPLC on Dionex DX-300 equipped with a Pulsed Amperometric Detector (Dionex Co.). The prepacked column was of Dionex CarboPac™ PA1 analytical column (4 x 250 mm) with CarboPac™ PA1 guard column (4 x 50 mm). The mobile phase was 15 mM [for $(GlcNAc)_2$] or 30 mM [for $(GlcNAc)_{3-6}$] NaOH. The flow rate was 1.0 ml min.$^{-1}$ Samples were diluted 80-fold with water and injection volume was 10 μl .

Purification of reaction products for FAB-MS analyses.- The enzyme was separated from the reaction solution by centrifugation in an Ultrafree C3-TGC (MILLIPORE Co.). The solution was then dialyzed through an electric dialyzer(AC-120-02 cartridge on Micro Acylizer G0, Asahikasei Kogyo Co. Ltd.), concentrated, and used for FAB-MS analysis.

FAB-MS analyses.- The FAB mass spectrum of each reaction product was obtained using JEOL JMS-SX102A. The positive ion mode was used for analyses and glycerol was used as matrix.

Estimation of heterogenic dimeric sugar by ^{1}*H-NMR analyses.-* The reaction solution (1.2 ml) using $(GlcNAc)_2$ as substrate was applied to a cation exchange column of Amberlite CG-120 (2ml; Orugano Co., Ltd.), washed with 6 ml of water and eluted with 8 ml of 0.5 N HCl. The free amino group from the reaction was detected by the method of Dische and Borenfreund [2]. Fractions (2ml) were collected and the fractions containing the product (No.4-7) were pooled, evaporated, and dissolved in D_2O (600 μl) for ^{1}H-NMR analyses. ^{1}H-NMR spectra were recorded on a Bruker DRX600 spectrometer at 303 K and sodium 3-(trimethylsilyl)-1-propane-sulfonate was used as the internal standard.

Results and discussion

Chitosan-oligosaccharides have been regarded as target materials for utilizing chitin because they have been reported to possess physiological activities such as antitumor activities [3] and phytoalexin-inducing activities [4,5]. Currently, chitosan-oligosaccharides are produced from chitin by thermochemical deacetylation followed by acid- or enzymatic hydrolysis. However, this conventional process including the thermochemical deacetylation step have problems such as (1) low recovery of oligomers caused by non-specific degradation of substrate, (2) production of a large quantity of alkaline wastes, and (3) contamination of partially acetylated chitosan oligosaccharides: complete deacetylation cannot be attained without a tremendous loss in product recovery.

Herein we propose an alternative method for producing chitosan-oligosaccharides using chitin deacetylase. Chitin deacetylase was first purified from *Mucor rouxii* to an electrophoretical homogeniety in 1993 [6], and several chitin deacetylases from Zygomycetes and Deuteromycetes have been purified and characterized using chitin, chitin derivatives, and chitin oligomers as the substrates [1, 6-9]. However, deacetylated products from chitin-oligosaccharides have not been well investigated from the viewpoint of industrial applications of chitin deacetylases. Enzymatic deacetylation methods have some advantages: (1) it never causes the degradation of sugar chains, (2) it produces no alkaline wastes, and (3) it may produce unique compounds which are hard to synthesize chemically. Here we have characterized chitin deacetylase from *Colletotrichum lindemuthianum*. This enzyme has some advantages over the chitin deacetylases of different origins: (1) it is secreted in the medium, (2) it is active toward relatively low molecular chitin oligomers (2 mer-), and (3) it retains relatively high activity in the presence of acetate [1].

The time course of HPLC profiles during the enzymatic deacetylation of $(GlcNAc)_6$ was shown in Fig 1. A broad peak for $(GlcNAc)_6$ at the retention time of approximately 17 min decreased gradually and disappeared after 240 minutes of the reaction. Under these conditions of HPLC the profile was recognized as three peaks eluting at approximately 13 min, 14 min and 17 min, respectively. The peak at the 14 minute retention time appeared after 60 min

310

and diminished after 180 minutes of the reaction. The peak at the 13 minute retention time increased linearly and formed a single peak after 300 minutes of the reaction. This peak showed a very similar retention time to that of chitohexaose. The shape and size of the peak at the 13 minute retention time did not change after prolonged incubation, therefore the whole solution was used for FAB-mass spectrometer analyses, following the removal of the enzyme and salts.

The profile of HPLC during the deacetylation process when $(GlcNAc)_{3\text{-}5}$ were used as the substrates were similar to Fig 1 finally giving a single peak on HPLC. As for $(GlcNAc)_2$, no intermediate peak could be detected on HPLC, however, a new peak increased in accordance with the decrease of the $(GlcNAc)_2$ peak (Figure 2). These end products were purified, concentrated and analysed on the FAB-mass spectrometer. The products derived from $(GlcNAc)_6$, $(GlcNAc)_5$, $(GlcNAc)_4$ and $(GlcNAc)_3$ showed $[M+H^+]$ pseudo-molecular ions at an m/z of 985, 824, 663 and 502, respectively. This indicated that all the acetyl groups in the substrate molecule had been removed to produce the corresponding chitosan-oligosaccharides. The product derived from $(GlcNAc)_2$ showed a main signal of $[M+H^+]$ pseudomolecular ion at 383 suggesting that either of two acetyl groups from the substrate molecule had been removed. Data from ^{1}H-NMR analysis confirmed that the acetyl group at non-reducing end of $(GlcNAc)_2$ was predominantly removed to produce 2-acetylamino-4-0-(2-amino-2-deoxy-ß-D-glucopyranosyl)-2-deoxy-D-glucose.

We have confirmed chitin deacetylase from *C. lindemuthianum* can be applied for producing thoroughly deacetylated chitosan-oligosaccharides from 3-mer to 6-mer, and here we propose an alternative method for the production of chitosan-oligosaccharides through the following steps; (1) hydrolysis of chitin into oligomers, (2) rough fractionation according to the molecular weight, (3) enzymatic deacetylation of chitin-oligosaccharides, and (4) separation of chitosan-oligosaccharides: this method does not include the step of thermochemical deacetylation. This enzyme can also be used as a tool for removing partially *N*-acetylated chitosan-oligosaccharides contaminated in chitosan-oligosaccharides. Furthermore, this enzyme efficiently produces a selectively-
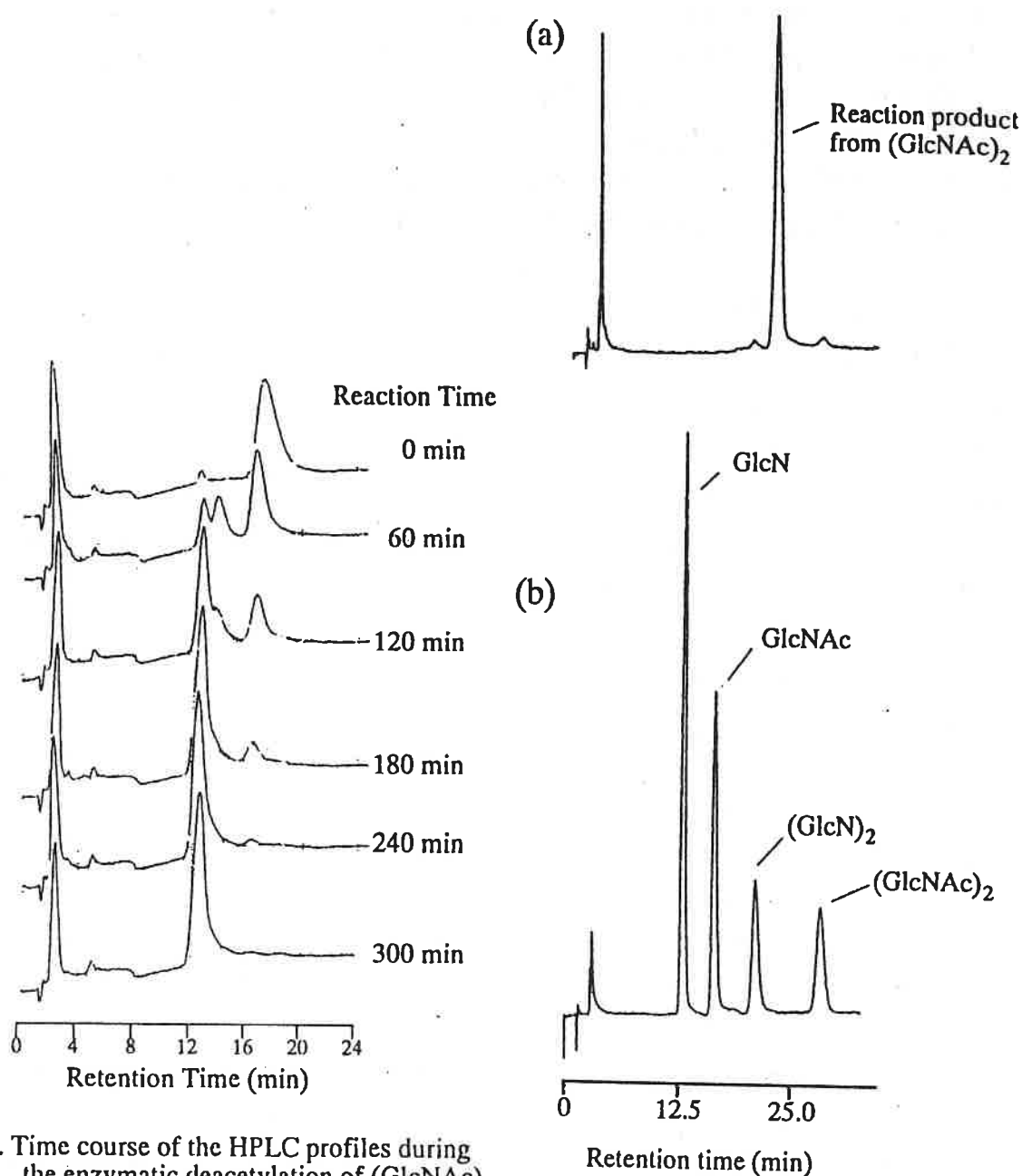
311

Fig 1. Time course of the HPLC profiles during the enzymatic deacetylation of (GlcNAc)$_6$

Fig 2. HPLC profiles of (a) the reaction product from (GlcNAc)$_2$ and (b) standards [GlcN, GlcNAc, (GlcN)2, and (GlcNAc)$_2$]

deacetylated unique compound, GlcN-GlcNAc from (GlcNAc)$_2$, which requires several steps if synthesized chemically. The mechanism of the recognition and deacetylation of substrates remains to be solved.

Conclusion

Chitin-oligosaccharides (2mer to 6mer) could be deacetylated by chitin deacetylase from *C. lindemuthianum*. According to the data from HPLC and FAB-MS analyses, $(GlcNAc)_{3-6}$ were fully deacetylated into corresponding chitosan-oligosaccharides. $(GlcNAc)_2$, however, was predominantly converted into a partially de-acetylated product. ^{1}H-NMR analysis confirmed the product was GlcN-GlcNAc. Thus this deacetylase has a high potential to be an excellent tool for producing chitosan oligosaccharides or unique compounds such as GlcN-GlcNAc.

References

[1] K. Tokuyasu, M. Ohnishi-Kameyama, and K. Hayashi, *Biosci. Biotech. Biochem.*, 60 (1996) 1598-1603.

[2] Z. Dische and E. Borenfreund, *J. Biol. Chem.*, 177 (1949) 751-766.

[3] M. Yoda, K. Shimoji, and J. Sasaki, Eur. Pat. 226, 381 (1987): *Chem. Abstr.*, 107 (1987) 237216.

[4] Y. Uchida, in M. Yabuki (Ed.), *Application of Chitin and Chitosan*, Gihodo Syuppan, pp 71-98 (in Japanese).

[5] M. G. Hahn, A. G. Darvill, and P. Albersheim, *Plant Physiol.*, 68 (1981) 1161-1169.

[6] D. Kafetzopoulos, A. Martinou, and V. Bouriotis, *Proc. Natl. Acad. Sci. USA*, 90 (1993) 2564-2568.

[7] X-D. Gao, T. Katsumoto, and K. Onodera, *J. Biochem.*, 117 (1995) 257-263.

[8] C. Alfonso, O. M. Nuero, F. Santamaría, and F. Reyes, *Curr. Microbiol.*, 30 (1995) 49-54.

[9] I. Tsigos and V. Bouriotis, *J. Bol. Chem.*, 270 (1995) 26286-26291.

GLUCOSAMINE 6-P SYNTHASE AND CONTROL OF CHITIN BIOSYNTHESIS IN CANDIDA ALBICANS

Agnieszka SZAJOWSKA, Kamilla NIEDZIELSKA and Sławomir MILEWSKI

Department of Pharmaceutical Technology & Biochemistry, Technical University of Gdańsk, 11/12 Narutowicza Str., 80-809 Gdańsk (POLAND), Fax: +48 58 47 26 94 E Mail: milewski@altis.chem.pg.gda.pl

Abstract

Glucosamine-6-phosphate (GlcN-6-P) synthase catalyses the first committed step in a chitin biosynthetic pathway in fungi. In the present communication we describe results of our studies on mechanisms of regulation of this enzyme in *Candida albicans*. UDP-GlcNAc inhibits GlcN-6-P synthase activity with $IC_{50} = 0.67$ mM but sensitivity to this inhibitor is modulated by glucose-6-phosphate. Kinetic investigations on interaction of UDP-GlcNAc with GlcN-6-P synthase revealed that the inhibitor binding site is separated from the enzyme active site.

It was found that the 3 - 6 fold increase in GlcN-6-P synthase activity during yeast-to-mycelia morphological transition, is accompanied by a rapid decrease of the enzyme sensitivity to a physiological feedback inhibitor - UDP-GlcNAc. Activity of the purified GlcN-6-P synthase increases upon the action of cAMP-dependent protein kinase.

Possible implications of these findings for understanding of mechanism of chitin biosynthesis regulation are discussed.

Keywords: GlcN 6-P synthase, feedback control, protein kinase, *Candida albicans*

Materials and Methods

Microorganisms *Candida albicans* 3153 Berkhout was obtained from the London Mycological Reference Laboratory. Recombinant *Saccharomyces cerevisiae* YRS65-2 strain overproducing the *C. albicans* GlcN-6-P synthase[1] was constructed by Dr. R.Smith (University of Aberdeen, UK)

Culture conditions. Inocula of YRS65-2 cells were propagated in defined media containing 2% glucose, 0.65% YNB w/o amino acids and appropriate supplements at 50 μg/ml, and then transferred to YPD medium (2% glucose, 2% bacterial peptone, 1% yeast extract) and grown at 28°C with vigorous shaking. *C. albicans* cells were cultivated in YPD medium at 28°C.

Morphological transformation *C. albicans* cells grown overnight in YPD were harvested, washed with saline and starved overnight in saline at 4°C. Starved cells were used to inoculate the YCB/BSA medium containing 1.17% YCB, 1% glucose and 0.2% bovine serum albumin. Yeast form cells grew efficiently in YCB/BSA, pH 4.5, at 28°C and Y→M transformation was performed in YCB/BSA, pH 6.5, at 37°C. Efficiency of the morphological transformation was assessed by cell counting in a Burker chamber.

Purification of GlcN-6-P synthase *C. albicans* GlcN-6-P synthase overproduced by YRSC-65 cells was purified to apparent homogeneity in a four-step procedure described earlier[2]. The enzyme from *C. albicans* 3153 was purified only partially, using first two steps from the above mentioned procedure.

