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Preparation of antibacterial chito-oligosaccharide by altering the degree of deacetylation of β-chitosan in a *Trichoderma harzianum* chitinase-hydrolysing process

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Abstract

BACKGROUND: Chito-oligosaccharide (COS) is generally known to possess many specific biological functions, especially antibacterial activity, depending on its size. To prepare a specific size range of COS, however, has proved difficult. The aim of this study was to establish a method for preparing a specific size range of antibacterially active COS by adjusting the degree of deacetylation (DD) of β-chitosan in a *Trichoderma harzianum* chitinase-hydrolysing process.

RESULTS: The molecular weight spectrum, elucidated by viscosity-average molecular weight, high-performance liquid chromatography and thin layer chromatography, of COS in chitosan hydrolysate was significantly related to the DD of its original chitosan. Compared with the original form, COS produced at 90% DD showed superior activity against most Gram-negative bacteria tested, with a minimum inhibition concentration (MIC) ranging from 55 ± 27 to $200 \pm 122\ \mu g\ mL^{-1}$. Conversely, most Gram-positive strains tested were less sensitive to COS (MIC $>880 \pm 438\ \mu g\ mL^{-1}$) than to its original form. Among the Gram-positive strains, *Staphylococcus xylosus* was the only exception in that it showed a high susceptibility to COS and had an MIC as low as $45 \pm 11\ \mu g\ mL^{-1}$.

CONCLUSION: The results indicate that the production of a specific size range of COS product is possible by altering the DD of chitosan in the chitinase-catalysed process. To produce various sizes of COS for versatile biological functions, as seen in this study to inhibit various types of bacteria, is made possible in this established process.
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Keywords: chito-oligosaccharide; chitinase; antibacterial activity; *Trichoderma harzianum*

INTRODUCTION

Chitosan, a partially N-deacetylated derivative of chitin, has attracted much attention owing to its broad range of applications and has been used in the food, agricultural, environmental protection, pharmaceutical and biomedical industries. The activity of chitosan and its derivatives against several species of bacteria is the primary utility in biological applications.[1] In addition to its antibacterial activity, chitosan as a dressing for wounds has proved to be effective in preventing bleeding and in the healing process.[2] Chitin, the raw material of chitosan, is composed of *N*-acetyl-D-glucosamine and exists in several forms depending on its crystalline structure. Two of these crystalline polymorphic forms are α-chitin and β-chitin. Commonly extracted from shrimp or crab shells, α-chitin has an antiparallel structure with strong, intermolecular hydrogen bonding. Principally obtained from squid or loligo pens, β-chitin has a parallel arrangement of fibres and exhibits weaker intermolecular hydrogen bonding.[3]

α-Chitin is the major form of chitin, and hence studies on chitin have been mostly carried out with this form. Actually, however, β-chitin exhibits higher chemical reactivity than α-chitin. It has been shown to be more easily de-ashed as well as less deacetylated after a simple two-step alkaline treatment. This is due to the low content of inorganic compounds in squid pens.[4] In addition, its easy manipulation makes it a potential candidate for various applications, particularly in the biomedical field.

Regardless of the source of chitosan, its antibacterial activity can be influenced by a number of factors, including the degree of polymerisation,[5–8] the species of micro-organism[9–11] and the degree of deacetylation (DD) that, when raised, will increase antibacterial activity.[12] Moreover, it has been established that enzymatically hydrolysed chitosan with a molecular weight between 5 and 27 kDa has effective antibacterial activity owing to its better solubility compared with its unaltered form.[9] Good solubility is of the greatest concern, since low solubility will limit chitosan's applications. For example, chitosans with molecular

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weights greater than 30 kDa cannot be used as antibacterial agents owing to their poor solubility in aqueous solutions at neutral pH.[13] However, partially hydrolysed chitosan was found to have a better antibacterial effect than chitosan submitted to extended hydrolysis.[11] In regard to specific species of bacteria, it has been shown that Gram-negative bacteria are more susceptible to chitosan than Gram-positive bacteria owing to the higher negatively charged surface of the cell wall.[14]

In order to break down the highly polymerised molecules of chitosan in creating chito-oligosaccharide (COS), generally considered with a viscosity-average molecular weight (M_V) of less than about 10 kDa,[1] both an acid hydrolysis process[15] and an enzymatic process[16] have been widely adopted. Acid hydrolysis usually results in a larger amount of monomeric D-glucosamine units instead of the desired size of COS.[11] The production of COS with an acceptable degree of polymerisation, however, was made possible by selectively breaking down suitably N-acetylated chitosan on an acetylated site with chitinases.[17] Chitinases have been used extensively for the purpose of generating fungal protoplasts[18] and are an attractive alternative to fungicides and insecticides as environmentally safe biocontrol agents in the inhibition of phytopathogens.[19,20]

This study set out to evaluate α-chitosan and β-chitosan at various DDs for their properties as substrates in the production of chitinase-catalysed hydrolysate. It was hoped to find a means to consistently create COS in the desired size range from both α-chitosan and, more importantly, since it has such untapped potential, β-chitosan. Further, the antibacterial effectiveness of α-chitosan, β-chitosan and their respective derived COS was determined against various food-spoiling bacteria and pathogens.

