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Characteristics of deacetylation and depolymerization of β-chitin from jumbo squid (*Dosidicus gigas*) pens

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ABSTRACT

This study evaluated the deacetylation characteristics of β-chitin from jumbo squid (*Dosidicus gigas*) pens by using strongly alkaline solutions of NaOH or KOH. Taguchi design was employed to investigate the effect of reagent concentration, temperature, time, and treatment step on molecular mass (MM) and degree of deacetylation (DDA) of the chitosan obtained. The optimal treatment conditions for achieving high MM and DDA of chitosan were identified as: 40% NaOH at 90 °C for 6 h with three separate steps (2 h + 2 h + 2 h) or 50% NaOH at 90 °C for 6 h with one step, or 50% KOH at 90 °C for 4 h with three steps (1 h + 1 h + 2 h) or 6 h with one step. The most important factor affecting DDA and MM was temperature and time, respectively. The chitosan obtained was then further depolymerized by cellulase or lysozyme with cellulase giving a higher degradation ratio, lower relative viscosity, and a larger amount of reducing-end formations than that of lysozyme due to its higher susceptibility. This study demonstrated that jumbo squid pens are a good source of materials to produce β-chitosan with high DDA and a wide range of MM for various potential applications.

1. Introduction

Chitin is a natural polysaccharide with a monomer of (1→4)-linked 2-acetamido-2-deoxy-D-glucose. It is known as the second-most abundant polymer after cellulose.[1] Chitin exists in three forms as α, β, and γ, in which α-chitin is mainly from shrimp, crab, and krill shells, β-chitin is from squid pens, and γ-chitin is usually from fungi and yeast with its morphology appearing as a combination of the α and β structures. β-Chitin is more reactive and has an affinity toward solvents due to its parallel structure and no inter-hydrogen bonds, which gives rise to loose binding between the molecules, while α-chitin has anti-parallel structure with inter-hydrogen bonds.[2,3]

Chitin is insoluble in most organic solvents due to the highly extended hydrogen-bonded semicrystalline structure dominated by the strong CO···HN hydrogen bonds that show a distance of about 0.47 nm.[4,5] The acetyl groups ($COCH_3$) in chitin are removed through a process called 'deacetylation' to obtain chitosan, that is, soluble in dilute acids. When the majority of the *N*-acetyl-D-glucosamine (NAG) units are converted to D-glucosamine (DG) units with free $-NH_2$ groups, the polymer becomes highly soluble in dilute acids. Several approaches may be employed to deacetylate chitin, including alkaline deacetylation,[6] intermittent water washing,[7] use of an organic solvent,[8] flash treatment,[9] and enzymatic deacetylation.[10] Among them, the alkaline deacetylation using strong alkaline reagents of NaOH or KOH has been mostly used, in which the specific method is usually named by the name of the principal researcher, such as the Broussignac method using KOH,[11] and Kurita method using NaOH.[12] Both alkaline treatments hydrolyze the acetyl groups from NAG, thus generating $-CH_3COO^-$ and $-NH_2$ groups on the polymer. However, using NaOH or KOH can result in different deacetylating processes, obtaining chitosan with different functional properties.[2,11,12] This may be due to the solubility differences between NaOH and KOH in organic solvents because of their different dielectric constants. For examples, the dielectric constant of NaOH is 80.1 in water, which is much higher than that of KOH, which is 25.3 in ethanol and 41.4 in ethylene glycol at 298 K when used for deacetylating chitosan. In the transition state of the deacetylation reaction, the development of ionic charges is initiated by the nucleophilic attack of the HO^- on the carbonyl groups of the amide functions and is favored by the high dielectric constant solvent.[2]

The functionality of chitosan is affected by several factors, including the source of the raw material, molecular mass (MM), degree of deacetylation (DDA), and its physical state (conformation, particle size, etc.).[13–15] Among them, MM and DDA may be most critical. Therefore, it is sometimes necessary to depolymerize native chitosan into polymers of lower molecular mass, a process called 'depolymerization'. Low MM chitosan has performed better for drug delivery because of its high solubility.[16] Chitosan