Determination of GlcN-6-P synthase activity. A standard incubation mixture consisted of : 7.5 mM D-fructose-6-phosphate (Fru-6-P), 10 mM L-glutamine, 1 mM EDTA, 1 mM dithiothreitol, 50 mM potassium phosphate buffer, pH 6.45, appropriately diluted enzyme preparation and inhibitors when necessary. In kinetic experiments, concentration of one of the substrates was fixed and that of the other one - variable. The reaction mixtures were incubated at 30°C for 30 min and terminated by heating at 100°C for 1 min.

The concentration of glucosamine-6-phosphate (GlcN-6-P) formed was determined by the modified Elson-Morgan procedure[3]. One unit of specific activity was defined as an amount of enzyme which catalysed the formation of 1 µmol GlcN-6-P/30 min/mg protein.

Other methods. SDS-PAGE discontinuous electrophoresis was performed by the method of Laemli[4] with 5% stacking gel and 7.5% separating gel. Protein was assayed by the Bradford procedure[5] with bovine serum albumin as a standard.

Results and discussion

GlcN-6-P synthase present in the crude extract obtained from *C. albicans* 3153 yeast form cells was inhibited by UDP-GlcNAc with $IC_{50} = 0.67$ mM. The same level of inhibition was observed in regard to the enzyme present in the crude extract prepared from the recombinant YRS65-2 cells. On the other hand, pure GlcN-6-P synthase was much less sensitive to inhibition ($IC_{50} > 5.0$ mM). We found that the enzyme lost the sensitivity already after the second step of purification, i.e. fractional precipitation with protamine sulfate. The same desensitisation was observed when crude extract was passed through the small Sephadex G-25 column, thus revealing that a presence of any low molecular weight component could be necessary for the effective inhibition. Since the previous literature data suggested possible involvement of sugar phosphates in modulation of GlcN-6-P sensitivity to UDP-GlcNAc[6], we compared the effects of different structurally related sugar phosphates on enzyme inhibition by UDP-GlcNAc and found that the presence of glucose-6-P (Glc-6-P) stimulates inhibition (Fig. 1).

Inhibition of *C. albicans* GlcN-6-P synthase by UDP-GlcNAc was non-competitive in respect to both substrates, i.e. L-Gln and Fru-6-P (graphs not shown), thus indicating that the UDP-GlcNAc binding site is separated from the enzyme active centre. It should be noted that the same situation was previously found for the enzyme from *Neurospora crassa*[7] however some other eukaryotic GlcN-6-P synthases behaved in a different way; for the enzyme from *Aspergillus nidulans*, the inhibition was uncompetitive in respect to both substrates[8] and for the enzymes from rat liver and *Blastocladiella emersonii* it was competitive in respect to Fru-6-P and non-competitive in respect to L-Gln[9, 10].

Previous literature data indicated that the chitin content in the *C. albicans* cell wall increases 4 - 5 fold during yeast-to-mycelia (Y → M) morphological transformation[11]. Chiew et al. observed similar increase in specific activity of GlcN-6-P synthase, using serum as an inducer of germ tubes formation and mycelial growth[12]. In our present studies we were able to confirm the latter observation, using defined growth media and conditions.

Fig. 2 shows that the activity of GlcN-6-P increased about 6-fold when Y → M transformation was performed in YCB/BSA medium, pH 6.5 at 37°C and this enhancement was correlated with a current content of mycelial cells. On the hand, the enzyme activity was practically constant when the cells were incubated in YCB/BSA, pH 4.5 at 30°C, i.e. under conditions ensuring yeast morphology. In that case, a small initial increase of the enzyme activity was followed by a quick return to the starting level.

GlcN-6-P synthase present in mycelial cells was much less sensitive to inhibition by UDP-GlcNAc (Table 1). Desensitisation of the enzyme was also correlated with a progress of Y → M transformation.
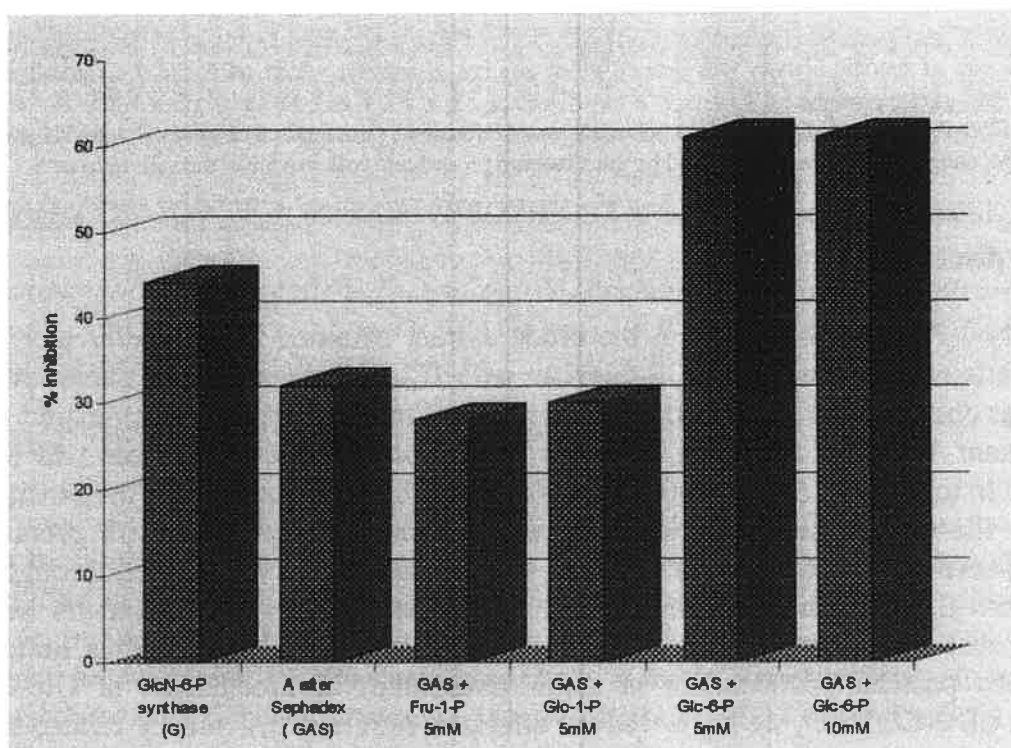
Fig. 1   Effect of sugar phosphates on inhibition of *C. albicans* GlcN-6-P synthase by 0.5 mM UDP-GlcNAc

Similar changes in the GlcN-6-P synthase activity and sensitivity to inhibition caused by UDP-GlcNAc was observed when morphological transformation was induced in the Lee's medium, widely used as a standard environment for the reproducible morphological transition. Transformation of the *C. albicans* 3153 cells in this medium was complete after 3 hrs, GlcN-6-P synthase activity increased 3 - 4-fold and substantial desensitisation of the enzyme to the feedback inhibitor was observed (detailed data not shown).
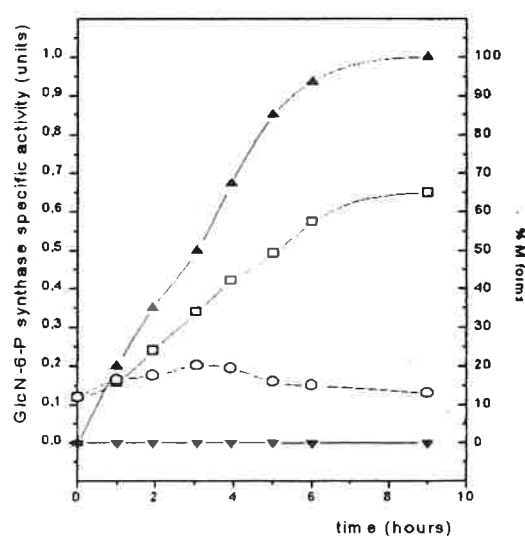

Fig. 2   GlcN-6-P synthase activity in cell free extracts prepared from germinating

(□, ▲ - YCB-BSA medium, 37°C)
and non-germinating

(O, ▼ - YCB-BSA medium, 28°C)
C. albicans 3153 cells.

(▼,▲) - percent of germinating cells;

(□, O) - GlcN-6-P synthase specific activity

GlcN-6-P synthase is coded in *C. albicans* by the *GFA1* gene. It was previously demonstrated that the *GFA1* mRNA level substantially increased at the beginning of yeast-to-mycelia shift, but then quickly fell down, despite further progress in morphological transformation[1]. This observation rather excludes a possibility of a transcriptional regulation of GlcN-6-P synthase level during morphogenesis.
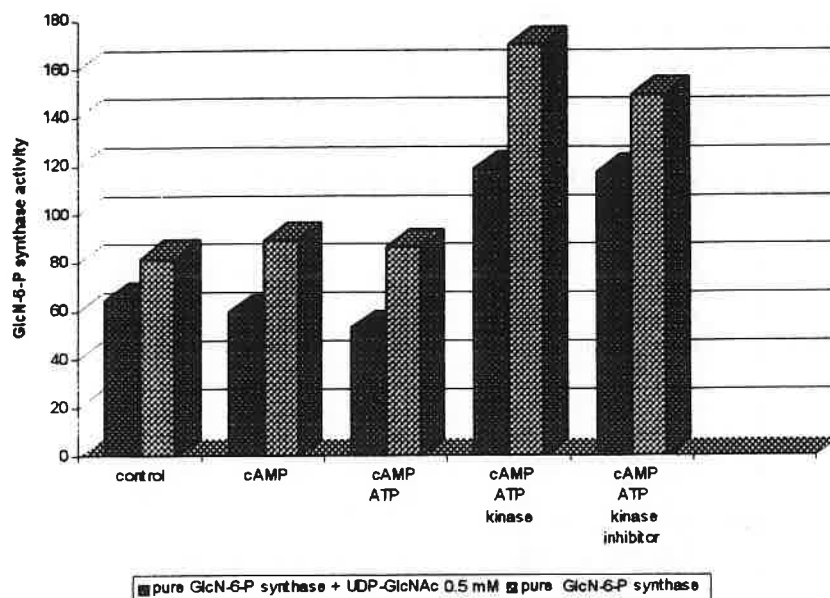
Time (hours)	% germination	% inhibition
0	0	45
1	23	12
3	64	5
5	87	5
7	100	8

Tab.1 Sensitivity of GlcN-6-P synthase present in crude extracts prepared from *C. albicans* 3153 cells, germinating in YCB-BSA medium at 37°C, to inhibition by 0.5 mM UDP-GlcNAc

Our present data shown in Fig. 2 and Table 1 provide evidence suggesting that *C. albicans* GlcN-6-P synthase is actually regulated at the posttranslational level, possibly by modification of catalytic properties of the existing protein molecules. One of the most likely mechanisms of such a change could be a phosphorylation/dephosphorylation mediated by protein kinase(s) and protein phosphatase(s). Several authors showed that different signal transduction systems involving cAMP-dependent and Ca^{2+}-dependent protein kinases, operate during Y $\rightarrow$ M transformation[13]. Dibutyryl cAMP effectively triggers germ tubes formation[14], while EDTA and trifluoperazine inhibit this process[15]. On the other hand, we previously demonstrated that GlcN-6-P synthase present in crude extract prepared from *C. albicans* Y cells could be quickly desensitised to inhibition caused by UDP-GlcNAc when the extract was incubated in the presence of Mg^{2+} ions and this effect was prevented by F⁻ anions, i.e. under conditions stimulating or inhibiting protein phosphatases, respectively[10].

C. albicans GlcN-6-P synthase contains a few consensus sequences which could be recognised by protein kinases. In order to check whether properties of the synthase could be changed upon the action of a protein kinase, we incubated the pure GlcN-6-P synthase, isolated from the recombinant YRS65-2 cells[2] with commercially available cAMP-dependent protein kinase from beef heart. Data shown in Fig 3 clearly demonstrate that such a treatment causes a substantial increase of GlcN-6-P synthase activity and some small desensitisation to UDP-GlcNAc.

Fig. 3 Changes in activity of *C. albicans* GlcN-6-P synthase and its sensitivity to UDP-GlcNAc after treatment with cAMP-dependent protein kinase

Pure GlcN-6-P synthase was preincubated for 30 min at 25°C with 10 μM cAMP, 1 mM ATP, cAMP-dependent protein kinase from beef heart (300 U/ml) and/or protein kinase inhibitor from rabbit muscle (200 U/ml), as indicated. Each sample contained 10 mM EDTA and 40 mM NaF. Samples were assayed for GlcN-6-P synthase activity and sensitivity to 0.75 mM UDP-GlcNAc

Changes in the GlcN-6-P synthase sensitivity to UDP-GlcNAc caused by phosphorylation/dephosphorylation of the enzyme molecules were previously demonstrated for *Blastocladiella emersonii* GlcN-6-P synthase[10] and suggested for the *Aspergillus nidulans* enzyme[8]. Results of our studies provide the first evidence for the possibility of existence of a still unknown mechanism of GlcN-6-P regulation , which is a direct enhancement of the enzyme activity upon the action of the cAMP-dependent protein kinase. However, we do not know if the GlcN-6-P synthase is actually a substrate for a *C. albicans* endogenous cAMP-dependent protein kinase.

On the other hand, it is clear that the sensitivity of *C. albicans* GlcN-6-P synthase to UDP-GlcNAc should also depend on the current intracellular level of Glc-6-P, which is known to be rather constant during Y → M transformation[16]. Therefore it is possible that any other, still unknown mechanism is responsible for the desensitisation to the feedback inhibitor, observed during the morphological shift of *C. albicans* cells.

It was previously estimated that the concentration of UDP-GlcNAc in *C. albicans* cells was about 1 mM[17]. If this level remains constant (data not known), this must mean that GlcN-6-P synthase in Y cells, containing less than 1% of chitin in their cell wall, remains in a semi-inhibited state. Changes in the molecular properties of the enzyme triggered at the beginning of Y → M transformation, enhance the catalytic efficacy of this protein, and, on the other hand, allow overcoming the feedback inhibition. Thus the modified molecules of GlcN-6-P synthase could be able to satisfy a requirement for the aminosugar - substantially enhanced in M cells containing 4 - 5 % of chitin in their walls.

Further studies, now on progress, are still necessary to understand the complexicity of GlcN-6-P synthase regulation in *C. albicans*.