MATERIALS AND METHODS

Materials

α-Chitin was purchased from Fluka (Buchs, Switzerland) and β-chitin was prepared according to the method of Galed *et al*.[21] *N*-Acetyl-D-glucosamine (NAG), tri-*N*-acetylchitotriose (NAG_3) and hexa-*N*-acetyl-chitohexaose (NAG_6) were obtained from Sigma (St Louis, MO, USA) and chitosan dimer to chitosan hexamer from Seikagaku (Tokyo, Japan). The thin layer chromatography plate (DC-Fertigplatten DURASIL-25UV_{254}) was purchased from Macherey-Nagel (Düren, Germany). Crude chitinase enzyme was prepared by inducing *Trichoderma harzianum* (BCRC 30 821) with a chitin-containing medium. This research also made use of Difco culture media (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Chemicals and solvents were all of analytical grade and purchased from Merck (Whitehouse Station, NJ, USA). The potassium bromide used for Fourier transform infrared (FTIR) measurement was of Spectrosol grade.

Micro-organisms

The response of 16 bacteria to the antibacterial activity of enzymatically prepared chitosan was tested. The Gram-positive strains included *Listeria monocytogenes* (BCRC 14 845), *Staphylococcus aureus* (BCRC 10 780), *Staphylococcus xylosus* (BCRC 12 930), *Streptococcus agalactiae* (BCRC 10 787), *Streptococcus bovis* (BCRC 14 729) and *Streptococcus pyogenes* (BCRC 14 758). The Gram-negative strains included *Alcaligenes faecalis* (BCRC 10 828), *Enterobacter aerogenes* (BCRC 10 370), *Enterobacter cloacae* (BCRC 10 401), *Escherichia coli* (BCRC 10 675), *Klebsiella oxytoca* (BCRC 13 985), *Pseudomonas aeruginosa* (BCRC 10 944), *Vibrio alginolyticus* (BCRC 12 829), *Vibrio harveyi* (BCRC 12 907), *Vibrio parahaemolyticus* (BCRC 10 806) and *Yersinia enterocolitica* (BCRC 13 999).

Culture of micro-organisms

Each micro-organism was cultured in an appropriate medium. *Staphylococcus aureus* and *S. xylosus* were cultured in tryptic soy agar (TSA), *V. alginolyticus* and *V. parahaemolyticus* in TSA containing 25 g L^{-1} NaCl, *S. agalactiae* and *S. pyogenes* in TSA containing 50 g L^{-1} defibrinated sheep blood, *S. bovis* and *L. monocytogenes* in brain heart infusion agar (BHIA) and *E. coli, K. oxytoca, P. aeruginosa* and *A. faecalis* in nutrient agar (NA) at 37 °C. *Enterobacter cloacae* and *E. aerogenes* were cultured in NA and *Y. enterocolitica* in BHIA at 30 °C. *Vibrio harveyi* was cultured in TSA containing 25 g L^{-1} NaCl at 25 °C.

Preparation and analysis of chitosan

Chitin was deacetylated as described by Chang *et al*.[22] with slight modification. Briefly, 3 g of pulverised chitin was added to 75 mL of 350 g L^{-1} NaOH and boiled in an oil bath at 140 °C. Partially deacetylated chitosan samples were taken after 1, 3 and 6 h of treatment, designated as $C_{\alpha n}$ and $C_{\beta n}$ ($n = 0$, 1, 3 and 6), and washed sequentially with distilled water to remove NaOH until pH 7 was reached. The washed samples were dried in an oven at 50 °C for 24 h. The DD of chitosan was determined by the FTIR spectral method[23] and a colloid titration method.[24]

The M_V of COS was determined based on its intrinsic viscosity, obtained in 0.25 mol L^{-1} acetic acid/0.25 mol L^{-1} sodium acetate at 25 °C, according to the Mark–Houwink–Sakurada (MHS) equation:[25] $[\eta] = 1.49 \times 10^{-4} M_V^{0.79}$.