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depolymerized by different enzymes showed different antimicrobial functions, in which chitosan is depolymerized by chitinase had stronger inhibition against Gram-negative bacteria, while lysozyme-depolymerized chitosan was more effective against Gram-positive bacteria.[17] In a MM range of 5×10^3–9.16×10^4, the antimicrobial activity of chitosan increased along with increased MM, while in the MM range of 9.16×10^4–1.08×10^6, it decreased as MM increased.[18] Chitosan products of different MM (1.74, 2.36, and 3.07 g/mol) also showed different antifungal activities against *Rhizopus stolonifer*, in which the lower MM was more active against mycelia growth, while the higher MM, products inhibited mold germination.[19] Chitosan with an MM of 71 kDa was less inhibitory against *Bacillus cereus* and *Escherichia coli* than those with MM values of 4.74–10 kDa.[20] Therefore, controlling the MM of chitosan is necessary for achieving the most effective antimicrobial activity. The degree of deacetylation (DDA) of chitosan also impacts its properties. Chitosan with 99% DDA showed the highest inhibition against the growth of both Gram-negative and Gram-positive bacteria,[21] and 90% DDA chitosan had higher reactive oxygen scavenging activity than that of 50% DDA chitosan.[22] In addition, increasing the DDA improved the mechanical properties (tensile strength, elongation, and Young's modulus) of β-chitosan-based films.[23]

Depolymerization may be achieved by enzymatic, chemical, or physical methods or combinations thereof. Chemical depolymerization has limited control over the extent of depolymerization due to its harsh conditions along with environmental concern of using high concentrations of chemical reagents.[24] The physical method, such as ultrasonically assisted treatment, consistently results in irregular molecular masses.[25] In contrast, the enzymatic method is more applicable due to its controlled extent of reaction.[26]

Previous studies on β-chitosan from squid pens were from squid species of *Loligo lessoniana*, *Loligo formosana*, *Loligo vulgaris*, *Ommasterphes bartrami*, and *Illex argentines*.[2,3,12,23,27] However, the catch of jumbo squid (*Dosidicus gigas*) had increased significantly during 1991–2002, and became the third largest amount of squid processed worldwide in 2002 (406,356 tons, 12.8%) after *Illex argentines* (511,087 tons, 16.1%) and *Todarodes pacificus* (504,438 tons, 15.9%). In spite of its increased production, studies using jumbo squid pens as material for producing chitin and chitosan have been scarce. As it is well known, the raw materials significantly impact the deacetylation process of chitin and the functionality of the resulting chitosan. Therefore, this study aimed to investigate the optimal deacetylation procedure of β-chitin from jumbo squid pens and the enzymatic depolymerization of the β-chitosan thus obtained to produce a series of low-MM chitosan material. Different factors potentially contributing to the deacetylation process of chitosan, including the type and concentration of alkaline reagents, reaction temperature, time, and treatment step were statistically considered. Based on our best knowledge, no study has reported the deacetylation and deploymerization characteristics of β-chitin from jumbo squid pens, where all these major contributing factors were statistically considered.

2. Results and discussions

2.1. Deacetylation of chitin by the Kurita method[12]

2.1.1. Characteristics of deacetylation

The DDA ranged from 45% to 99%, and the MM from 5362 to 11,684 kDa (Table 1). High DDA values (>90%) for chitosan were all obtained at 90 °C treated with either 40% or 50% NaOH for at least 4 h, in which relatively low MM was observed, indicating that the severe treatment conditions removed more acetyl groups from chitin, resulting in higher DDA values and further degradation of the chitosan molecules. The MM range obtained in this study was similar to that by Chandumpai et al. (9110–10,240 kDa) in which 0.17 M acetic acid solution was used to measure MM,[3] but was highly different from the results by Tolaimate et al. (450–595 kDa) in which 0.3 M acetic acid/0.2 M sodium acetate solution was used for measuring MM.[28] As stated in the experimental section, the molecular mass measured in this study was 'viscosity-average molecular mass' and is highly related to the solubility of chitosan in solvents, and in turn, the type of solvent used for dissolving the chitosan samples. Previous studies indicated that MM values measured by dissolving chitosan in acetic acid/sodium chloride and acetic acid/sodium acetate were much lower than that by dissolving in diluted acid solution,[2,3,28] probably due to the electrostatic repulsion of chitosan as the polycationic polymer in an acidic solvent. The resolving process of chitosan is initiated by the binding between the hydrogen ions and free amine group to form a cation ion (NH_3^+) when pH is below its pK_a. Therefore, the amount of cation ions is important in determining the solubility and viscosity due to their electrostatic repulsions. The viscosity of a solution is increased along with the increased amount of NH_3^+, because it makes larger spaces between the polymers for a water trap, thus forming longer linear polymers that are more stretched out.[29] However, anions, such as Cl^- or CH_3COO^-, as in sodium chloride or sodium acetate could block the electrostatic repulsion between cations in chitosan, thus decreasing its intrinsic viscosity.[30] The relatively low MM observed in low-DDA samples might also be due to the rigid crystal structure of the chitosan samples, resulting in lower solubilities.[31,32]