Conclusions

- UDP-GlcNAc, the final product of the cytoplasmic part of the chitin biosynthesis pathway in *C. albicans* inhibits activity of GlcN-6-P synthase - the first enzyme which is unique for this pathway. Glc-6-P stimulates the enzyme sensitivity to the feedback inhibitor
- Activity of GlcN-6-P synthase increases 3 - 6 fold and sensitivity of this enzyme to UDP-GlcNAc substantially decreases during Y $\rightarrow$ M transition of *C. albicans* cells. Both effects are correlated with the progress of transformation
- The action of cAMP-dependent protein kinase enhances an activity of *C. albicans* GlcN-6-P synthase
- GlcN-6-P synthase seems to be the crucial point of regulation of chitin biosynthesis in *C. albicans*

Literature

1. Smith, R.J., Milewski, S., Brown, A.J.P. and Gooday, G.W., *J. Bacteriol.*, 1966, **178**, 2320
2. Milewski, S., Smith, R.J., Brown, A.J.P. and Gooday, G.W., in: *Chitin Enzymology*, Atec Edizioni, Italy ,1996, **vol. 2**, 56
3. Kenig, M., Vandamme, E. and Abraham, E.P., *J.Gen.Microbiol.*, 1976, **94**, 46
4. Laemli, U.K., *Nature*, 1970, **227**, 680
5. Bradford, M.M., *Anal.Biochem.*, 1976, **72**, 248
6. Winterburn, P.J. and Phelps, C.F., *Biochem.J.*, 1970, **121**, 721
7. Endo, A., Kakiki, K. and Misato, K., *J. Bacteriol.*, 1970, **103**, 588
8. Borgia, P.T., *J.Bacteriol.*, 1992, **174**, 384
9. Kornfeld, R., *J.Biol.Chem.*, 1967, **242**, 3153
10. Frisa, P.S. and Sonnenborn, D.R., *Proc.Natl.Acad.Sci.USA*, 1982, **79**, 6289
11. Chattaway, F.W., Holmes, M.R. and Barlow, A.J.E., *J.Gen.Microbiol.*, 1968, **51**, 367
12. Chiew, Y.Y., Shepherd, M.G. and Sullivan, P.A., *Arch.Microbiol.*, 1980, **125**, 97
13. Gow, N.A.R. and Gadd, G.M., *The Growing Fungus*, Chapman & Hall, London, 1995
14. Niimi, M., *Fung.Gen.Biol.*, 1996, **20**, 79
15. Paranjape, V. Roy, B.G. and Datta, A., *J.Gen.Microbiol.*, 1990, **136**, 2149
16. Sullivan, P.A., Chiew, Y.Y., Molloy, C. and Templeton, M.D., *Can..J.Microbiol.*, 1983, **29**, 1514
17. Gooday, G.W. in: *Biochemistry of Cell Walls and Membranes in Fungi*, Kuhn, P.J., Trinci, A.P.J., Jung, M.J. and Goosey, M.W. eds, Springer Verlag, Berlin 1990, 61

Acknowledgement

PREPARATION AND EVALUATION OF NOVEL TYPES OF CHITIN DERIVATIVES

Keisuke KURITA

Department of Industrial Chemistry, Faculty of Engineering, Seikei University, Musashino-shi, Tokyo 180 (JAPAN). Fax: +81-422-37-3871, E-Mail: kurita@chgw.ch.seikei.ac.jp

Abstract

Efficient modification reactions to prepare chitin derivatives with well-defined structures are reviewed. *N*-Phthaloyl-chitosan proved to be a convenient precursor because of the marked solubility and easy deprotection after modification, and some sugar branches including α-mannoside groups could be introduced regioselectively at C-6 by glycosylation reaction with the corresponding orthoesters. Reaction with an oxazoline of *N*-acetylglucosamine gave rise to the introduction of β-glucosaminide branches. Deprotection of these products gave branched chitosans and the subsequent N-acetylation resulted in the formation of branched chitins. Regioselective introduction of carboxymethyl groups became also possible with *N*-phthaloyl-chitosan. High chemical reactivity of β-chitin was confirmed, as compared with the reactivity of α-chitin, in tritylation and trimethylsilylation reactions, and fully substituted derivatives could be prepared under appropriate conditions. Some properties of the resulting derivatives are also discussed.

Keywords: chitin, chitosan, chemical modification, *N*-phthaloyl-chitosan, nonnatural polysaccharide, branched chitin, branched chitosan, carboxymethylation, tritylation, trimethylsilylation

Introduction

Even though chitin is quite attractive as a specialty biopolymer with distinctive physicochemical properties and biological activities, it is currently utilized to only a limited extent. The delay in application study is partly ascribable to the difficulty in controlled modifications because of the insoluble nature and multi-functionality of chitin. Many kinds of modification reactions have been exploited thus far [1,2].

In order to fully explore the high potential, however, it is necessary to further develop various modes of efficient modification reactions, which would make possible sophisticated molecular design. Chitosan and partially deacetylated chitin are convenient starting materials for N-substitution. Some organosoluble derivatives such as N-phthaloylated chitosan have proved to be useful for preparation of derivatives with well-defined structures [3]. In view of possible facile modifications under mild conditions, β-chitin is considered important because of the weak intermolecular forces. Here, a recent progress of modification reactions based on the soluble precursors and β-chitin along with some properties of the resulting derivatives will be discussed.
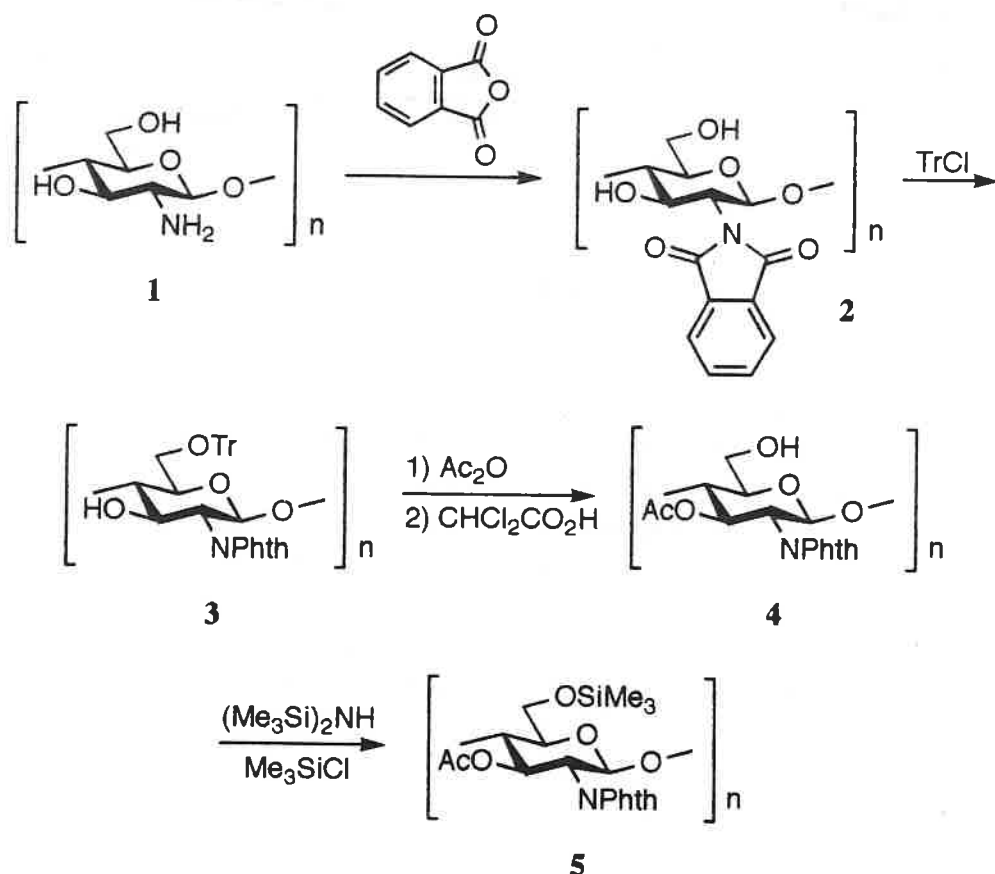
Methods, results, and discussion

Regioselective introduction of sugar branches

Branched polysaccharides occur in nature, and some of them are attracting much attention owing to their biological functions including antitumor and immunoadjuvant activities [4,5]. Synthesis of such polysaccharides is considered quite significant, but it has been difficult to introduce glycosyl groups into linear polysaccharides such as cellulose and curdlan at a specific position [6]. Ring-opening polymerization of anhydro sugars enabled the preparation of branched polysaccharides through post-glycosylation of synthetic dextran [7] or polymerization of an anhydro disaccharide [8]. Due to the presence of free amino groups in chitosan, various sugar groups could be introduced by reductive alkylation, though not by glycosidic linkages [9].
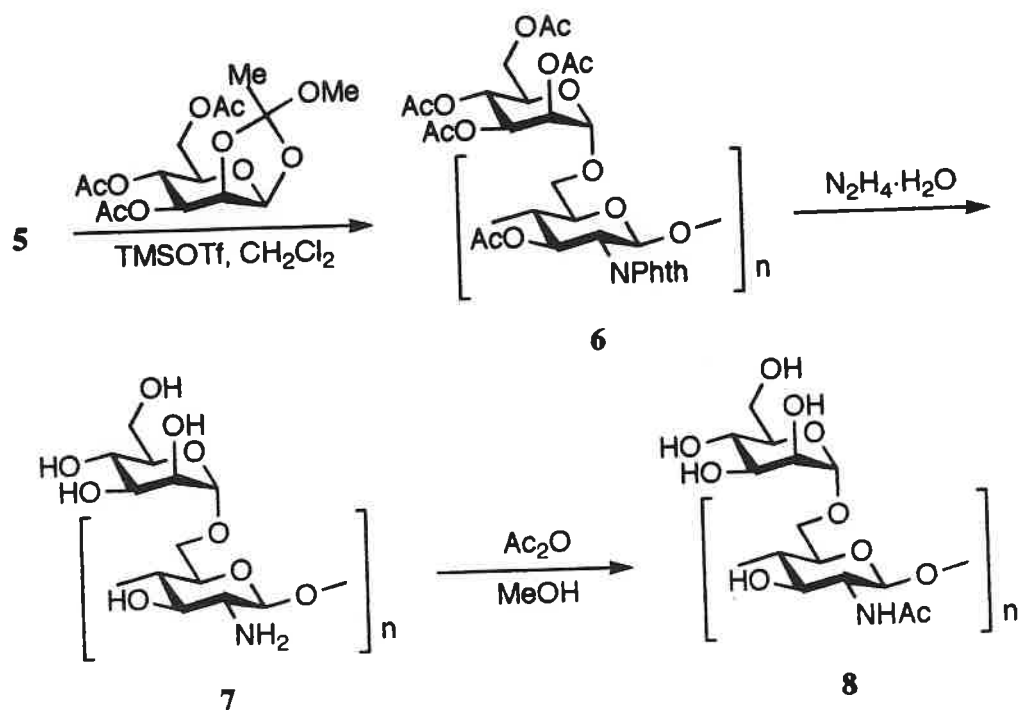
Our interest is in the preparation of branched polysaccharides by regioselective introduction of sugar groups into chitin and chitosan. N-Phthaloyl-chitosan (2) would be a versatile key intermediate for preparing nonnatural branched polysaccharides, since it is soluble in common organic solvents and easily deprotected to regenerate the free amino groups, resulting in effective discrimination of the three kinds of functional groups.

For substitution at C-6, 2 was converted into 3-O-acetyl-2-N-phthaloyl-chitosan (4), which was then trimethylsilylated to the corresponding silyl derivative (5) to further improve the solubility (Scheme 1).

Scheme 1.

321

The reaction of **5** with an orthoester of D-mannose proceeded smoothly at room temperature in dichloromethane in the presence of trimethylsilyl trifluoromethanesul-fonate (TMSOTf) as the catalyst, giving rise to a fully protected product having α-mannoside branches (**6**) (Scheme 2) [10]. The degree of substitution was dependent on the amount of the orthoester and reached about 0.6. Deprotection with hydrazine hydrate gave chitosan having α-mannoside branches (**7**). Subsequent N-acetylation afforded the corresponding chitin (**8**).
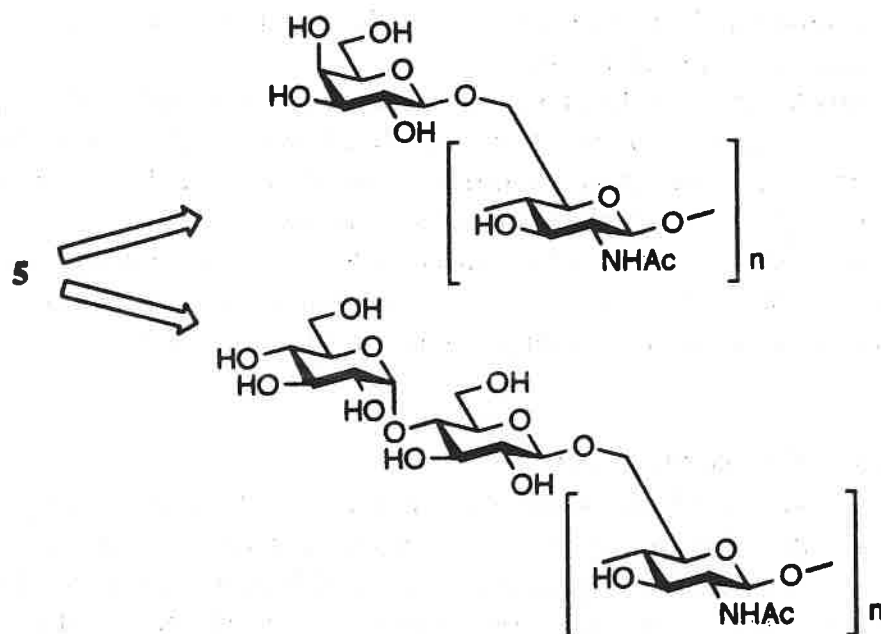

Scheme 2.
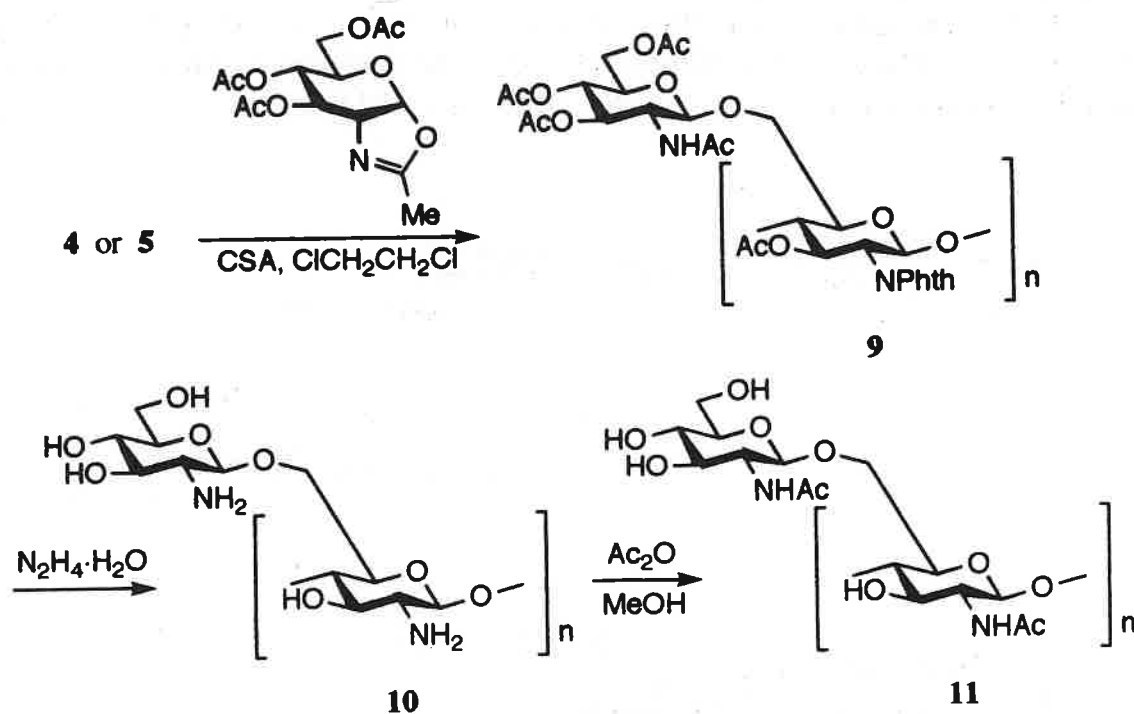
Possibility of introducing β-sugar branches into chitin was then examined. With orthoesters of D-galactose and D-maltose, branched products having β-D-galactoside and β-D-maltoside groups were obtained in a similar manner (Scheme 3).