Preparation of crude chitinase enzyme complex

Chitinase was prepared according to Felse and Panda.[26] The seed culture medium contained 10 g dextrose, 4.2 g $(NH_4)_2SO_4$, 6.9 g NaH_2PO_4, 2 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 1 g peptone, 10.5 g citric acid monohydrate and 0.3 g urea L^{-1}. The medium was inoculated with *T. harzianum* spores (~5×10^5 spores mL^{-1}) from a 120-h-old slant. The chitinase induction medium contained 10 g colloidal chitin,[27] 4.2 g $(NH_4)_2SO_4$, 6.9 g NaH_2PO_4, 2 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.2 g Tween 80, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.0016 g $MnSO_4$, 0.0014 g $ZnSO_4$ and 0.002 g $CaCl_2 \cdot 2H_2O$ (pH 5). The induction medium was inoculated with 50 mL L^{-1} 48-h-old seed culture and cultured for 5 days at 30 °C to obtain an optimal yield of chitinases. The enzyme activity of colloidal α-chitin-induced crude chitinases was assessed by the 3,5-dinitrosalicylic acid (DNS) method to determine the amount of reducing sugar. The chitinolytic activity was determined by mixing 10 g L^{-1} colloidal α-chitin solution (pH 4) with crude chitinases at 42 °C for 1 h. The enzymes were then inactivated by heating at 100 °C for 10 min. The optical density of the supernatant at 590 nm (OD_{590}) was determined after centrifugation of the reaction mixture. One unit (1 U) of enzyme activity represented 1 µmol equivalent of NAG produced h^{-1}.

Preparation and analysis of enzymatic chitosan hydrolysate

A 10 mL aliquot of 11 g L^{-1} chitosan dissolved in 100 mmol L^{-1} sodium acetate buffer (pH 4) was mixed with 1 mL of crude chitinases (1 U mL^{-1}) and incubated at 42 °C. A 0.5 mL aliquot of the mixture was withdrawn at the required time, designated as H_m (m = hydrolysing time), and boiled for 10 min to stop the enzymatic reaction. Chitosan hydrolysate samples were kept at −20 °C until use.

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Size exclusion column high-performance liquid chromatography (SEC-HPLC)

The molecular weight profile of hydrolysate samples was determined using a TSKgel G3000PW SEC (7.5 mm × 300 mm; TOSOH Co., Tokyo, Japan). The HPLC system consisted of an isocratic pump (Spectra Series P100, Thermo Quest, San Jose, CA, USA) and a differential refractive index detector (Shodex RI-101, Showa Denko Co., Tokyo, Japan). The mobile phase was 100 mmol L^{-1} sodium acetate buffer (pH 4) at a flow rate of 1 mL min^{-1}. NAG_3, NAG_6 and 10 kDa dextran were used as standard markers.

Thin layer chromatography (TLC) analysis

TLC was performed according to Thamthiankul *et al.*[28] and Yoon *et al.*[29] with some modification. Enzymatic hydrolysates were spotted on a silica plate and developed with a solvent system comprising *n*-propanol and 300 g L^{-1} ammonia water (2 : 1 v/v). The results were obtained by spraying with 200 g L^{-1} H_2SO_4 in ethanol and heating the plate at 150 °C for 10 min. NAG, NAG_3 and NAG_6 were used as standard markers.

Minimum inhibition concentration (MIC) test

A 100 µL aliquot of each 16-h-old test micro-organism (∼5 × 10^6 colony-forming units (cfu) mL^{-1}) was mixed with 100 µL of sterilised chitosan hydrolysate samples, which were twofold serially diluted in advance with a suitable medium buffered with 100 mmol L^{-1} phosphate buffer (pH 7) to the designated concentration. A corresponding mixture containing no test strain was used as a control. The mixture was then applied to each well of a 96-well microplate and incubated at the appropriate temperature for each micro-organism for 48 h. Growth of the test micro-organism was monitored by determining OD_{590} every 6 h for 48 h using an enzyme-linked immunosorbent assay (ELISA) reader (Biolog, Hayward, CA, USA). All treatments were conducted five times. MIC was ultimately defined as the lowest concentration of sample required to inhibit bacterial growth for over 48 h beyond that of the control sample.

Statistical analysis

Mean values and standard deviations were calculated from the data of tests performed five times per sample. Results were compared by the least significant difference (LSD) test using SAS Version 8.01 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Deacetylation and analysis of chitosan

Chitosan was defined as chitin with a DD higher than 40%.[30] According to the results shown in Table 1, both α- and β-chitin were deacetylated into chitosan form in 1 h. It is apparent that β-chitin, with a less crystallised structure, was much more susceptible to the deacetylation process in that it took only 1 h to reach a DD of 70%, whereas it took α-chitin 6 h to achieve a comparable level (74%). A similar result was obtained by Kurita *et al.*[31] In general, without considering molecular weight, chitosan can dissolve in weak acid solution only at high DD (≥70%). Each of the prepared C_β groups dissolved well in acetate buffer. However, it was observed that the C_α groups, even $C_{\alpha6}$ (DD 74%), were only partially dissolved in acetate buffer, so their M_Vs, which were determined by intrinsic viscosity, were not measured. According to the results, the C_β with higher DD had lower M_V. This could be caused by alkaline degradation, which could be avoided by adding an oxygen scavenger such as thiophenol.[32]