Though there was no statistical difference on MM among nine treatment conditions except the 1st and the 9th runs (Table 1), the MM generally decreased in the severe treatment conditions of using a higher NaOH concentration (50%) or longer reaction time at 90 °C (Table 1). Similarly, Chandumpai et al. reported that the MM of chitosan gradually decreased along with increased treatment time from 2 to 8 h in 50% NaOH at 100 °C.[3] Tolaimate et al. found that β-chitin deacetylated by 40% NaOH at 80 °C for 6 h has a larger MM than that treated under the same conditions for 9 h.[28] Hasegawa et al. also indicated that the MM decreases along with increased concentrations of reagents and temperatures.[33] Therefore, the higher concentration of deacetylation reagent and longer reaction time served to further degrade the polymer. In contrast, Ottey et al. did not show further polymer degradation by extended reaction time.[34]

2.1.2. Optimal deacetylation conditions

Through the Taguchi design method the average values of three measured parameters (DDA, intrinsic viscosity, and MM) and the rank of each contribution factor on these parameters were obtained (Table 1). Since the intrinsic viscosity directly related to MM, it is not separately discussed here. The R_i value for the NaOH concentration was the lowest among all tested contributing factors on MM and DDA, R_i values of temperature and time on DDA were, respectively, ranked first and second, and were second and first on MM, respectively. ANOVA results indicated that the NaOH concentration had no significant effect on all measured parameters, but both temperature and time significantly affected DDA, but not MM (P <0.05). Therefore, regardless of the treatment factors and their levels applied in this study while using the Kurita method, no polymer degradation (change of MM) occurred in the deacetylation process. Based on this study, it may be concluded that the optimal deacetylation conditions to obtain chitosan with DDA values over 95% and without significant polymer degradation is to use 40% NaOH at 90 °C for 6 h in three divided steps (2 h + 2 h + 2 h) or use 50% NaOH at 90 °C for a period of 6 h.

Table 1
Intrinsic viscosity, molecular mass and degree of deacetylation of deacetylated chitin using the Kurita method and estimated parameters from the Taguchi design method

Run		Measured parameters		
		Intrinsic viscosity (mL/g)	Viscosity-average molecular mass (kDa)	Degree of deacetylation (%)
1		3281[b+]	5362[b]	52.7[cd]
2		5708[ab]	9730[ab]	94.2[ab]
3		4923[ab]	8328[ab]	98.8[a]
4		3957[ab]	6583[ab]	62.7[c]
5		4362[ab]	7288[ab]	95.6[ab]
6		4963[ab]	8392[ab]	45.0[d]
7		6109[a]	10464[ab]	81.0[b]
8		5502[ab]	9357[ab]	90.0[ab]
9		6766[a]	11684[a]	83.2[ab]
Factors	Levels			
A	K_{A1}	[++A]5357	[A]9114	[A]82.0
	K_{A2}	[B]4696	[A]7905	[A]73.3
	R_i[+++]	661	1209	8.7
B	K_{B1}	[A]4577	[A]7700	[A]60.3
	K_{B2}	[A]5452	[A]9277	[B]92.4
	R_i	875	1577	32.0
C	K_{C1}	[A]4582	[A]7703	[B]62.5
	K_{C2}	[A]5477	[A]9332	[AB]80.0
	K_{C3}	[A]5131	[A]8693	[A]91.8
	R_i	896	1629	29.3
D	K_{D1}	[A]4803	[A]8111	[A]77.2
	K_{D2}	[A]5593	[A]9529	[A]73.4
	K_{D3}	[A]4794	[A]8089	[A]83.8
	R_i	799	1440	10.4
	Rank[++++]	C > B > D > A	C > B > D > A	B > C > D > A

[+] Means followed by the lowercase letter in the same column within nine treatments were not significantly different (P >0.05).
[++] Means preceded by the same capital letter in the same column within each factors were not significantly different (P >0.05).
[+++] R_i was the difference between the highest and lowest values.
[++++] Ranks were based on the order of R_i values.