Introduction of D-glucosamine into chitosan and N-acetyl-D-glucosamine into chitin is particularly interesting, since the resulting branched polysaccharides have the same sugar units in the main chains and branches. To introduce glucosaminide branches in the β-form, an oxazoline derived from N-acetylglucosamine was expected to be suitable. The reaction of **5** with the oxazoline in dichloroethane solution in the presence of camphorsulfonic acid (CSA) at 80 °C gave the protected branched product (**9**) (Scheme 4).

When **4** was used instead of **5**, the reaction mixture was heterogeneous in the initial stage in dichloromethane but became homogeneous as the glycosylation reaction proceeded. The degree of substitution was up to 0.6. Deprotection with hydrazine gave branched chitosan (**10**), which was transformed into branched chitin (**11**) by N-acetylation.

Scheme 3.

9

4 or 5 $\xrightarrow{\text{CSA, ClCH}_2\text{CH}_2\text{Cl}}$

10

$\xrightarrow{\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}}$

$\xrightarrow[\text{MeOH}]{\text{Ac}_2\text{O}}$

11

Scheme 4.

Properties of branched chitosans and chitins

All the branched products having protecting groups were soluble in common organic solvents such as dichloromethane, while the deprotected chitosan and chitin derivatives having sugar branches were readily soluble in neutral water in sharp contrast to the

insoluble linear chitin and chitosan. They were also highly hygroscopic, and exhibited high absorption and retention of moisture.

The branched polysaccharides prepared here are nonnatural, and the influence of sugar branches on the biodegradation is considered of interest. Susceptibility of the products to lysozyme was thus examined, and they were found to be readily enzymatically degradable despite the nonnatural structures.

Antimicrobial activity of chitosan is quite attractive, and the activity of the branched chitosan 10 against some bacteria was examined in comparison with that of chitosan. The results suggested a high potential of 10 as a water-soluble antimicrobial agent.

Introduction of carboxymethyl groups

Carboxymethyl-chitin is soluble in water in the sodium salt form and may be a useful derivative. Carboxymethylation of chitin has been carried out with chloroacetic acid and may occur even at the C-3 hydroxy groups though C-6 hydroxy groups are the preferred reaction position. During the reaction, moreover, deacetylation takes place to a considerable extent due to the strongly alkaline reaction conditions. The carboxymethyl-chitin prepared by the conventional method is therefore structurally ambiguous, and it is necessary to prepare carboxymethyl-chitins with well-defined structures to discuss the structure-properties relationship.

In contrast, reductive alkylation of chitosan with glyoxalic acid gave structurally well-defined N-carboxymethyl-chitosan [11]. Similarly, N-carboxybutyl-chitosan was prepared and confirmed to be useful as a biomedical material [12].

Scheme 5.

324

In order to prepare two kinds of chitin derivatives having the carboxymethyl group regioselectively at C-3 and C-6, reactions of **3** and **4** with methyl bromoacetate were carried out in pyridine, and the substituted products were deprotected and N-acetylated to give 3-*O*-carboxymethyl-chitin (**12**) and 6-*O*-carboxymethyl-chitin (**13**), respectively (Scheme 5).
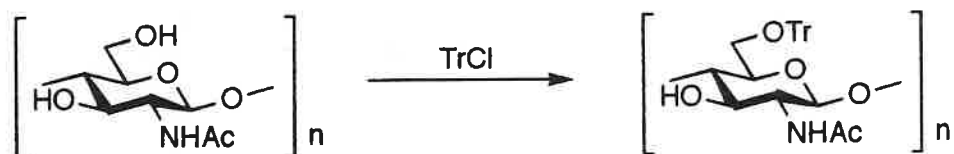
The effect of substitution position on the biodegradation was elucidated in terms of the susceptibility to lysozyme. The results indicated that **12** was degraded at a similar rate to that of chitin, while **13** turned out to be much more susceptible.

Reactivity characteristics of β-chitin

Although β-chitin is much less abundant than α-chitin, it is available in quantity from squid pens and quite attractive as another form of chitin. β-Chitin is characterized by weak intermolecular forces owing to the parallel arrangement of the chitin molecules unlike ordinary α-chitin with antiparallel arrangement [13].

Since chitin molecules are packed loosely in β-chitin, it would be biodegraded more facilely than α-chitin. Actually, β-chitin was much more susceptible to lysozyme. The extent of deacetylation markedly affected enzymatic degradation, and the rate of degradation increased with an increase in the degree of deacetylation (dd). It reached a maximum at dd about 0.5 and then decreased. This indicates that partial deacetylation resulted in substantial disturbance of packing of chitin molecules.
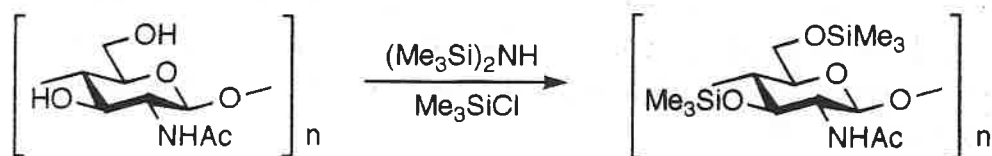
As suggested from the high affinity for solvents and biodegradability, β-chitin was expected to exhibit high reactivity in chemical modifications. The reactions such as deacetylation [14], acetylation, tosylation [15], and acetolysis [16] actually proceeded much more facilely with β-chitin than α-chitin. The degrees of substitution of β-chitin were much higher than those for α-chitin under the same conditions. Furthermore, full tritylation could be possible with β-chitin in one step (Scheme 6), whereas the same derivative was prepared only through five-step reactions starting from α-chitin. It is interesting to note that even the derived chitosan showed higher reactivity in N-phthaloylation than chitosan from α-chitin [17].

Scheme 6.

It was considered of interest to prepare trimethylsilylated chitin to enhance solubility without sacrificing the chemical reactivity. The high reactivity of β-chitin therefore prompted us to examine the possibility of direct trimethylsilylation. The reaction with a mixture of hexamethyldisilazane and trimethylsilyl chloride proceeded smoothly in pyridine, and even a fully substituted derivative could be prepared under appropriate conditions (Scheme 7). The resulting silylated chitin was soluble in acetone, and solution casting was possible to give films. Desilylation of the films with aqueous acetic acid gave chitin films.

Scheme 7.

Conclusion

Because of the high potential of chitin as a specialty biopolymer due to the unique structure and properties, preparation of the derivatives with well-defined structures is becoming increasingly important. Efficient chemical modifications based on some appropriate key precursors have proved advantageous to design complicated molecular environments and would be useful for developing various advanced functions based on this attractive amino polysaccharide.

Acknowledgments

References

1. Muzzarelli, R. A. A. *Chitin*, Pergamon, Oxford (1977)

2. Roberts, G. A. F. *Chitin Chemistry*, Macmillan, London (1992)

3. Nishimura, S., Kohgo, O. and Kurita, K. *Macromolecules* 1991, **24**, 4745

4. Chihara, G., Maeda, Y., Hamuro, J., Sasaki, T. and Fukuoka F. *Nature* 1969, **222**, 687; Chihara, G., Maeda, Y. and Hamuro, J. *Int. J. Tissus. React.* 1982, **4**, 207

5. Mitani, M., Ariga, T., Matsuo, T., Asano, T. and Saito, G. *Int. J. Immunopharmacol.* 1980, **2**, 174

6. Matsuzaki, K., Yamamoto, I., Sato, T. and Oshima, R. *Makromol. Chem.* 1985, **186**, 449; 1986, **187**, 317

7. Ito, H. and Schuerch, C. *J. Am. Chem. Soc.* 1979, **101**, 5797

8. Kuzuhara, H., Ichikawa, Y., Sakairi, N., Uryu, T. and Yoshida, T. XIVth Int. Carbohydr. Symp. (Stockholm), Abstr. p. 201 (1988)

9. Hall, L. D. and Yalpani, M. *J. Chem. Soc., Chem. Commun.* 1980, 1153; Yalpani, M. and Hall, L. D. *Macromolecules* 1984, **17**, 272; Yalpani, M., Hall, L. D., Tung, M. A. and Brooks, D. E. *Nature* 1983, **302**, 812

10. Kurita, K., Kobayashi, M., Munakata, T., Ishii, S. and Nishimura, S. *Chem. Lett.* 1994, 2063

11. Muzzarelli, R. A. A., Tanfani, F., Emanuelli, M. and Mariotti, S. *Carbohydr. Res.* 1982, **107**, 199

12. Muzzarelli, R. A. A., Weckx, M., Filippini, O. and Lough, C. *Carbohydr. Polym.* 1989, **11**, 307; Muzzarelli, R. A. A., Tarsi, R., Filippini, O., Giovanetti, E., Biagini, G. and Varaldo, P. E. *Antimicrob. Agents Chemotherapy* 1990, **34**, 2019

13. Blackwell, J., Gardner, K. H., Kolpak, F. J., Minke, R. and Classey, W. B. *ACS Symp. Ser.* 1980, **141**, 315

14. Kurita, K., Tomita, K., Tada, T., Ishii, S., Nishimura, S. and Shimoda, K. *J. Polym. Sci., Part A: Polym. Chem.* 1993, **31**, 485

15. Kurita, K., Ishii, S., Tomita, K., Nishimura, S. and Shimoda, K. *J. Polym. Sci., Part A: Polym. Chem.* 1993, **32**, 1027

16. Kurita, K., Tomita, K., Ishii, S., Nishimura, S. and Shimoda, K. *J. Polym. Sci., Part A: Polym. Chem.* 1993, **31**, 2393

17. Kurita, K., Tomita, K., Tada, T., Nishimura, S. and Ishii, S. *Polym. Bull.* 1993, **30**, 429

Triphenylsilylchitin: a new chitin derivative soluble in organic solvents

Marc Vincendon

CEA, Centre Etudes Nucleaires, DRFMC, F-38054 Grenoble Cedex 9, France

Abstract

Triphenylsilylchitin, a silylated derivative of chitin has been prepared with an average degree of substitution of 1.5. This new derivative of chitin is found to be soluble in most organic solvents, such as dioxan, tetrahydrofuran, chloroform, toluene.
The characterization of this new polymer has been achieved by using spectroscopic methods. The ^{13}C NMR spectroscopy characterizes a statistical derivative of chitin, mainly substituted on the C-6 position. Low values of intrinsic viscosities polymer solutions are observed. This silylated derivative of chitin is found stable towards water hydrolysis.
The thermal analytical methods indicate that triphenylsilylchitin is softened at 225/240°C, and is thermally degraded above 250°C. This derivative can thus be processed at 230°C.

Keywords : chemical modification, chitin, triphenylsilylation, IR, NMR and thermal analysis.

Chitin is a linear polymer difficult to dissolve in polar solvents. Due to the high density of hydrogen bonds in its solid state, chitin does not show a melting temperature. For these reasons, this natural polymer is difficult to process. It has been shown, since a long time that via the reaction of the hydroxyl reactive groups of polysaccharides, it was possible to induce major changes in their physicochemical, thermal and mechanical properties. The hydroxyl groups of polysaccharides can react with alkyl and acyl halides or with isocyanates to yield ethers [1, 2], esters [3, 4] or carbamates derivatives [5, 6]· These chemical derivatives are generally less polar than the original polysaccharide, and consequently more soluble, and possibly thermoplastic.
Little attention has been given to silylated derivatives of chitin. Partially substituted trimethylsilylchitin has been obtained [7], which did not show an important increase in solubility. On the other hand, trimethylsilyl derivatives of polysaccharides present a high sensitivity towards water molecules which readily cleave the TMS

groups. In this work we have synthetized a triphenylsilyl derivative of chitin, in order to improve its solubility in organic solvents and its stability towards hydrolysis.

Materials and methods

α chitin powder (100-500 μm), extracted from crab shells, was obtained from Aber Technologies, France.
Regenerated α chitin was obtained by a rapid dissolution of chitin powder in 85% phosphoric acid, within 40 minutes at room temperature, precipitation in 1M sodium hydroxide solution, filtration, neutralization and drying [8]. β chitin was extracted from squid pens.
Preparation of the triphenylsilylchitin: 1g of α chitin (4.92 mmole) was dried for 2 hours at 110°C. To the dried powder 40mL of anhydrous pyridine were added, and the suspension was stirred for 2 hours at 100/110°C. Then 5.8 g (19.7 mmole,i.e., 4 moles per N-acetyl-D-glucosamine unit) of triphenylchlorosilan (Fluka) were added. The suspension was stirred at 100/110°C for 20 hours. An amber solution was obtained, which was cooled and precipitated in 400 mL of ethanol. The white precipitate was filtered and washed. The solid was extracted in a Soxhlet apparatus using ether to eliminate the triphenylsilanol. The remaining solid was then extracted with chloroform, and after evaporation of the solvent,1.8 g of polymer was obtained.
Microanalysis:
Mono(triphenylsilyl)chitin,$(C_{26}H_{27}O_5NSi)n$ C:67.8; H:5.65; N:3.04 Si:6.08
Bis(triphenylsilyl)chitin $(C_{44}H_{41}O_5NSi_2)n$ C: 73.5; H: 5.7; N: 1.94; Si: 7.79
Experimental,   C: 70.5; H: 5.8; N: 2.3; Si: 7.1

The IRFT spectra were obtained on film samples by using a Perkin Elmer 1600 spectrophotometer.
The NMR spectra were obtained on a Bruker AM 400 spectrometer having a frequency of 400MHz for proton and 100MHz for ^{13}C.
Thermogravimetry analysis was carried out on a Setaram TGL 85 thermobalance by using a heating rate of 2°C/minute.
Intrinsic viscosities of triphenylsilylchitin in chloroform solutions, were determined at 25°C by using an Ubbelhode viscosimeter.

Results and discussion

The triphenylsilyl substituent, like the trityl (triphenylmethyl) is one of the bulky groups which will present important steric interactions on a polymer chain. It has been shown that trityl chloride reacts on the primary hydroxyl group of chitin

and that the β polymorph is more reactive than the α one [9]. However, it has also been shown in the case of cellulose - a polysaccharide with a structure similar to that of chitin - that reaction with a large excess of reagent, at high temperature, can also affect the secondary hydroxyl groups [10]. On the basis of these experiments, the reaction of the chitin hydroxyl groups (OH-3 and OH-6) with the triphenylsilylchloride has been investigated.

Synthesis of triphenylsilylchitin :

Chitin samples from different origins were used in order to vary the accessibility of the polymer chain towards the reagent. The accessibility of the hydroxyl groups increases from the α chitin of crab shells to the β chitin from squid pens and to regenerated chitins. Regenerated chitin samples were obtained by dissolution in N,Ndimethylacetamide containing lithium chloride or in concentrated phosphoric acid [8] and precipitation in non-solvents. The accessibility of chitin is revealed by its solubility, which increases from α chitin to β chitin, and to regenerated chitin, and by the X-ray diffraction diagrams which show a decrease of the crystallinity index.

Table 1 gives the experimental results of the synthesis, conducted in all cases in a pyridine suspension at 100/110°C during 20 hours.