Effect of DD on degradation of chitosan

In order to discern the effects of DD on the behaviour of chitinase in the hydrolysing process of chitosan, both C_α and C_β with different DDs were treated with crude chitinases for 7 days to produce various enzymatic products. Our results indicated that β-chitosan with higher DD could bring about higher M_V of COS products: $C_{\beta1}H_{24}$ (<10 kDa) < $C_{\beta3}H_{24}$ (14.2 ± 0.5 kDa) < $C_{\beta6}H_{24}$ (26.5 ± 0.7 kDa). According to Aiba,[17] an increase in DD could reduce the digestibility of chitosan by chitinase owing to the existence of fewer NAG sites. Our results seem to confirm this. The viscosity of the highly hydrolysed COS products (≥72 h), except $C_{\beta6}H_{72}$ (22.2 ± 0.8 kDa), was far too low for M_V determination. Unlike the C_β groups, the COS obtained from any of the C_α groups did not dissolve completely, so their M_Vs were not measured.

The digestibility of chitosan can also be investigated via the release of reducing sugar in the hydrolysing process. It can be seen in Fig. 1 that the reducing sugar content increased with hydrolysing time for each group of C_α and C_β. Further, the content of reducing sugar increased steadily as the DD increased for α-chitosan hydrolysates obtained from corresponding hydrolysing times at various DDs, e.g. from 5 mmol L^{-1} ($C_{\alpha0}H_{168}$) to 20 mmol L^{-1} ($C_{\alpha6}H_{168}$) (Fig. 1). This is attributed to enhancement of the solubility of chitosan substrate by increased DD (data not shown). However, a contrary result was observed for β-chitosan hydrolysates obtained from corresponding hydrolysing times: here the reducing sugar content fell dramatically as the DD increased, e.g. from 68 mmol L^{-1} ($C_{\beta0}H_{168}$) to 19 mmol L^{-1} ($C_{\beta1}H_{168}$) and finally to 10 mmol L^{-1} ($C_{\beta6}H_{168}$). This phenomenon cannot be explained by the increased solubility of the substrate, but instead by the decrease in chitinase cleavage sites, i.e. NAG units, existing along the chitosan fibre (Fig. 2). The decrease in the number of cleavage sites could also decrease the production of reducing sugar.

Additionally, comparing the two native form chitosans, $C_{\alpha0}$ and $C_{\beta0}$, the total amount of reducing sugar obtained from $C_{\alpha0}$ hydrolysates was much lower than that from $C_{\beta0}$ hydrolysates. It was also observed that the enzymatic reaction was nearly complete after 168 h for $C_{\beta6}$, the 24, 72 and 168 h hydrolysates of which showed no significant intra-group differences, but not for any of the C_α groups, where the hydrolysing reaction was far from complete even after 168 h. The M_V result for $C_{\beta6}H_m$ (Table 1)

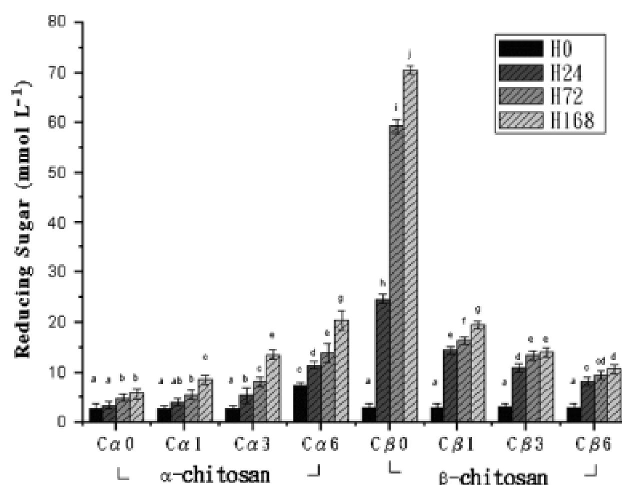

Figure 1. Amount of reducing sugar produced during hydrolysing process of α- and β-chitosan after 0, 24, 72 and 168 h. Values are expressed as mean ± standard deviation; different letters above bars indicate significant differences ($P < 0.05$) for varieties.