2.2. Deacetylation of chitosan using the Broussignac method[11]

2.2.1. Characteristics of deacetylation

Our preliminary studies found that when using the Broussignac method, samples treated at 60 °C were visibly insoluble in 0.1 M acetic acid (data not shown); thus increasing the reaction temperature to 90 and 120 °C was necessary. The DDA and MM ranged between 57% and 99% DDA and 34 and 11,934 kDa, respectively (Table 2). The higher MM chitosan products were obtained at 90 °C for at least 4 h in 1 or 3 steps (run number of 5, 6 and 8 in Table 2), while the MM from the treatment at 120 °C was significantly (P <0.05) lower than those at 90 °C except for the one for 2 h (P >0.05). The MM (1797 kDa) obtained at 90 °C for 2 h in two divided steps was significantly lower than that for the other samples treated at the same temperature (P <0.05), probably owning to the lower solubility of chitosan as shown by the low DDA of 57%. Similar to what was observed in the Kurita method, the rigid structure with hydrogen bonds in low-DDA chitosan might induce a lower solubility in the chitosan, thus affecting the measurement of MM. The MM at 90 °C for 4 h with three divided steps (1 h + 1 h + 2 h) was significantly (P <0.05) higher than that from the same conditions with one straight step. Similarly, the MM of chitosan deacetylated for 6 h in three divided steps (2 h + 2 h + 2 h) was higher than that with two divided steps. This might be explained as the multiple-step treatment helped prevent polymer degradation. In the Kurita method, the highest MM was also obtained at 90 °C for 4 h. Hence, the treatment conditions to obtain the highest MM were at 90 °C for 4 h in both methods. Compared with the Kurita method, the MM range obtained from the Broussignac method was wider, in which the higher treatment temperature may contribute to the significant degradation of chitosan as evidenced by a decrease in MM.

In respect to DDA, 90 °C for 4 h was a minimal condition required to obtain chitosan with DDA over 90%, and the multiple-step process helped increase the DDA as shown by the fact that 4 h with three divided steps (1 h + 1 h + 2 h) gave a 93% DDA versus 87% DDA obtained through a single 4-h treatment. The DDA was over 90% when samples were treated at 120 °C, and there was no significant difference in DDA in all 120 °C treated samples regardless of time or a multiple-step process (P >0.05). The lowest DDA (57%) was observed on samples treated at 90 °C for 2 h with two divided steps. Similar to our results, Tolaimate found that a 2-h treatment at 120 °C results in a DDA over 96%, confirming the important role of temperature, and a multi-step process resulted in a higher DDA compared to a single step process for the same treatment time.[2] In this study, samples treated at 120 °C for 6 h with three divided steps had a 99% DDA, which was close to the value for a fully deacetylated chitosan.

2.2.2. Optimal deacetylation conditions

The R_i value for temperature was the highest among all contributing factors (Table 2), and temperature significantly affected MM and DDA (P <0.05). These results indicated that while the high temperature treatment increases the DDA to a value over 95%, chitosan depolymerization simultaneously occurred. Time was the second-most contributory factor on DDA, but did not show a significant effect on the MM. Hence, controlling treatment time in the Broussignac method may provide better control on the MM and DDA of chitosan than temperature as it could satisfy our goal to obtain chitosan with a high DDA, but with less polymer degradation. A multiple-step process was the least contributing factor on DDA, and the second-most contributory effect on MM. It did not affect the DDA (P >0.05), but the MM, which was different from the results of the Kurita method. This result was also inconsistent with

Table 2
Intrinsic viscosity, molecular mass, and degree of deacetylation of deacetylated chitin using the Broussignac method and estimated parameters from the Taguchi design method

Run		Measured parameters		
		Intrinsic viscosity (mL/g)	Viscosity-average molecular mass (kDa)	Degree of deacetylation (%)
1		859^{cd+}	1273^{c}	98.7^{a}
2		111^{d}	143^{c}	96.1^{ab}
3		122^{d}	157^{c}	99.1^{a}
4		1797^{c}	2850^{c}	57.2^{c}
5		6903^{a}	11934^{a}	93.2^{ab}
6		6207^{ab}	10645^{ab}	94.3^{ab}
7		116^{d}	149^{c}	94.0^{ab}
8		4789^{b}	8067^{b}	87.4^{b}
9		30^{d}	35^{c}	97.9^{a}
Factors	Levels			
A	K_{A1}	$^{++B}$248	^{B}352	^{A}97.2
	K_{A2}	^{A}4924	^{A}8375	^{B}83.0
	$^{+++}R_i$	4676	8023	14.2
B	K_{B1}	^{A}924	^{A}1424	^{A}83.3
	K_{B2}	^{A}3934	^{A}6715	^{A}92.2
	K_{B3}	^{A}2120	^{A}3613	^{A}97.1
	R_i	3010	5291	13.8
C	K_{C1}	^{A}3951	^{A}6662	^{A}93.5
	K_{C2}	^{B}646	^{B}1010	^{A}83.7
	K_{C3}	^{AB}2380	^{AB}4080	^{A}95.5
	R_i	3305	5652	11.7
	$^{++++}$Rank	A > C > B	A > C > B	A > B > C