TABLE 1

	Moles of reagent	Soluble fraction(%)	$[\eta]$ g/dL	Si (%)
α chitin	4	67	0.76	7.1
β chitin	4	78	0.47	6.9
Regenerated chitin	2	95-100	0.62	7.4

In order to obtain a noticeable reaction of the triphenylsilylchloride on the hydroxyl groups of the chitin chain, we used four moles of chloride per monomer unit, i.e., two moles per hydroxyl group. For regenerated chitins which present a higher accessibility to the reagent,, we used a stoechiometric ratio - one mole per hydroxyl group.

The regeneration by precipitation of the modified polymer yields an heterogeneous polymer, as chitin is not uniformly substituted. This is particularly examplified by the solubility of the chitin silyl derivative, which varies with the degree of substitution (DS). The solvent extraction of the solid modified polymer, using chloroform, gives an organic soluble fraction which increases from α chitin (67%) to β chitin (78%), and to regenerated chitin (100%). One can notice that the higher the accessibilty of the starting polymer, the higher the solubility. The insoluble part in classical organic solvents corresponds to weakly substituted chitin.

All the following characterizations have been achieved on the soluble part in organic solvents.

Characterization of the triphenylsilylchitin :

Microanalysis of these new silyl derivatives of chitin gives an indication of the DS value (maximum DS = 2) through the elemental silicon determination. Values of the order of 7% of silicon are found for the three analysed samples (TABLE 1), which corresponds to an average DS value of 1.5.

The IRFT spectrum, obtained on a film prepared from a chloroform solution, is shown in Figure 1 and characterizes an aromatic silicon derivative of chitin. One can particularly observe a decrease in the hydroxyl vibration band intensity at 3500 cm^{-1}, the presence of aromatic substituent characteristics (3050,1950-1750, 750-700 cm^{-1}) and the Si-C and Si-O vibration bands at 1240, 1080 and 850-810 cm^{-1}.

The ^{1}H NMR spectrum has been obtained in a CDCl$_3$ solution and is poorly resolved. This is due to the noticeable viscosity of the solution and the presence of aromatic rings as substituents which very often broaden the proton NMR signals. Nevertheless, the aromatic signals resonating between 7 and 7.8 ppm and the cyclic proton signals of the N-acetyl-D-gucosamine repeating unit, resonating between 3 and 4.7 ppm allow the determination of an average degree of substitution. The signal at 1.8 ppm, due to the methyl protons of the N-acetyl group, clearly indicates that the etherification reaction is conducted without elimination of this group.

The ^{13}C NMR spectrum obtained in the same solvent (Figure 2), is typical of statistical polysaccharide derivatives. In the case of regularly substituted polysaccharides, each type of carbon nuclei appears on the spectrum as a single signal, while in the case of statistically substituted polysaccharides the same carbon nuclei appear as a multiplet signal depending on the possible cases (4 for chitin). One can obtain signals from : 1 - unsubstituted monomeric units; 2 - monosubstituted units at C3; 3 - monosubstituted units at C6; 4 - disubstituted units The multiple broad signals obtained for each type of carbon atom of the N acetyl-D-glucosamine unit of this chemically modified polymer, clearly chararacterize a statistical derivative. Most of the resonances observed on the spectrum, appear as pairs of broad signals due to the influence of the neighbouring carbon atoms either substituted or not. As an example, the C2 signal appears on the spectrum as a doublet at 54 and 55.2 ppm, depending or not on the substitution of the hydroxyl group linked to C3.

All the results obtained from the different analytical methods used here, indicate that the average 1.5 triphenylsilyl substituents found per repeating unit are statistically distributed over the two hydroxyl groups in position C3 and C6, the primary hydroxyl C6 being the more substituted one.

Thermal properties and stability of this polymer :

When the triphenylsilylchitin is heated at a high temperature a softening transformation temperature at 225°C is obtained. The Differential Scanning Calorimetry curve shows no major transformation points. This is related to the amorphous state of this chitin derivative which does not show a melting temperature.

The thermogravimetry analysis yields the curves shown in Figure 3 for chitin (A) and triphenylsilylchitin (B). The triphenylsilyl derivative curve indicates that this modified polymer is less sensitive to the hydration than the original polymer, while surprisingly it is more affected at high temperature : an extrapolated onset temperature of 250°C (Td) is found for the triphenylsilylchitin, whereas a value of 290°C is found for chitin. The thermal properties of this new derivative of chitin reveal a softening state, ranging from 225 to 245 °C without a noticeable degradation.

The intrinsic viscosity value [η] is an indication of the molecular weight of this new polymer. It could be also an indication of the degradation arising during the etherification reaction. Figure 4 shows the extrapolated values [η] for three different samples of triphenylsilylchitin, in a chloroform solution at 25°C. All the samples of triphenylsilylchitin have low intrinsic values, in the range of 0.45 to 0.8 dL/g. These values indicate that derivatisation of chitin proceeds with an important decrease in the molecular weight. This was also observed for the synthesis of butyrylchitin which is a chitin derivative highly soluble in organic solvents [11].

The trimethylsilyl derivatives of polysaccharides are sensitive to hydrolysis, particularly in acidic media and even in water [7, 12]. We have tested the stability of triphenylsilyl groups regarding hydrolysis, by refluxing a triphenylsilylchitin sample in water (pH = 6) for 24 hours. No significant loss of weight has been observed (93% of the initial weight is recovered). The Infrared spectrum of the recovered polymer is identical to the original one (Figure 1). The degree of substitution determined on the basis of the silicon elemental analysis (%Si = 7.4) has exactly the same value as that of the starting polymer. It can be concluded that contrarily to TMS groups, the triphenylsilyl groups linked on polysaccharides via ether bonds are stable towards water hydrolysis at a high temperature.

Conclusion

Despite the bulkiness of the triphenylsilyl group, it is shown in this work that more than one group can be linked per repeating unit of the chitin chain via a reaction of etherification. In comparison to the triphenylmethyl (trityl) group [9], the triphenylsilyl group is found to exhibit a higher reactivity towards

chitin. The solubility in organic solvents of this new polymer is obtained for an average DS above 1. It can be more easily obtained with chitin of lower crystallinity - and therefore of higher accessibility to the reagent - e.g. regenerated chitin. The triphenylsilylchitin is one of the only chemically modified chitin derivatives soluble in organic solvents; butyryl chitin is also soluble when highly substituted (DS = 2) and when the degree of polymerization is low [11].

The substitution of 1.5 hydroxyl groups among the two possible ones, largely modifies both the physicochemical and thermal properties. The modification of the polarity induced by the substitution of the hydroxyl polar groups - via triphenylsilyl non polar groups - explains the important decrease in water adsorption observed on the TGA diagram. Chitin, as already mentioned, is a non thermoplastic polymer, due to the high density of hydrogen bonds. The substitution of the reactive hydroxyl groups lowers this density and allows the modified polymer to soften, and thus to be processed at high temperatures. However, the thermal stability is not enhanced as it could be expected with the introduction of silicon atoms.

References

[1] Somorin, G., Nishi, N., Tokura, S., Noguchi, J. *Polym. J.* 1979; **11**: 391.

[2] Tokura, S., Yoshida, J., Nishi, N., Hiraoki, T. *Polym. J.* 1982;**14**: 527.

[3] Kaifu, K., Nishi, N., Komai, T. *J. Polym. Sci. Chem.* 1981; **19**: 2361.

[4] Kurita, K., Yoshino, H., Yokota, K., Ando, M., Inoue, S., Ishi, S., Nishimura, S. *Macromolecules* 1992; **25**: 3786.

[5] Terbojevich, M., Carraro, C., Cosani, A., Marsano, E. *Carbohydr. Res.* 1988; **180**: 73.

[6] Vincendon, M. in "*Advances in Chitin and Chitosan*", (E. J. Brine, P. A. Sandford, J. P. Zizakis, Eds.) Elsevier, Amsterdam, 1992, p. 556.

[7] Harmon, R. E., De, K. K., Gupta, K. *Carbohydr. Res.* 1971; **31**: 407.

[8] M. Vincendon in "*Chitin World*" (Z. S. Karnicki, M. Brzeski Edits.) WirtschaftsVerlag, Bremerhaven, 1995, p. 91.

[9] Kurita, K., Ishi, S., Tomita, K., Nishimura, S., Shimoda, K. *J. Polym. Sci.* 1994; **A 32**: 1027.

[10] Green, W. J. in "*Methods in Carbohydrate Chemistry*" (Whistler R. L. Edit.) Acad. Press, 1963, Vol. 3, p. 327.

[11] Szosland, L. in "*Chitin World*" (Z. S. Karnicki, M. Brzeski, Edts.) WirtschaftsVerlag, Bremerhaven, 1995, p.209.

[12] Hashimoto, K., Imanishi, S. I., Okada, M., Sumimoto, H. *J. Appl. Polym.Sci.* 1991; **29**:1271.

Preparation and Characterization of Functionalized Chitosan Fibers

Piotr Kulpinski and Shin-Ichiro Nishimura
Division of Biological Sciences, Graduate School of Science,
Hokkaido University, Sapporo, 060 JAPAN

Seiichi Tokura
Graduate School of Enviromental Earth Science,
Hokkaido University, Sapporo, 060 JAPAN

Abstract

Synthesis of high performance biomaterials from chitin and chitosan have been ever increasing attention because of their biocompatibility, applicability to materials science with large scale preparation. As part of ongoing projects on the synthesis of biologically active glycoconjugates and related biomaterials, we present herein a novel and simple strategy for converting chitosan into new functional materials having specific affinities based on protein-carbohydrate interactions. In the present communication, we designed and prepared new type of chitosan fibers bearing branched α-galactoside residues through an appropriate spacing from C-2 amino groups of glucosamine residues.

For recent decades it is observed still fast growing interest in carbohydrate and bio-polymer chemistry. It is not coincident that the biggest sciences centers on the world are involved in those two domains of science. In fact those areas have somehow common background. Day by day the knowledge of the roles played by carbohydrates in the living systems becames wider and wider.

The carbohydrates not only take part in storage and supply of energy but also play main role many biological processes as cell-cell recognition, growth, differentiation and

334

infection.

For example the adhesion of bacteria to host cells constitues the first step in many infection. The kind of sugar residue and its conformation located on the cell surface seems to be essential factor recognizing by virus or bacteria.

Proteins show high affinities to the glycopolymers having specific sugar branches. Therfore the affords toward synthesis of new class of biologically active glycoconjugates including neoglycoproteins, neoglycolipids, cluster and glycopolymers leeds to understunding of the most basic interactions in the living systems Glycocongjugates have been designed as: models in carbohydrate-protein interaction studies [1], vaccines, inhibitors of cell adhesion by viruses, bacteria, mycoplasma and toxins [2], ligand in affinity chromatography, diagnostic reagents, probes, targeted drug-delivery systems [3] or to confer upon enzymes and proteins improved thermal and protolytic stabilities.

At the present the development of carbohydrate chemistry demonstrate how important in the living systems are glycoconjugates, aspecially glycoproteins and glycolipids.

Chitosan is a well-known biocompatibile natural polymer. It has found a wide range of application as a biomedical material. For example chitosan is used as a wound healing dressing. It also found new application in cosmetics and technics as well. This biodegradable and nontoxic polysaccharidecan be also introduce to organize as a pharmaceutical carier.

Chitosan - the multifunctional polymer therefore can be easily transform to various derivatives[4,5]. Its chemical modification leads to obtain soluble chitosan in common organic solvents. It gives good oportunity to design a novel types of functional materials such polymeric drugs and biocompatible artificial organs with high specifity.

The part of ongoing projects on the design and synthesis of biologically active compounds is devoted to work out of new strategy for converting chitosan into new functional materials.

Combining polysaccharides eg. chitosan with biological active carbohydrate compounds seems to be very interesting from point of view of application in materials science. Therefore it could be possible to obtain new classes of biomaterials having completely new properties. In synthesis of glycoconjugates usually can be applied several different strategies.

The one of very usefull way is show on the fig. 1.

In this manner, some typical compounds which structures are shown in fig. 2 can be obtain.
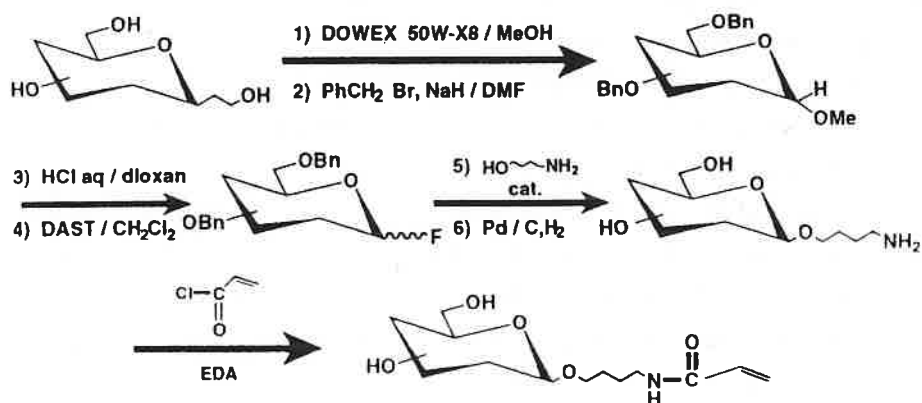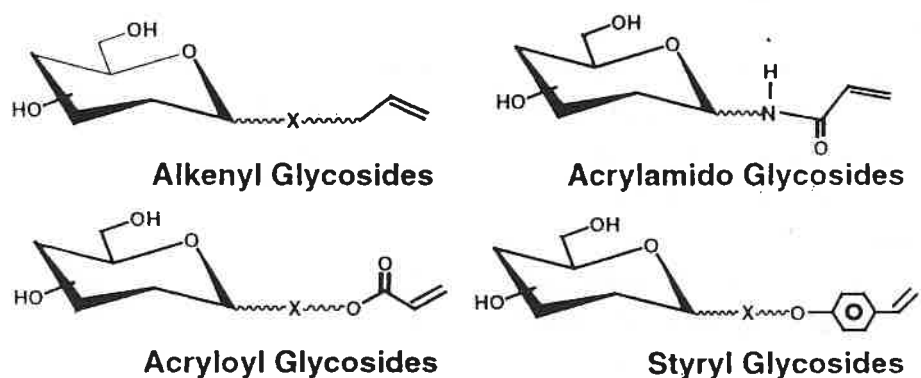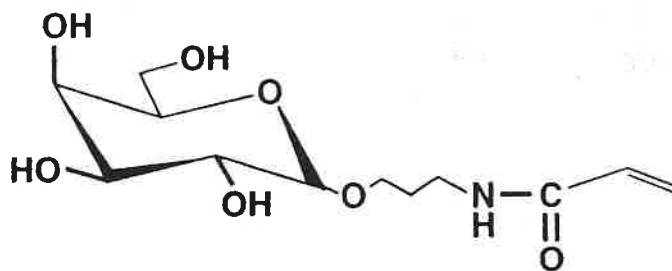
Fig. 1

Alkenyl Glycosides

Acrylamido Glycosides

Acryloyl Glycosides

Styryl Glycosides

Fig 2

Also synthesis of 3-(acrylamido)propyl α-galactopyranoside (shown in fig. 3) was succesfully carried out.

3-(acrylamido)propyl α-galactopyranoside

Fig. 3

336

Double bond in those compounds allows to their polymerizatoin in presence or absence of comonomers. Glycopolymers can be prepared with different shares, sized, sugar residues and different sugar residues densites.