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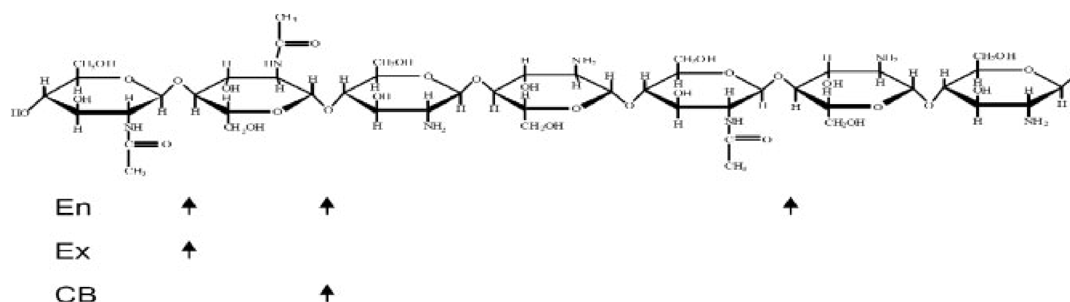

Figure 2. *N*-Acetyl-D-glucosamine units on chitosan can be recognised as cutting sites by a chitinase mixture: ↑, cutting site; En, endochitinase (EC 3.2.1.14); Ex, *N*-acetylglucosaminidase (EC 3.2.1.52); CB, chitobiosidase (EC 3.2.1.30).

Table 1. Physical characteristics, DD and M_V, of differently deacetylated α-chitosan and β-chitosan and their hydrolysates

			M_V (kDa)[a]			
			Hydrolysing time (h)			
Sample	Deacetylation time (h)	DD (%)	0	24	72	168
α-Chitosan						
$C_{\alpha 1}$	1	47	UM	ND	ND	ND
$C_{\alpha 3}$	3	61	UM	ND	ND	ND
$C_{\alpha 6}$	6	74	UM	ND	ND	ND
β-Chitosan						
$C_{\beta 1}$	1	70	156.5 ± 8.2a	–	–	–
$C_{\beta 3}$	3	83	140.8 ± 11.3a	14.2 ± 0.5c	8.8 ± 1.5e	–
$C_{\beta 6}$	6	90	120.4 ± 10.2b	26.5 ± 0.7d	22.2 ± 0.8f	20.5 ± 1.7f

[a] Values are mean ± standard deviation. Means followed by different letters are significantly different ($P < 0.05$). UM, unmeasured owing to partial solubility; ND, not done; – , not determined owing to extremely low viscosity.

also confirmed this point. The incomplete hydrolysis of C_α groups is attributed to the high crystallisation of α-chitin, which causes uneven deacetylation and thus increases local steric hindrance to the enzymatic reaction. Similar results, obtained using lysozyme, reported by Kurita *et al*.[31] indicate that β-chitin is degraded much more readily than α-chitin owing to its weak intermolecular forces; besides, their results also reveal that the degradation rate can be affected markedly by the extent of deacetylation.

Molecular weight distribution profiles of chitosan hydrolysates

To further reveal the influence of DD on the hydrolysing process, the prepared hydrolysates were analysed by TLC and SEC-HPLC for their molecular size distribution. TLC, with limited resolving power in the separation of oligosaccharide molecules ranging from monomer to hexamer, was used to analyse the $C_{\alpha 6}$ and $C_{\beta 6}$ hydrolysates obtained during the hydrolysing process for their oligosaccharide profiles. The major component in both groups of hydrolysates was the dimer, which increased as the hydrolysing time increased (Fig. 3). However, the second most abundant component was the monomer for $C_{\alpha 6}$ (Fig. 3(A)) but the trimer for $C_{\beta 6}$ (Fig. 3(B)). Moreover, the spots located between tetramer and hexamer were far fewer for the $C_{\alpha 6}$ group than for the $C_{\beta 6}$ group. This result was consistent with the DNS assay, which found that β-chitosan hydrolysates contained more reducing sugar and larger molecules. It has been emphasised that COS has the ability to improve food quality and promote human health. Hexamer to octamer in particular have been found to be most valuable for their immunomodulating function.[33] However, it has been difficult to manufacture COS with desired molecular weights. Our results suggest that the production of a suitably sized product is possible by adjusting the DD of chitosan substrate in the chitinase-hydrolysing process.

To reveal the influence of varying the DD on the size distribution of chitosan hydrolysate, the 168 h hydrolysates obtained from $C_{\beta 0}$, $C_{\beta 1}$ and $C_{\beta 6}$ were used as examples in SEC-HPLC analysis, which can demonstrate the transformation of the size distribution profile of hydrolysates in response to various DDs of chitosan. To locate the distribution of COS molecules in hydrolysates, NAG, NAG_6 and 10 kDa dextran were used as molecular weight standard markers (Fig. 4). It can be clearly seen in Fig. 5 that the molecular weight profiles became wider and larger as the DD increased, which reveals that an increase in DD can increase the resistance of chitosan to the degradation caused by chitinase. It can also been seen in Table 1 that the M_V of $C_{\beta 6}H_m$, as compared with others, was slightly changed along the hydrolysing course to the end. Again, this was due to the fewer chitinase cleavage sites provided by higher DD of chitosan. $C_{\beta 0}H_{168}$ had a uniform peak centred at the monomer and dimer of the NAG molecule because of $C_{\beta 0}$'s low DD and it being susceptible to the degrading action of chitinase. $C_{\beta 6}H_{168}$ had a broader size distribution and accounted for the greatest amount of product distributed in the size range between hexamer and 10 kDa dextran, which is considered as COS according to the definition provided by Kim and Rajapakse[1] and could have good potential biofunctionality.