$^{+}$ Means followed by the lowercase letter in the same column within nine treatments were not significantly different (P >0.05).
$^{++}$ Means preceded by the same capital letter in the same column within each factors were not significantly different (P >0.05).
$^{+++}$ R_i was the difference between the highest and lowest values.
$^{++++}$ Ranks were based on the order of R_i value.

the findings by Tolaimate et al., in which the multi-step treatment prevented the depolymerization of MM when the temperature was at 80 °C.[2] The difference might be due to the low solubility of some of the chitosan samples, which affects the accuracy in MM measurement as previously stated. In future work, the solubility of deacetylated chitosan should be considered as a contributing factor in the Taguchi design to obtain a more accurate result.

Based on the results from this study, it may be concluded that the Broussignac method was more applicable when lower MM and higher DDA chitosans were targeted, while the Kurita method was more suitable for obtaining chitosan with higher MM and higher DDA. This conclusion was similar to that reported by Tolaimate who compared the Kurita and Broussignac methods in the deacetylation of β-chitin from *Loligo vulgaris* squid pens.[2] However, it should be noted that the KOH used in the Broussignac method is more expensive than the NaOH used in the Kurita method; thus it might be of less interest in commercial applications.

2.2.3. Depolymerization of chitosan by commercial enzymes

Enzyme type (ET), initial MM (IM), and depolymerization time (DT) were all significant (P <0.05) factors affecting depolymerization of chitosan, and there were significant (P <0.05) interactions between ET and DT, and IM and DT (P <0.05) (Table 3). High molecular mass (HMM) (2100 kDa) and low molecular mass (LMM) (594 kDa) chitosan had about 89% and 56% degradations, respectively, by cellulase (EC3.2.1.4) in the first hour, another 15–20% degradation in the following hour, but no further degradation after 2 h (Fig. 1). Lysozyme (EC 3.2.1.17) resulted in about 56% and 68% degradation in HMM chitosan during the first and second hour treatment, but did not induce further degradation after that. While the reaction on LMM chitosan was significantly slower, no degradation occurred until after 5 h (15% and 47% degradation at 5 and 6 h, respectively). Therefore, chitosan depolymerization by enzymes was significantly induced during the first 1–2 h of the reaction except for lysozyme treatment on LMM chitosan. The initial

Table 3
Analysis of variance (ANOVA) results (P = 0.05) for analyzing independence or interactions among treatment factors during the deplymerization of chitosan

Source of variation	Molecular mass (MM, kDa)			Degree of deacetylation (DDA, %)		
	df	F value	P value	df	F value	P value
Linear terms						
Enzyme types (ET)	1	111.57	<.0001	1	0.06	0.8158
Initial MM (IM)	1	54.73	<.0001	1	28.42	<.0001
Depolymerized time (DT)	7	56.75	<.0001	7	0.69	0.6759
Interaction terms						
ET X IM	1	3.17	0.0829	1	5.72	0.0217
ET X DT	7	31.09	<.0001	7	0.31	0.9428
IM X DT	7	2.42	0.0368	7	0.92	0.5020
Model	24	33.39	<.0001	24	1.99	0.0274
Error	39			39		
Corrected total	63			63		

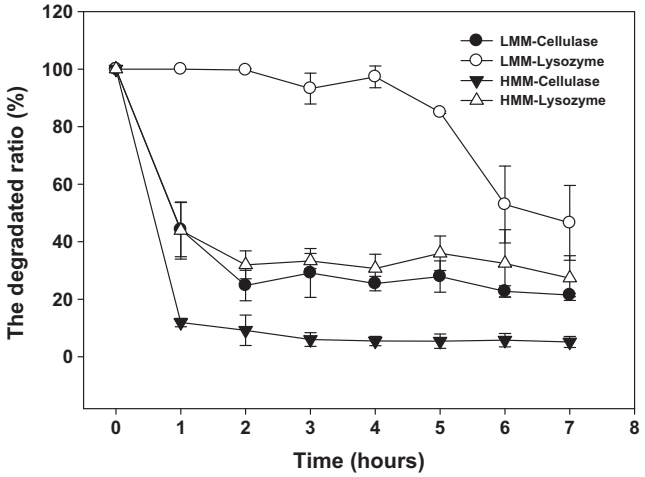

Figure 1. The ratio of the depolymerization in chitosan treated with 1% cellulose or lysozyme for 7 h ($n = 2$). Data were mean of two replications. HMM = higher molecular mass; LMM = lower molecular mass.