The presence of double bond makes good possibility to attach glycoconjugates to compounds having eg. NH_2 groups. For this purpose can be used chitosan with its abudance of amino groups. Synthesis could be realized in Micheal addition reaction[6] Fig. 4.

This reaction can be carried out on the surface of chitosan fibers.
The fibers were obtaining by means of method worked out by prof. Tokura[7] Thus

Fig. 4

chitosan Flonac-C (DAC 70) was mixed with water and after 30 min acetic acid was added. Mixture was stired during 24 hours. After that time dope was filtrated and left overnight to degasify. Concentration of chitosan in dope was 12.5%. Spinning was carried out using 50 holes nozzle. Precipitation bath consisted of water solution of amonia cupprum. Washing bath was solution of methanol in water (1:1). Fibers were washed in hot water solution of EDTANa till loss the color than washed with destilated water untill complete neutralization and dried up in room temperature.
The schema of spinning aparatus is shown on fig. 5.

Prepared in this way chitosan fibers can be easily modified with glycosides monomer in Micheal addition reaction. The conjugated bouble bond of the saccharide
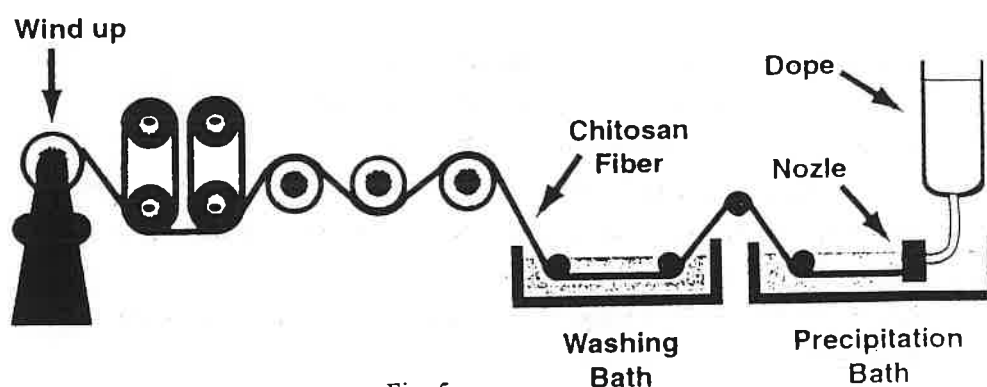

Fig. 5

337

compound can serve as Michael acceptor for the nucleophilic addition of chitosan amino groups. The big advantage of this type of reaction is relatively easy selection of parameters of reaction which allow to control of distribution of attached glycosides along chitosan chain. Later succesful binding of proteins are significantly depent on sugar densieties and their orientations.

Certain selection of reaction parameters and selection of appropriate glycosides as well allow to obtain for example selective chitosan filter traping only required biological compound. It makes good possibility for inventing new kind of "inteligent" and biocompatible fibers which can be applied in the various areas of medicine and technics.

Conclusion

1. Chitosan as a multifunctional polymer can be easily modified by biologically active compounds. One of promissing methods is the Micheal addition reaction. This process can be easily carried out in the middle reaction condition.
2. Owing to special properties of chitosan it is possible to obtain its functionalized derivatives in different shapes eg.: fibers, membranes, granules, powder, etc.
3. Functionalized chitosan can be used as efficient toxin trap.
4. It is possible to obtain high bio-selective chitosan using different glycoside modifiers.

References:

1. R. U. Lemieux, Chem. Soc. Rev., 18, 347 (1989).
2. J. C. Paulson, The Receptors, Acad. Press. New York, vol. 2. P. 131 (1985).
3. J. Kopecek, J. Controlled Relase, 11, 279 (1990).
4. Long-Di Ren, Yasumoto G., Hiroaki K., et. Al., Polym. Int., 35, 303, (1994).
5. Shin-Ichiro Nishimura, Osamu Kohgo, Keisuke Kurita, Macromolecules, 1991. 24. 4745.
6. Roy, R. A. Pon, F. D. Tropper, F. O. Anderson, J. Chem. Soc., Chem. Commun.. 1993, 264.
7. S. Tokura, N. Nishi, Kagaku To Seibutsu, 1977, 15(12), 766.

HYDROPHOBIC DERIVATIVES OF CHITIN : SYNTHESIS, CHARACTERIZATION AND PROPERTIES

Jacques DESBRIERES and Marguerite RINAUDO

CERMAV-CNRS, affiliated with Joseph Fourier University, BP 53, 38041 GRENOBLE Cedex, FRANCE, e-mail : desbrier@cermav.cnrs.fr

Abstract : Hydrophobic derivatives of chitin or chitosan are synthetised by reductive amination to graft alkylchains on the amino group. Their structure is determined from n.m.r. spectroscopy and some properties are studied. Particularly rheological, fluorescence and surface active properties are considered and the influence of parameters such as the polymer concentration, temperature and salt content is demonstrated. Finally the complexes formed between these hydrophobic polyelectrolytes and surfactants of opposite charge are shown to be more efficient than the pure surfactant to decrease the surface tension of aqueous solutions.

Keywords : chitin derivatives, hydrophobic interactions, rheology, fluorescence, surface activity, surfactant

Introduction

Associating water-soluble polymers represent a new class of industrially important macromolecules. Their intriguing rheological properties (for example) in aqueous media are due to their amphiphilic character. They are of great interest as thickening materials giving high viscosity even in very dilute solutions and with moderate molecular weight; they can be used as thickening additives in many aqueous-based industrial formulations such as latex paints, drilling muds, flocculants, foods, cosmetics. The general picture emerging from the studies is that above a certain polymer concentration the interchain aggregation of hydrophobic groups, or domains, dominates which can result in a formation of a thermoreversible three-dimensional network. The junctions can break and recombine, therefore the associating systems can exhibit a complicated phase diagram.

This paper concerns experimental results on hydrophobic derivatives of chitin.

Materials and methods

The original chitosan samples were from Protan (Norway), called Chitosan I, or Aber Technologies (Plouguerneau, France), called Chitosan II. The degree of acetylation (DA) was determined by [1]H n.m.r. considered to be the most sensitive technique using an AC300 Bruker spectrometer [1]. From previous viscometric data the average viscometric molecular weight of these samples were determined as Mv values around 190000 g/mol. Carboxymethylchitin-sodium salt (CMCh) was synthetised using the improved method [2] consisting in the treatment of crab chitin with NaOH followed by the reaction with monochloroacetic acid in water-isopropanol media. The substitution degrees on carboxyl and N-acetyl groups were estimated from [13]C n.m.r. [3]. The degree of acetylation of CMCh is 0.85. Alkylation reactions were carried out on chitosan and CMCh as described further.

The solutions of the alkylated derivatives were prepared by mechanical stirring at low temperature (5°C) to reduce the hydrophobic interactions.

The anionic surfactant, sodium dodecylsulfate (SDS), and the cationic surfactant, tetradecyltrimethylammonium bromide (TDAB) were from Fluka. These surfactants were used without further purification.

The viscosity measurements were performed with an Ubbelohde capillary viscometer (ϕ = 0.5 mm). A low shear viscometer (LS30 from Contraves) was also used for testing the effect of shear rate on the viscosity measurements. A cone-plate rheometer (CS50 from Carrimed) was used for dynamic experiments in a 10-60°C temperature range. The cones we have used have 4 or 6 cm diameter and an angle of 3°59' with a cap to avoid vaporization. The rate of the temperature ramp from 10 to 60°C was 1.8 degree per minute. The cone displacement was of 1° and the frequency equal to 1 Hz.

The fluorescence of pyrene used a marker for hydrophobic domains was determined using a Perkin Elmer LS50B spectrofluorimeter. Pyrene was added at a concentration of 10^{-7} mol.L^{-1}. The studied parameter was the I_1/I_3 ratio of the intensities of first and third peaks of fluorescence spectrum of pyrene in chitosan derivative solutions.

Surface tension was measured using the Wilhelmy plate method with K-10 tensiometer from Krüss with the accuracy of $\pm$

$0.3 \text{ mJ} \times \text{m}^{-2}$. The alkylated carboxymethylchitin solutions were prepared using bidistilled water followed by exposure for one day. The surface tension measurements are carried out at 25°C.

Results and discussion

Alkylation reaction

The alkylated chitosan derivatives were obtained by reductive amination following the procedure described by Yalpani [4]. Generally the reaction of substitution on polysaccharides proceeds in heterogeneous conditions with preferential reactivity of accessible zones thus giving a blockwise distribution of substituents. Hence the derivative is structurally inhomogeneous. We have developed a procedure for performing homogeneous chemical reactions based on the swollen structure of chitosan directly modified after precipitation by basic neutralisation, which allows a better accessibility of reactive sites [5]. Such a reaction is carried out on chitosan or ionic derivatives of chitin such as carboxymethylchitin.

The reaction is performed in mild conditions with no modification of the degree of acetylation nor of the polymerization degree. On the basis of chitosan, one gets cationic amphiphilic polymers in acidic medium (due to the amine protonation); from highly carboxylated carboxymethylchitin, alkylated derivatives are negatively charged and water soluble for pH larger than 4.

The prepared derivatives are named according the alkyl chain length which is grafted (CC8 is a chitosan substituted with C_8 alkyl chain from a C_8 aldehyde, CMChC14 is the carboxymethylchitin substituted with C_{14} alkyl chain).

Characterization of alkylchitosans

The structural characterization of such derivatives was performed from n.m.r. spectroscopy and microanalysis. These techniques allow us to determine the average degree of substitution of the macromolecular chain and the presence of mono or disubstituted units [6]. The characteristics of synthetised derivatives are given in table 1.

From the data given in table 1, it is demonstrated that the modification is controlled by the stoechiometric conditions meaning that the reaction is complete in the experimental conditions adopted.

Table 1 :   Characterization of amphiphilic alkylchitosans

	Degree of acetylation DA	Monomolar ratio NH_2/aldehyde (effective degree of substitution τ)	$[\eta]$ (mL/g)	k'
Chitosan I	2		1100[*]	0.70
CC8	2	1/0.1 (1/0.12)	1250[*]	1.50
CC10	2	1/0.1 (1/0.12)	1200[*]	1.92
Chitosan II	1 2		765[**]	0.33
CC12	1 2	1/0.05 (1/0.04)	1990[**]	1.52

[*] : solvent AcOH 0.3M / AcONa 0.05M, T = 20°C
[**] : chlorhydrate form, solvent water, T = 5°C

Characterization of the alkyl carboxymethylchitin

The molecular weight of the investigated fraction of CMCh has been determined by the diffusion-sedimentation method and it was found M_w equal to 140000 g/mol. The total degree of carboxylation is 1.1, and it is demonstrated that 90% of carboxymethyl substituents are located at C-6 atoms of monomeric units [7]. The carboxylated polymer was then alkylated with C_{12} or C_{14} aldehyde through the reductive amination reaction. The degree of alkylation, determined by [1]H n.m.r. was τ=0.016 with C_{12} chain and τ=0.044 with C_{14} chain.

Properties

Rheology

The hydrophilic-hydrophobic balance will influence the properties of such chemicals. It may be adjusted by modifying the hydrophobic character of the product through the length and the number of grafted alkyl chains or the hydrophilic character through the ionization of the amino group (modification of the pH) or the content in ionic groups such as carboxylic groups in CMCh. We have demonstrated that a minimum of six C atoms is necessary on the alkyl chain for observing a hydrophobic effect (gelation, increase in viscosity...) [6].

The role of hydrophobic interactions on the viscosity of solution appears even in dilute solutions (table 1). Even the intrinsic viscosity of alkylated chitosans increases slightly

compared with the original polymer; in the same time, when the reduced viscosity is plotted against the polymer concentration, the Huggins constant (k') obtained is larger than 1.5 indicating the presence of interactions even at low polymer concentration. This is confirmed when the specific viscosity is plotted versus the overlap parameter (c[η]) (figure 1). There is only one curve for non modified chitosans and another one for all the modified polymers. In the latter case this is not a straigth line meaning the large increase in k' values. This divergence appears for low values of the overlap parameter (around 0.07). This indicates the presence of intermolecular interactions even at low concentrations.

Figure 1 : Influence of the overlap parameter on the specific viscosity

For larger polymer concentrations the roles of the polymer concentration, of the degree of alkylation and of the length of the alkyl chains are clearly demonstrated [8]. An example is given in figure 2a for CC12. This increase in the viscosity clearly indicates interchain interaction up to a gel-like behaviour previously demonstrated by dynamic rheological measurements [6]. The hydrophobic nature of these interactions is confirmed from fluorescence spectroscopy experiments (figure 2b). The two series of data point out the existence of a critical concentration $C_{crit} \sim 0.2$ g/L (lower than the overlap concentration c^*) over which viscosity increases rapidly and the fluorescence indicates that hydrophobic domains are formed where the pyrene is located; this effect is specific of the presence of the alkyl chains. The junction points for the 3D-network formation are constituted of alkyl aggregates looking like a micellisation. These interactions were demonstrated as promoted by increase of the temperature [9] or

addition of external salt [10]. Especially in the presence of external salt, it is well known that for polyelectrolytes the viscosity decreases due to the screening of electrostatic repulsions; it is the situation of protonated chitosan.
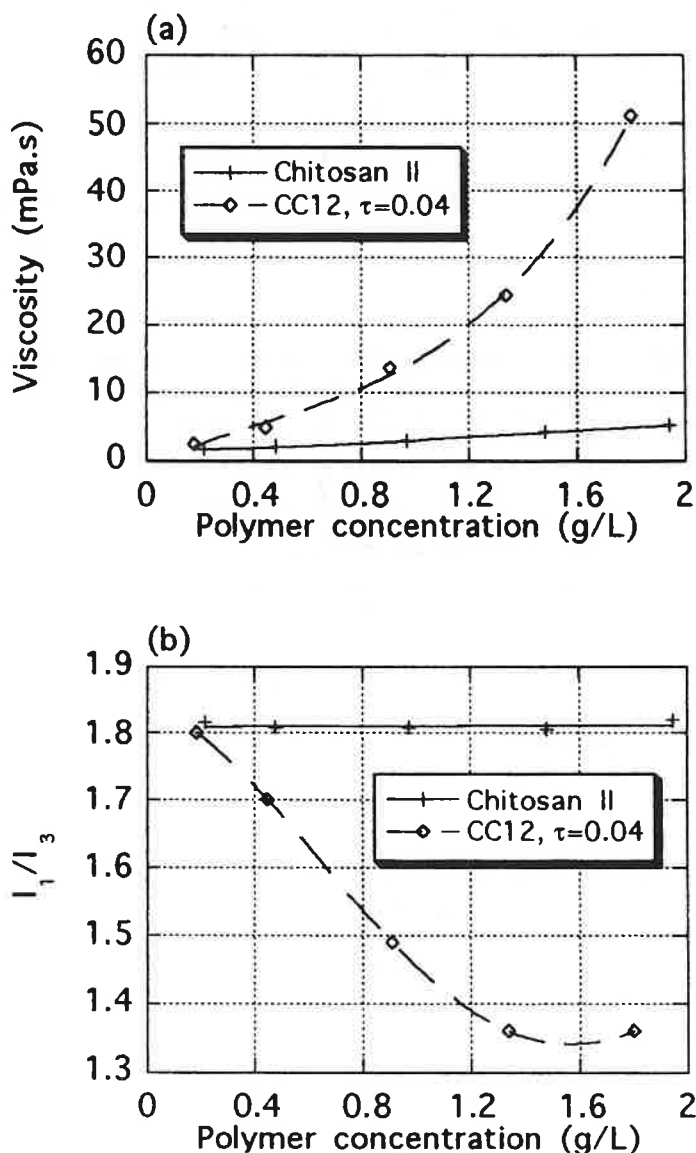

Figure 2 : Influence of polymer concentration on the viscosity (a) and the fluorescence (b) of chitosan solutions (chlorhydrate form in water, T = 5°C, [pyrene] = 10^{-7} M) (reference 8)

In the presence of external salt due to the decrease of the electrostatic repulsions and the enhancement of the hydrophobic interactions, the gel-like properties are reinforced [6].