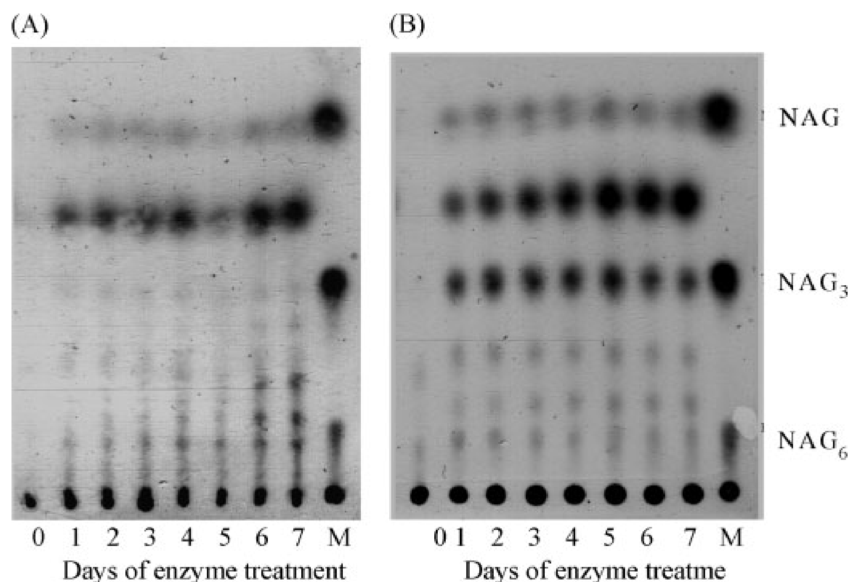

Figure 3. TLC profiles of hydrolysates obtained from (A) $C_{\alpha 6}$ and (B) $C_{\beta 6}$ treated with chitinase for various periods of time (0–7 days). M, *N*-acetyl-chito-oligosaccharide markers.

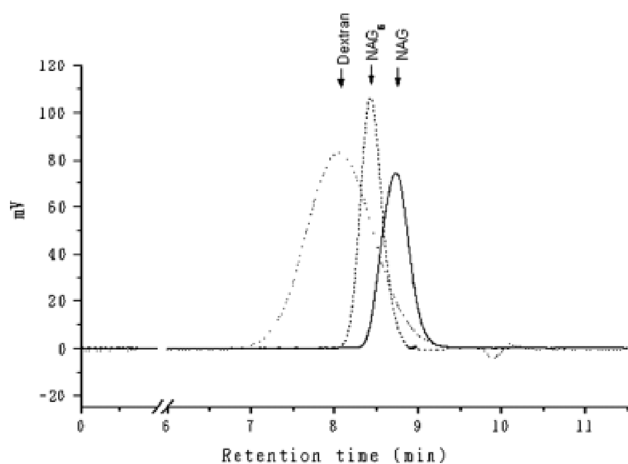

Figure 4. HPLC profiles of molecular weight standard markers: dextran, 10 kDa; *N*-acetyl-D-glucosamine (NAG), 221 Da; hexa-*N*-acetylchitohexaose (NAG_6), 1237 Da.

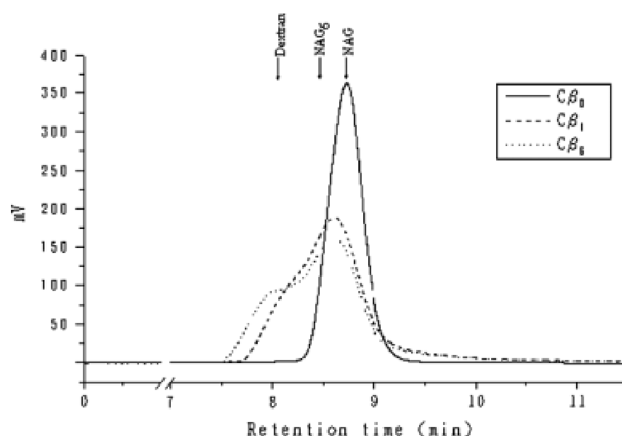

Figure 5. Molecular weight profiles of 168 h hydrolysates obtained from $C_{\beta 0}$, $C_{\beta 1}$ and $C_{\beta 6}$. Dextran, NAG and NAG_6 were used as standard markers indicating molecular weights of 10, 221 and 1237 Da respectively.