fast degradation was probably due to the predominant endo-action of cellulase and lysozyme that broke internal bonds (the (1→4)-glycosidic linkage of the polysaccharide) of chitosan.[17] Previous studies reported that chitosan depolymerized by cellulase shows 83.5% degradation in only 5 min, and 95.3% degradation in 4 h with a final MM of 24 kDa.[35]

The types of enzyme had significantly different impact on the depolymerization of HMM and LMM chitosan (P <0.05). MM values of lysozyme-treated samples were significantly (P <0.05) higher than those from cellulose-treated ones regardless of the difference in the initial MM (Table 4). According to Lin, lysozyme had less susceptibility than cellulase in terms of chitosan degradation since it only recognizes the site existed $NAG_{(3-5)}$ site of *N*-acetyl-D-glucosamine (NAG), while cellulase can randomly cleave the (1→4)-glycosidic linkages of chitosan regardless of the type of monomers.[17] Kurita found that lysozyme had higher susceptibility in 57% DDA chitosan than in higher DDA chitosan.[27] The initial DDA values of HMM and LMM chitosan were about 85% and 95%, respectively, and may explain the diminished degradation observed in LMM chitosan when depolymerized by lysozyme.

DDA values were measured during the polymerization process to investigate the potential impact of the treatment factors (Table 5). The initial MM was the only factor affecting the DDA (P <0.05), clearly due to the initial difference in DDA for the HMM and LMM chitosans. After one hour of deplymerization, the difference in DDA no longer existed among products of the different treatment conditions. A significant (P <0.05) interaction between enzyme type and initial MM on DDA was observed (Table 3). This interaction is probably attributable to the different susceptibility of the enzyme to chitosan that had different MM and DDA values.

2.2.4. Physicochemical properties of depolymerized chitosan

Changes of relative viscosity and reducing-end formation during the depolymerization of chitosan are illustrated in Figures 2 and 3, respectively. Initial viscosity of HMM (2137 kDa) and LMM (594 kDa) chitosan were 2048 cP and 48 cP, respectively. While the MM in HMM chitosan was about four times higher than that in LMM, the viscosity of HMM chitosan was about 50 times higher than that of LMM chitosan. Similar to the changes in MM, the relative viscosity of HMM chitosan decreased much faster than that of LMM chitosan, showing an approximate 95% reduction in the first 10 min and no change after that. This was observed on HMM chitosan treated by either cellulose or lysozyme. On the other hand, the relative viscosity of the LMM chitosan depolymerized by cellulase or lysozyme gradually decreased during the first hour, reaching about 73% and 37% reduction at 1 h, respectively, and was stable after that. These observations were similar to those from a previous study that showed that the viscosity change was mostly induced during the initial 10–20 min as a result of enzymatic action.[25] The reduction of relative viscosity was faster and higher in LMM chitosan treated by cellulase than that by lysozyme since the high DDA in LMM showed lower susceptibility on lysozyme treatment.

Theoretically, the relative viscosity is proportional to MM, concentration, and the chain entanglement of chitosan,[36] and can be highly increased when MM is higher than a certain critical molecular mass (M_C) of a polymer. The strong dependence of viscosity in the high MM samples can be explained as the effect of chain entanglement in the polymer.[37] For example, the viscosity is proportional to $MM^{3.4}$ if $MM > M_C$ and to $MM^{1<a<2.5}$ if $MM < M_C$, and no distinct M_C could be up to MM of 1000 kDa.[37,38] Therefore, the relative viscosity of HMM chitosan was significantly higher than that of LLM chitosan due to its stronger dependence on MM.[37] The way the viscosity is computed may also attribute to the high viscosity value in HMM chitosan as the relative viscosity was expressed as the viscosity at sampling time relative to the initial viscosity of chitosan prior to enzyme treatment. The initial viscosity of HMM chitosan was considerably higher than that of LMM chitosan, resulting in a higher decreasing rate even though the same number of viscosity reduction occurred in both HMM and LMM chitosan.