Phase diagram

The degree of charge was decreased by controlled addition of NaOH; then the roles of the fraction of net charge α and of the temperature were investigated (figure 3). This allows to modulate the electrostatic - hydrophobic balance. When the charge is decreased at low temperature, the viscosity increases when $\alpha < 0.5$ and the hydrophobic attractions dominate; the viscosity passes through a maximum and then decreases to phase separation when $\alpha \sim 0.1$-0.2 for CC12 polymer. When temperature increases, the hydrophobic attractions are reinforced, the viscosity is less affected and phase separation occurs more and more rapidly; a more tightly interacting system is formed. From these data, one deduces the phase diagram for one of our polymer (CC12) at 0.9 g/L (figure 4). This curve is largely dependent on the polymer concentration and on the substitution.
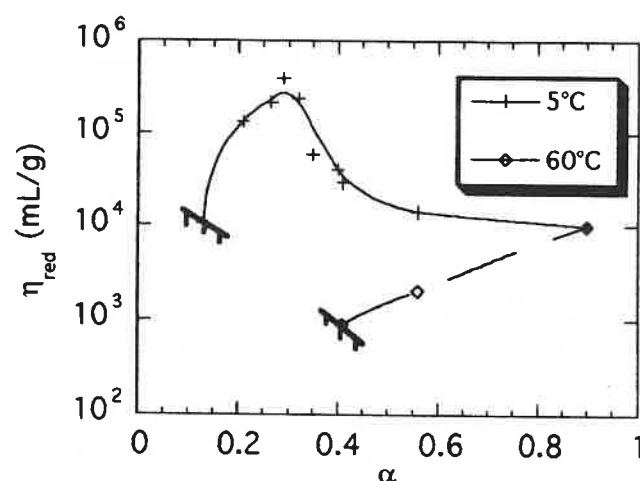
Figure 3 : Influence of charge density and temperature on reduced viscosity of CC12 solutions (chlorhydrate form in water, $\tau = 0.04$, c = 0.9 g/L)

Surface active properties of alkylated CMChitins

Carboxymethylchitin has no tensioactive properties as well as cationic polyelectrolyte chitosan (figure 5). This corresponds to the known property of weakly hydrophobic polyelectrolytes to manifest poor adsorption activity at both oil-water and air-water interfaces at relatively high degree of ionization [11]. The alkylation of CMCh improves the adsorption properties and the larger effect is observed when the alkyl chain is longer [7]. This is coherent with the fact that with increasing the hydrophobicity of the polysoaps their adsorption at the air-water interface is expected to increase.
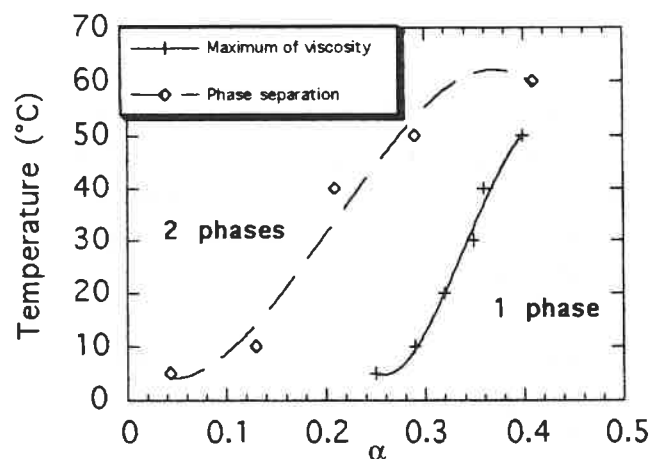
Figure 4 : Phase diagram of CC12 alkylchitosan
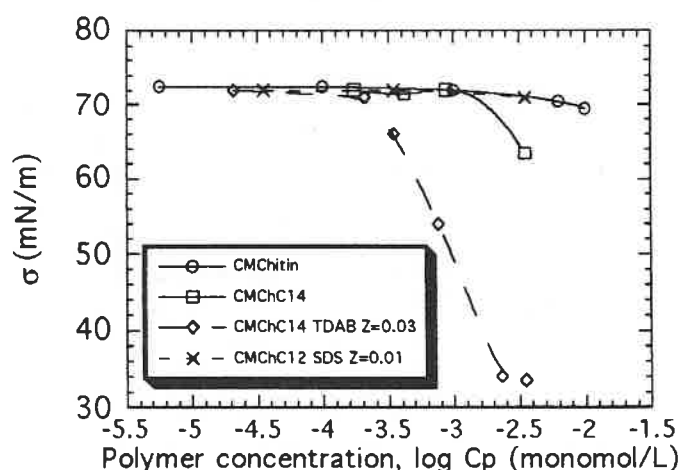(chlorhydrate form in water, $\tau = 0.04$, c = 0.9 g/L) (reference 8)

Figure 5 : Surface activity of hydrophobic derivatives of chitin and complexes with surfactants

The surface activity of polyelectrolytes may also be increased by the formation of dynamic association (called SPEC) with the oppositely charged surfactants. It may be demonstrated [7] that from the point of view of their effect on the surface activity the SPECs reveal to be much more efficient that the alkylated CMCh. There is a breakthrough at a concentration similar to a critical aggregation concentration which is almost 100 times smaller than the critical micellar concentration (CMC) of the pure surfactant (when the surfactant concentration $C_S = Z \, C_p$ is considered, Z being the stoechiometric ratio), giving a much better efficiency. The same type of results is obtained when the alkylchitosan is mixed with an anionic surfactant. This allows to define a large range of polymeric surfactants by modifying the length of the alkyl chain

346

and/or the Z ratio. When the carboxymethylchitin derivative is mixed with surfactants having the same ionic charge, one can conclude that hydrophobic interactions occur increasing the net charge and then the solubility.

Conclusion

In this work chemical modifications of chitin or chitosan to reinforce their hydrophobic character are presented and examples of their original properties are discussed. In particular, the viscosity of aqueous solutions of such hydrophobic derivatives increases slighthly when the polymer concentration is larger than a threshold value but hydrophobic interactions exist already at low concentration. From the influence of parameters such as the temperature, the ionic charge... the phase diagram of these derivatives is determined. Finally surface activity is demonstrated, their efficiency being improved by the formation of surfactant-polyelectrolyte complex (SPEC) of opposite charge.

References

[1] Rinaudo, R., Le Dung, P., Gey, C., Milas, M. Int. J. Biol. Macromol. 1992; 14; 121.

[2] Vikhoreva, G.A., Gladisev, D.Y., Batz, M.R., Barkov, V.V., Gal'Braich, L.S. Cellulose Chem. and Technol. 1992; 26; 663.

[3] Desbrieres, J., Rinaudo, M., Babak, V., Vikhoreva, G. Polymer Bull. 1997; 39; 209.

[4] Yalpani, M., Hall, L.D. Macromolecules 1984; 17; 272.

[5] Le Dung, P., Milas, M., Rinaudo, M., Desbrieres, J. Carbohydr. Polym. 1994; 24; 209.

[6] Desbrieres, J., Martinez, C., Rinaudo, M. Int. J. Biolog. Macromol. 1996; 19; 21.

[7] Babak, V., Lukina, I., Vikhoreva, G., Desbrieres, J., Rinaudo, M. 213th ACS National Meeting, San Francisco (USA), April 1997

[8] Desbrieres, J., Rinaudo, M., Chtcheglova, L. Macromol. Symp. 1997; 113; 135.

[9] Ben Aim, A "Hydrophobic Interactions", Plenum Press, New York, 1980.

[10] Barley, F.E., Callard, R.W. J. Appl. Polym. Sci. 1959; 1; 56.

[11] Ishimuro, Y., Ueberreiter, K. Colloid Polym. Sci. 1980; 258; 928.

THE MODIFICATION OF CHITOSAN IN VIEW OF ELABORATING NEW POLYMER ELECTROLYTES. 2. POLYETHER-BASED NETWORKS USING OXIPROPYLATED CHITOSAN

Patricia VELAZQUEZ-MORALES, Jean-François LE NEST and Alessandro GANDINI

Ecole Française de Papeterie et des Industries Graphiques (INPG) ,
BP 65, 38402 St. Martin d'Hères, France

Abstract

In order to improve the film-forming properties of polymer electrolytes based on polyether networks, we have been studying the chemical incorporation of polysaccharidic structures into these materials. After an investigation dealing with cellulosics, we recently switched to using chitosan. Reactions of model compounds with isocyanates were successful, but chitosan itself proved intractable. This communication describes first the synthesis and characterization of oxipropylated chitosans and then their reaction with oligoether-based di-isocyanate developed in our laboratory. This double operation enabled the synthesis of crosslinked materials made up of polysaccharide backbones bridged by PEO chain and bearing also PPO branches. These networks were fully characterized and then loaded with lithium perchlorate to test their ionic conductivity, which was indeed comparable to those measured on more conventional networks prepared in our previous studies. The major conclusion of this study is that one can indeed prepare thin films with chitosan-based polyether networks and maintain at the same time all the optimized properties of the best polymer electrolytes, including good mechanical performances and a high ionic conductivity.

Keywords: Polymer electrolytes, oxipropylated chitosan, oligomeric isocyanates, condensation reactions, polyether networks, ionic conductivity.

Introduction

Among the various structures based on polyethers which have been tested in the last couple of decades in order to prepare polymer electrolytes with a good overall performances [1,2], we have chosen to study crosslinked topologies [3]. This deliberate strategy is justified because creep problems are eliminated and, if the length of the polyether chains in the network is kept reasonably low, cristallization is minimized.

The study of these networks with and without an added salt (mostly lithium salts of strong acids) using a variety of probing techniques has allowed a considerable insight into their fundamental properties and a unified theory based on free-volume considerations has been put forward to rationalize their physico-chemical, mechanical and electrochemical features [3-5].

Having reached what we consider as being a good achievement in terms of stable and highly conductive polymer electrolyte, it became interesting to move onto a more applied problem, viz. the possibility of improving the film-forming properties of these networks in order to optimize the elaboration of thin devices (battery, electrochromic

windows,...). The idea was of course to keep the good overall performance already attained and add the new capability of processing the material into films as thin as possible. Some polysaccharides and their synthetic derivatives are well known for their film-forming aptitude and we moved therefore in that direction. The first investigation was conducted with cellulosic ethers which were branched and crosslinked with PEO or PPO chains using condensation reactions based on urethane chemistry [3, 6-8]. Results were satisfactory because we were able to conserve the good mechanical and electrical properties while improving the possibility of casting thin films.

Having proved the viability of this new approach, we decided to explore another family of polysaccharides, namely chitin derivatives because it was thought that the exceptional film-forming properties of polymers like chitosan could improve further this aspect of our search. Reactions of model compounds with isocyanates were successful, but when we switched to chitosan, we discovered that unfortunately the high cohesive energy of this type of polymer did not allow the diffusion of the isocyanate reagent inside its particules, no matter what medium we employed. Therefore only the surface of the chitosan powder was modified, but the resulting materials remained insoluble in all aprotic solvents [9].

The chitosan was therefore modified with propylene oxide in order to disrupt the high structural regularity and the ensuing high-energy interchain hydrogen bonding and thus produce a less cohesive material still possessing its potential reactivity through the chain-extended OH groups. The purpose of this communication is to describe the details of this study and the elaboration and characterization of a new polymer electrolyte based on modified chitosan branched and crosslinked with short polyether chains.

Experimental

Two commercial samples of chitosan (from *Aber Technologies* and *France Chitine*) were used in this work. Their molecular weights were 80000 and 200000 with 65 and 91% degree of deacetylation, respectively. Propylene oxide was a high-purity commercial sample. PEO-based di-oligoisocyanate (PEODI, Mn=650) was synthesized from the corresponding commercial Jeffamine (O,O'-bis(2-aminopropyl)polyethylene glycol 500), following our previously developed procedure [5,7,10].

The oxipropylation reactions were carried out in bulk with KOH as catalyst using a stainless steel autoclave. Pressures reached about 10 bars and temperatures varied from 60 to 145°C. Reactions were followed by the pressure drop and stopped when all the propylene oxide had reacted, which required from a few hours to a few days depending on the operating conditions. The condensation reactions of the oxipropylated chitosans with the oligoether isocyanates were made in THF and DMAc solutions at room temperature in the presence of traces of dibutyltin dilaurate as catalyst. Membranes were obtained by pouring the viscous solution in a mould immediately after mixing the reagents. After crosslinking, the materials were thoroughly dried and extracted with CH_2Cl_2 to determine the sol/gel ratio. The introduction of lithium salt $(LiClO_4)$ in the membranes was conducted by adding it to the solution of oxipropylated chitosan before crosslinking and the concentration is given per volume the polyether isocyanate.

The materials obtained at the various stages of this study were characterized by FTIR, thermal analysis, ionic conductivity (using the complex impedance technique) and dynamic-mechanical analysis.

Results and Discussion

Oxipropylation Reactions

The product of this system was always a mixture of chain-extended chitosan and low-DP homopolymer of propylene oxide. The initial composition of the reaction mixture was varied in order to optimize the amount of the former product. Thus, when the proportion of chitosan was increased from 10 to 30% w/w, the amount of homoPPO decreased from 90 to 30%. A further increase in the relative amount of chitosan led to excessive viscosities and impractical conditions. Moreover, the higher the chitosan content, the more difficult became the temperature control and above 100°C degradation problems occurred. The other parameter which played an important role was the type and quantity of catalyst. It was found that a strong base is necessary to catalyze the reaction at a reasonable rate as shown by the fact that KOH worked adequately, whereas triethylamine and 1,4,-diazobicyclo[2.2.2]octane gave very sluggish reactions. However, KOH was also found to degrade chitosan if used in too high concentrations under the conditions of the oxipropylation. The optimum amount of this catalyst was therefore established at 5% w/w to chitosan.

The reaction products, consisting of grafted chitosan and homoPPO, were stirred into an excess of ether for 24 h at room temperature in order to separate the two components, since low-DP PPO is soluble in this solvent. The modified chitosans isolated in this way were characterized by FTIR spectroscopy and thermal analysis. In the FTIR spectrum the relevant feature is the fact that the relative intensity of the bands just below 3000 cm^{-1} (aliphatic CH, CH$_2$ and CH$_3$) increased very considerably after the reaction, proving that propylene oxide chains had been grafted onto the polysaccharide. Of course, the most important evidence that chain extension had indeed occurred in these reactions was the very fact that the product which was insoluble in ether (removal of homoPPO) was a paste-like mass soluble in numerous common solvents like THF, methylene chloride, water and acetone. This clearly pointed to the formation of a much less organized polymer than the starting material, which was totally insoluble in the above solvents.