Antibacterial activity of chitosan hydrolysates

The effective dose of antibacterial function that chitosan can exert is influenced by its DD and molecular size. The 24 h hydrolysates produced from $C_{\beta 1}$, $C_{\beta 3}$ and $C_{\beta 6}$ with DDs of 70, 83 and 90% respectively were tested for their activity against several Gram-negative and Gram-positive strains of bacteria, as were their original forms. The results for the original forms demonstrate that the lower deacetylated chitosan showed less inhibition than the higher deacetylated chitosan at the concentration used in this study (Tables 2 and 3). The antibacterial action of chitosan is believed to be mediated by electrostatic forces between the protonated NH_2 group in chitosan and negative residues on the cell surfaces.[12] Since the number of protonated NH_2 groups increases with increasing DD, higher deacetylated chitosan exhibits more effective antibacterial activity.

The hydrolysates $C_{\beta 1}H_{24}$ and $C_{\beta 3}H_{24}$ derived from $C_{\beta 1}$ (DD 70%) and $C_{\beta 3}$ (DD 83%) respectively were much less effective antibacterially than their original forms. It was reported that partially hydrolysed chitosan was more effective as an antibacterial agent than highly hydrolysed chitosan; besides, Park *et al.*[10] observed that the antibacterial activity of COS increased as the molecular weight increased. As mentioned earlier, since lower acetylated chitosan can be degraded into smaller molecules, $C_{\beta 1}H_{24}$ therefore has a lower molecular weight spectrum distributed towards the less effective size range in comparison with that of $C_{\beta 6}H_{24}$. This not only explains why the hydrolysed form was less effective than its native form but also explains the antibacterial intensity results: $C_{\beta 1}H_{24} < C_{\beta 3}H_{24} < C_{\beta 6}H_{24}$. The effective molecular weight range has been widely studied. It has been reported that COS with an average molecular weight of less than 2.2 kDa is not capable of suppressing microbial growth but that COS with a molecular weight around 5.5 kDa suppresses growth according to dosage.[34] Further, it has been established that enzymatically hydrolysed chitosan with a molecular weight range between 5 and 27 kDa exhibits effective antibacterial activity.[9] The relation-

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Table 2. Comparison of activities of three differently deacetylated β-chitins, $C_{\beta1}$ (DD 70%), $C_{\beta3}$ (DD 83%) and $C_{\beta6}$ (DD 90%), and their 24 h enzymatically treated hydrolysates, $C_{\beta1}H_{24}$, $C_{\beta3}H_{24}$ and $C_{\beta6}H_{24}$, against Gram-negative bacteria

	MIC (μg mL^{-1})[a]					
Tested strain	$C_{\beta1}$	$C_{\beta1}H_{24}$	$C_{\beta3}$	$C_{\beta3}H_{24}$	$C_{\beta6}$	$C_{\beta6}H_{24}$
A. faecalis	180 ± 45a	360 ± 89b	180 ± 0a	320 ± 110b	140 ± 55a	120 ± 45a
E. aerogenes	720 ± 179a	1440 ± 358b	360 ± 89cd	480 ± 160ac	240 ± 89d	110 ± 55e
E. cloacae	720 ± 179a	1280 ± 438b	400 ± 0cee	720 ± 179a	200 ± 0d	140 ± 55e
E. coli	320 ± 110a	360 ± 89a	200 ± 0b	240 ± 89ab	110 ± 45c	55 ± 27d
K. oxytoca	360 ± 89a	1600 ± 0b	320 ± 110a	880 ± 438c	200 ± 122d	180 ± 45d
P. aeruginosa	400 ± 0a	1440 ± 358b	200 ± 0c	400 ± 0a	180 ± 45c	200 ± 122c
V. harveyi	240 ± 89a	320 ± 110a	110 ± 45b	200 ± 0ae	100 ± 0b	60 ± 22c
V. parahaemolyticus	220 ± 110abc	360 ± 89a	180 ± 45b	200 ± 0c	120 ± 45b	55 ± 27d
Y. enterocolitica	1440 ± 358a	NI	880 ± 438ab	NI	720 ± 179b	880 ± 438ab

[a] Values are mean ± standard deviation. Means in the same row followed by different letters are significantly different ($P < 0.05$). NI, no inhibition.

Table 3. Comparison of activities of three differently deacetylated β-chitins, $C_{\beta1}$ (DD 70%), $C_{\beta3}$ (DD 83%) and $C_{\beta6}$ (DD 90%), and their 24 h enzymatically treated hydrolysates, $C_{\beta1}H_{24}$, $C_{\beta3}H_{24}$ and $C_{\beta6}H_{24}$, against Gram-positive bacteria