The absorbance value at 420 nm indicates the amount of reducing ends formed by the depolymerization of chitosan (Fig. 3). The absorbance value in chitosan depolymerized by cellulase was significantly higher than that by lysozyme in both HMM and LMM chitosan. There was no significant difference in the absorbance between HMM and LMM chitosan (P >0.05). The absorbance increased fast in the initial 30 min and then increased slowly after that. The difference in the reducing end formation of chitosan depolymerized by cellulase or lysozyme was probably due to different specificities of enzymes for cleaving at different active sites.[17,27] As discussed in the changes of MM and relative viscosity, the DDA values of chitosan impacted the specificities of enzymes on the formation of the reducing ends that were formed by the

Table 4
Effect of initial molecular mass of chitosan, type of enzyme, and depolymerization time on the molecular mass of chitosan during depolymerization

	Initial MM[+++] (IM)	Enzyme types (ET)	Depolymerization time (h)								*P* value
			0	1	2	3	4	5	6	7	
	HMM	Cellulase	[+]$^{A}2100^{a}$	$^{B}298^{b}$	$^{B}165^{b}$	$^{B}137^{b}$	$^{B}145^{b}$	$^{B}139^{b}$	$^{B}133^{b}$	$^{B}124^{b}$	<.0001
		Lysozyme	$^{A}2100^{a[++]}$	$^{A}670^{b}$	$^{A}633^{b}$	$^{A}533^{b}$	$^{A}645^{b}$	$^{A}625^{b}$	$^{A}718^{b}$	$^{A}611^{b}$	0.0003
	LMM	Cellulase	$^{B}594^{a}$	$^{B}264^{b}$	$^{B}148^{b}$	$^{B}175^{b}$	$^{B}152^{b}$	$^{B}167^{b}$	$^{B}136^{b}$	$^{B}128^{b}$	<.0001
		Lysozyme	$^{B}594^{a}$	$^{A}686^{a}$	$^{A}604^{a}$	$^{A}553^{a}$	$^{A}584^{a}$	$^{A}505^{ab}$	$^{B}317^{b}$	$^{B}279^{b}$	0.0012
P value			0.0180	0.0036	0.0001	0.001	0.0003	0.0080	0.0012	0.0023	

[+] Means preceded by the same capital letter in the same column within each treatment were not significantly different among different treatments (P >0.05).
[++] Means followed by the lowercase letter in the same row within each treatment were not significantly different among different depolymerization times (P >0.05).
[+++] HMM = higher molecular mass, LMM = lower molecular mass.

Table 5
Effect of initial molecular mass of chitosan, type of enzyme, and depolymerization time on the degree of deacetylation (DDA) of chitosan during depolymerization

	Initial MM[+++] (IM)	Enzyme types (ET)	Depolymerization time (h)								*P* value
			0	1	2	3	4	5	6	7	
	HMM	Cellulase	[+B]85.5[a++]	[A]87.2[a]	[A]83.1[a]	[A]88.8[a]	[A]87.6[a]	[A]85.0[a]	[A]87.0[a]	[A]82.9[a]	0.2107
		Lysozyme	[B]85.5[a]	[A]90.6[a]	[A]89.9[a]	[A]89.7[a]	[A]89.7[a]	[A]87.0[a]	[A]89.0[a]	[A]85.3[a]	0.1885
	LMM	Cellulase	[A]95.1[a]	[A]92.2[a]	[A]95.4[a]	[A]95.3[a]	[A]91.5[a]	[A]90.1[a]	[A]94.2[a]	[A]93.1[a]	0.3451
		Lysozyme	[A]95.1[a]	[A]90.0[a]	[A]85.2[a]	[A]90.9[a]	[A]92.1[a]	[A]92.2[a]	[A]90.4[a]	[A]91.1[a]	0.8213
P value			0.0001	0.8167	0.0521	0.1274	0.7391	0.2036	0.2550	0.1969	

[+] Means preceded by the same capital letter in the same column within each treatment were not significantly different among different treatments ($P > 0.05$).
[++] Means followed by the lowercase letter in the same row within each treatment were not significantly different among different depolymerization times ($P > 0.05$).
[+++] HMM = higher molecular mass, LMM = lower molecular mass.