The DSC tracings of these grafted chitosans showed glass transition temperatures going from -40 to -10°C (the Tg of amorphous PPO is around -70°C) as a function of the composition, namely the higher the extent of grafting, the lower the Tg. The role of these short propylene oxide chains is therefore to induce the plasticization of chitosan.
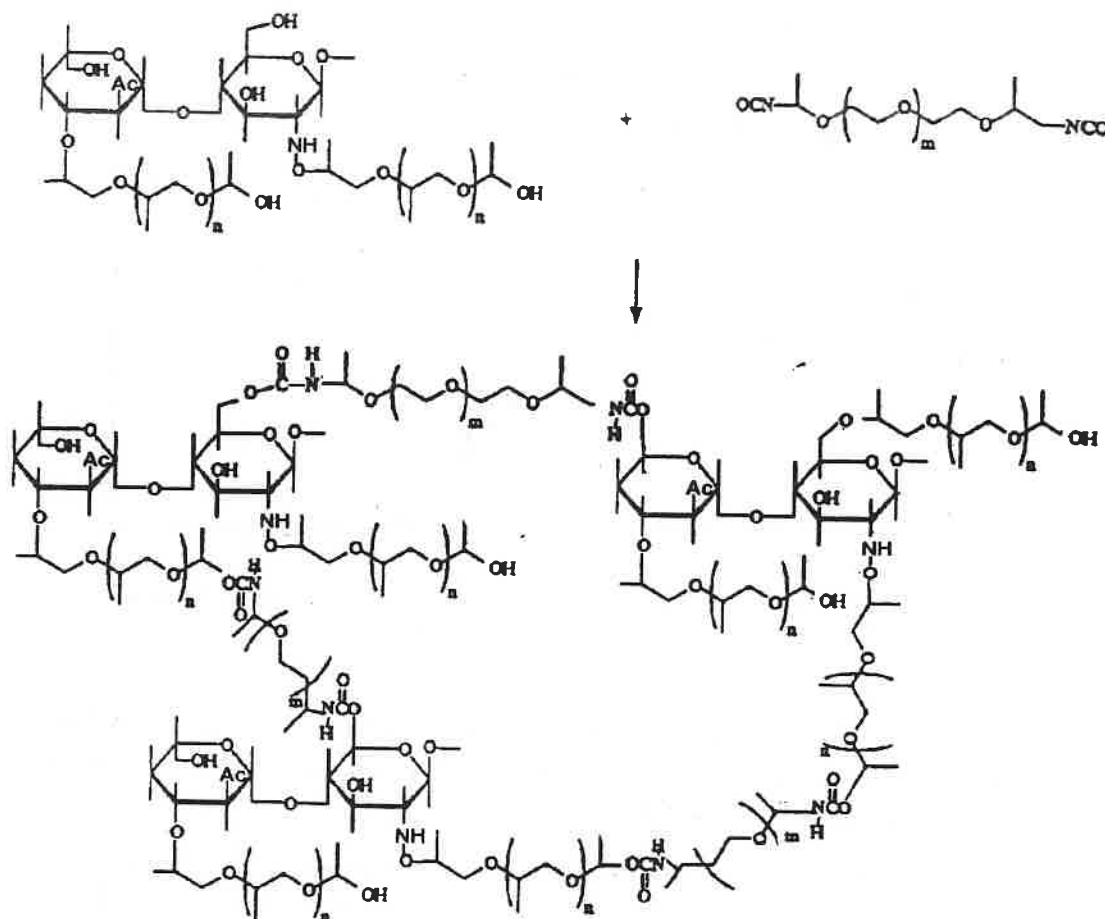
A quantitative analysis of the grafted chitosans without homoPPO, based on the measurement of the hydroxy content and the mass gain, indicated that the molar substitution, viz. the average number of propylene oxide units attached to each saccharidic moiety, varied between 1 and 6 depending on the operating conditions.

It can be concluded that oxiranes react readily with chitosans under the effect of a strong base at relatively low temperatures, giving viscous polyols soluble in many media and therefore open to further chemical modification.

Crosslinking Reactions

Condensing the oxipropylated chitosans with the oligoethylene oxide diisocyanate (PEODI) resulted in the corresponding polyeyher/polysaccharide networks. When the mixture of oxipropylated chitosan and homoPPO was used for these reactions, we obtained a high proportion of soluble product, which was essentially a linear polyurethane formed by

350

the reaction of PEODI with the homoPPO. When we isolated the oxipropylated chitosan, its condensation with PEODI gave a much lower proportion of soluble product. Clearly the separation based on the selective dissolution in ether is not quantitative and some homoPPO always remained incorporated into the modified chitosan. However, membranes prepared under conditions close to the stoichiometric situation, viz. [NCO]/[OH]=1, gave less than 30% of sol, after soxhlet extraction with CH_2Cl_2. The following reaction scheme illustrates the formation of the crosslinked structure :

These networks exhibited Tg values around -45°C with sharper transitions than those of their oxipropylated precursors. These values of Tgs reflect two opposite trends introduced by the crosslinking reaction, viz. (i) an increase in segmental mobility introduced by the presence of the oligoethylene oxide chains and (ii) a stiffening effect introduced by the crosslink moieties. However, -45°C is quite a viable glass transition temperature for the use of these materials as polymer electrolytes. The networks were stable up to 210°C (TGA).

Addition of $LiClO_4$ into these membranes provided the first polymer electrolytes of this new family. The increase in salt concentration translated into an elevation of the glass transition temperatures because of the strong interactions between the Li^+ cations and the oxygen atoms of the polyether chains.

The classical behaviour of the dynamic mechanical properties of these elastomeric networks can be seen in Figure 1, namely the drop of modulus associated with the glass transition and the convergence of the values of the modulus on the rubbery plateau. Addition of the lithium salts provoked an displacement in the glass transition (increase of Tg). The free-volume treatment of the data through master curves (Figure 2) provided very satisfactory values for the $C_1(Tg)$ and $C_2(Tg)$ constants of the WLF equation, viz. 15 and 42 K, respectively.

The ionic conductivity of these new polimeric electrolytes was measured as a function of temperatures and $LiClO_4$ concentration. The value obtained at room temperature was about 3×10^{-5} S/cm, which is close to the optimum conductivities displayed by optimized PEO-based networks with salt. Figure 3 shows Arrhenius-type plots for different salt concentrations. The typical non-linearity stems from the well known feature attributing the ionic mobility to segmental motions, viz. a free volume behaviour. The corresponding treatment of the conductivity data according to WLF equation provided $C_1(Tg)$ and $C_2(Tg)$ values of 7 and 33 K, respectively.
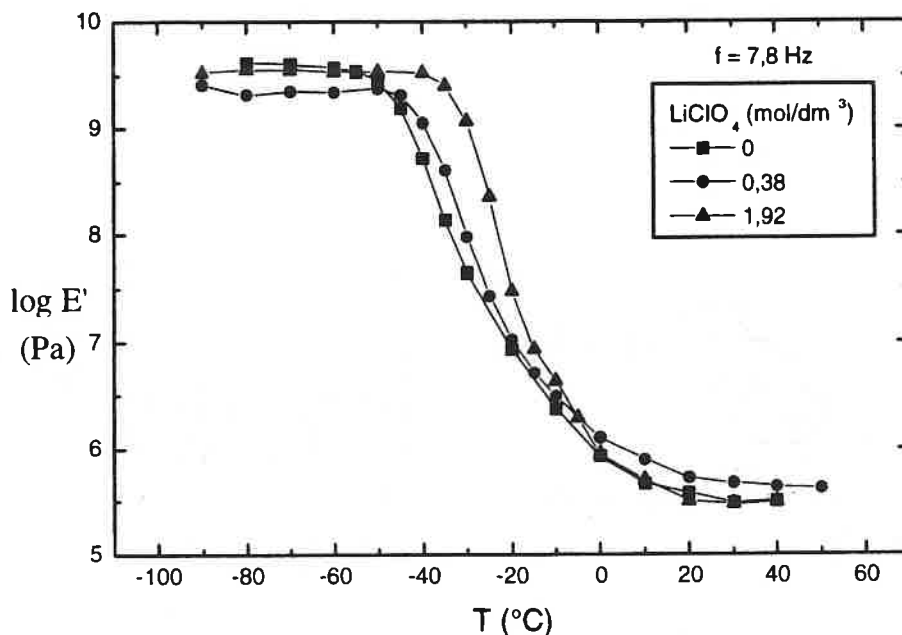

Figure 1. Variation of the elastic modulus as a function of temperature at 7.8 Hz for oxipropylated chitosan + PEODI networks ([NCO]/[OH]=1) containing increasing amounts of $LiClO_4$.
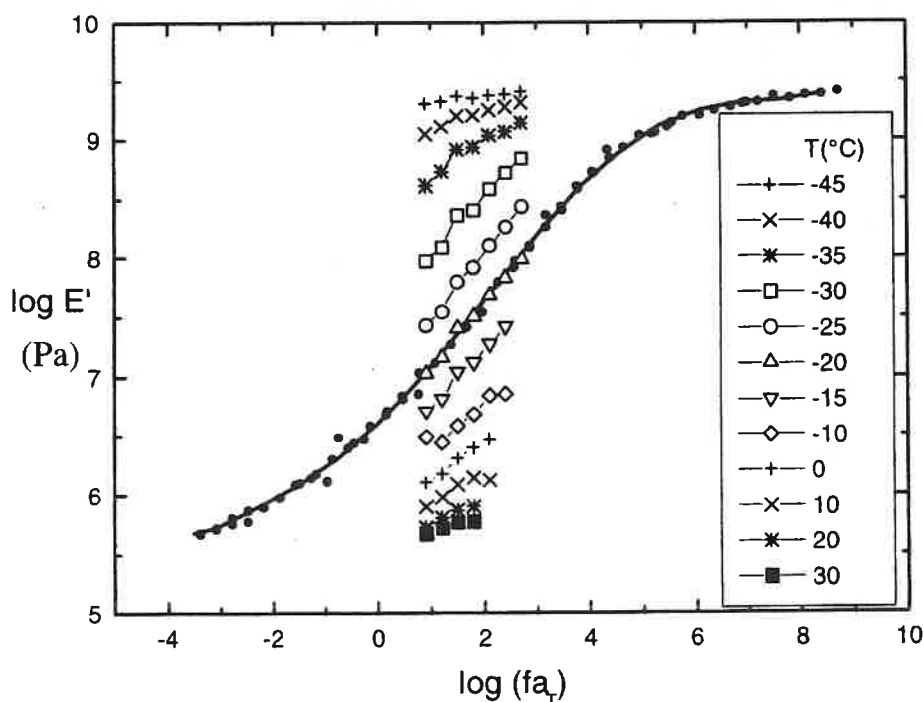

Figure 2. Master curve based on the WLF equation for an oxipropylated chitosan + PEODI network ([NCO]/[OH]=1) containing [LiClO₄]=0,38 mol/dm³.
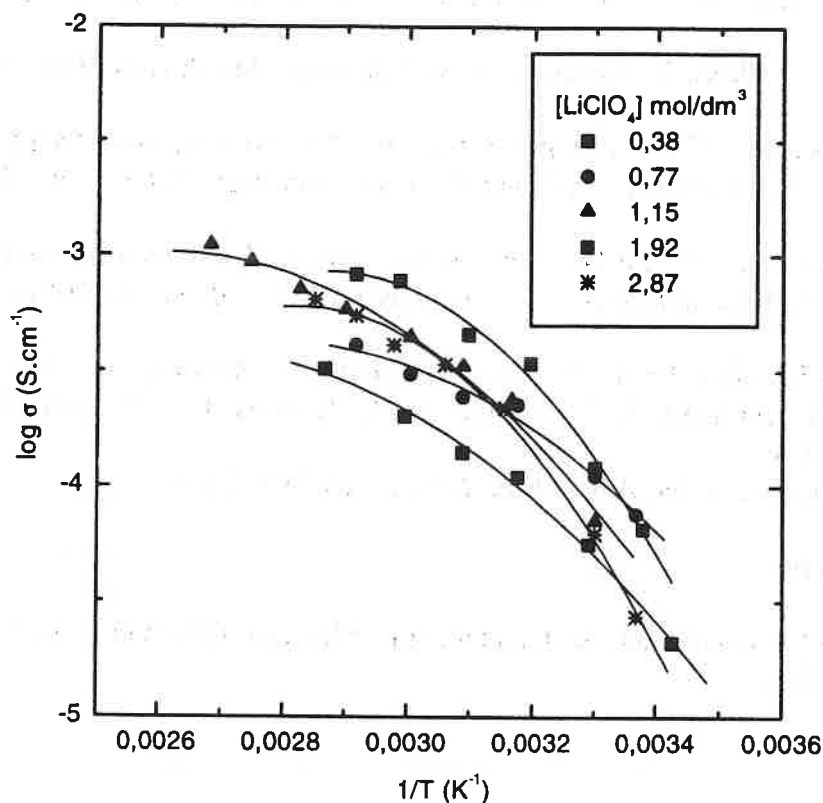
Figure 3. Arrhenius-type plots of the ionic conductivity of oxipropylated chitosan + PEODI networks ([NCO]/[OH]=1) containing increasing amounts of $LiClO_4$.

Conclusion

This study has opened various possibilities of obtaining crosslinked and branched polymer electrolytes based on polyethers and chitosan. In particular two approaches seem interesting: (i) the use of chitosans as starting materials for grafting with propylene oxide and the subsequent branching and crosslinking with the macroisocyanates. (ii) The addition of salts into the networks obtained with the oxipropylated chitosan and the oligoether isocyanates represented a new family of polymer electrolytes with the goods conductivity and mechanical properties.

We remarked that the presence of the chitosan backbone gave a very good film-forming aptitude to these materials, obviously much better than with PEO-based networks, but also better than those elaborated from cellulosic ethers.

References

1. *Second International Symposium on Polymer Electrolytes* (Scrosati, B., ed) Elsevier (1990).

2. *Electrochim. Acta, Special Issue Devoted to the Third International Symposium On Polymer Electrolytes,* **37**, 1469-1745 (1992).

3. J.F. Le Nest, A. Gandini and C. Schoenenberger, *Trends Polym. Sci.* **2**, 432 (1994).

4. Le Nest, J.F., Gandini, A. and Cheradame, H., *Br. Polym. J.*, **20**, 253 and refs. therein (1988).

5. Le Nest, J.F., Callens, S., Gandini, A. and Armand, M., *Electrochim. Acta*, **37**, 1585 (1992).

6. C. Schoenenberger, J.F. Le Nest and A. Gandini, *Electrochim. Acta*, **13-14**, 2281 (1995).

7. J.F. Le Nest, A. Gandini, L., XU and C. Schoenenberger, *Polym. Adv. Technol.*, **4**, 92 (1993).

8. C. Schoenenberger, J.F. Le Nest and A. Gandini, in *The chemistry and processing of wood and plant fibrous materials*, (J.F. Kennedy Ed.), Woodhead Publishess, London, 377 (1995).

9. P. Velazquez-Morales, J.F. Le Nest and A. Gandini, in *Advances in Chitin Science*, Vol. 1 (A. Domard, C. Jeuniaux, R. Muzzarelli and G. Roberts, Eds) J. André Publisher Lyon France, 236 (1996).

10. H. Eckert and B. Forster, *Ang. Chem. Int. Ed.*, **26**, 894 (1987).

Acknowledgments