	MIC (μg mL^{-1})[a]					
Tested strain	$C_{\beta1}$	$C_{\beta1}H_{24}$	$C_{\beta3}$	$C_{\beta3}H_{24}$	$C_{\beta6}$	$C_{\beta6}H_{24}$
L. monocytogenes	800 ± 0a	NI	560 ± 196b	1440 ± 358c	360 ± 89d	880 ± 438abc
S. agalactiae	880 ± 438a	NI	480 ± 160ab	1440 ± 358a	360 ± 89b	800 ± 0a
S. bovis	1440 ± 358a	NI	1120 ± 392ab	NI	720 ± 219b	1440 ± 358a
S. pyogenes	1660 ± 0a	NI	1120 ± 392b	NI	320 ± 110c	960 ± 358b
S. aureus	NI	NI	NI	NI	880 ± 438	NI
S. xylosus	220 ± 110a	NI	100 ± 0b	200 ± 0a	70 ± 24 c	45 ± 10c

[a] Values are mean ± standard deviation. Means in the same row followed by different letters are significantly different ($P < 0.05$). NI, no inhibition.

ship between antibacterial activity and M_V in our results mostly agrees with these rules. However, a significantly opposite result was observed in the higher deacetylated form $C_{\beta6}$ (DD 90%). Here the 24 h hydrolysate ($C_{\beta6}H_{24}$) was more effective than its original form against most of the Gram-negative strains, although not against the Gram-positive strains. By increasing the frequency of contact with micro-organisms, the increased solubility may be the reason why $C_{\beta6}H_{24}$ has a higher antibacterial capability than its original form. Since the NAG units existing on the chitosan fibre are recognised and cleaved by chitinase, the COS thus produced potentially has a higher DD than its original form. The increase in DD, in addition to the increase in solubility and suitable particle size of chitosan molecules, also plays a role in the enhancement of antibacterial activity of $C_{\beta6}H_{24}$.

An opposite result was observed for Gram-positive strains of bacteria. Here the hydrolysate ($C_{\beta6}H_{24}$) lost 50% of antibacterial activity as compared with its original form ($C_{\beta6}$). It is possible that Gram-positive bacteria are less sensitive than Gram-negative bacteria to smaller chitosan molecules.

The three *Enterobacter* strains *E. aerogenes*, *E. cloacae* and *E. coli*, found as normal microflora in animal intestines, had an MIC of 100 μg mL^{-1} to $C_{\beta6}H_{24}$. This implies that the use of this hydrolysate as a food additive could greatly affect the gut microflora composition, which could be a double-edged sword, since the health of the gastrointestinal tract is directly related to the balance of intestinal microflora. The two *Vibrio* strains *V. harveyi* and *V. parahaemolyticus* are the most significant food-borne pathogens in Taiwan, Japan and other coastal countries in Asia. Both strains were very susceptible to $C_{\beta6}H_{24}$ and had an MIC as low as 50 μg mL^{-1}, which suggests that $C_{\beta6}H_{24}$ could be used as a pharmaceutical agent to stop the growth of pathogens.

Pseudomonas aeruginosa has been reported to be resistant to low-molecular-weight chitosan (5–27 k Da), possibly owing to the production of mucus.[9] In the present study, however, *P. aeruginosa* was very susceptible to $C_{\beta6}H_{24}$ and had an MIC as low as 200 μg mL^{-1}. This could be due to strain variation.

It has been verified by many researchers[9,10] that chitosan possesses activity mostly against Gram-negative bacteria. Similar results were found in the present study, with Gram-negative bacteria having a much lower MIC range (Table 2) than Gram-positive bacteria (Table 3). The charge distribution of the bacterial cell wall seems to play a considerable role in the observed antibacterial activities of COS. According to Chung *et al*.,[14] there is a close relationship between the hydrophilicity and negative charge distribution of the bacterial cell surface. The distribution of negative charge on the cell surface of Gram-negative bacteria was found to be higher than that of Gram-positive bacteria and led to a higher hydrophilicity and a greater interaction with chitosan. This can clearly explain why most Gram-negative bacteria were more sensitive to COS. In addition, the negative charge distribution on the cell surface also varies among Gram-negative and Gram-positive bacteria. Therefore the sensitivity of cells to COS is normally in the order of higher negatively charged Gram-negative bacteria to less negatively charged Gram-positive bacteria. This

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could be one of the reasons why *S. xylosus*, a typical Gram-positive strain, exhibited a strong sensitivity (MIC 50 μg mL^{-1}) to chitosan. *Staphylococcus xylosus* may carry a unique cell surface characteristic that could make it an ideal candidate for studying how chitosan exerts its antibacterial effectiveness.

Controlled deacetylation of β-chitin would make possible finely tuned biodegradation, which could be useful for some advanced applications, particularly in the biomedical field. COS thus produced from chitinase-catalysed β-chitin displayed a unique antibacterial activity on several strains of food spoilage micro-organisms, food-borne pathogens and intestinal microflora. These results suggest that the COS thus produced has great potential for use as a safe and natural preservative and antibiotic in the food industry and as a food supplement for the improvement of intestinal health.

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