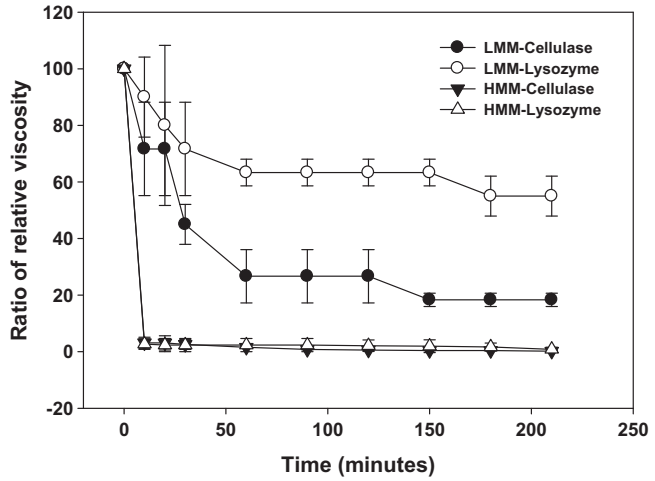

Figure 2. The decreasing ratio of viscosity in 1% (w/v) chitosan solution treated with 1% cellulose or lysozyme for 210 min. The decreasing ratio was expressed as the viscosity at the sampling time relative to the initial viscosity of the higher or lower molecular mass chitosan without enzyme treatment ($n = 2$). HHM = higher molecular mass; LMM = lower molecular mass.

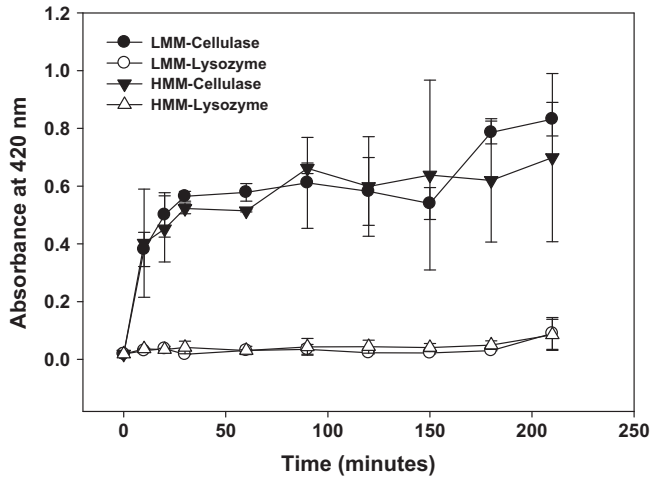

Figure 3. The reducing end formation from 1% chitosan solution treated with 1% cellulose or lysozyme for 210 min ($n = 2$). Ferricyanide absorbance corresponded to the amount of the reducing ends as a function of time. HHM = higher molecular mass; LMM = lower molecular mass.

enzymatic depolymerization of polysaccharide. It is known that the active site of cellulase is in (1→4)-glycosidic linkages regardless of the type of monomers, while lysozyme cleaves the active site occupied by the $NAG_{(3-5)}$ bindings. The initial DDA of chitosan was about 85% in HMM and 96% in LMM. Because of the relatively higher DDA in both HMM and LMM chitosan with fewer NAG monomers, chitosan depolymerized by lysozyme produced a smaller amount of reducing ends due to lower susceptibility, compared with chitosan depolymerized by cellulase. Cellulase is one of enzymes with the highest chitosan depolymerizing activity.[26] Studies of chitosan depolymerized by pepsin, cellulase, lipase A, and chitosanase also showed the fast generation of the reducing ends during the first hour.[26] The number of reducing ends was more dependent on the type of enzyme than the initial properties of the chitosan, probably due to the relatively higher DDA in both LMM and HMM chitosan.

3. Experimental

3.1. Materials

The β-chitin from jumbo squid (*Dosidicus gigas*) pens was provided by Dosidicus LLC, USA. Samples were ground into about 18 mesh (ASTM) size by a grinder (Glenmills Inc., USA) and stored in a desiccator until deacetylation treatment was carried out. NaOH and KOH were purchased from the Mallinckrodt Chemicals Co., (USA). *N*-Acetyl-D-glucosamine and monoethylene glycol were from Sigma–Aldrich Chemical Co., (USA). Toluidine Blue indicator and 1/400 potassium polyvinyl sulfate (PVS) were from Wako Chemicals (USA). Hen egg-white lysozyme and cellulase from *Aspergillus niger* were obtained from Fordras S.A. (SWISS) and TCI America (USA), respectively. All chemicals were of reagent grade.

3.2. Deacetylation of chitin

In this study, two different alkaline deacetylation processes were carried out: the method of Kurita et al. using NaOH as reagent[12] and the method of Broussignac using KOH[11] Depending on the type of chitin and alkaline reagents employed, the deacetylation process may respond differently. Using NaOH might result in β-chitosan with higher DDA and MM under moderate treatment conditions (40% NaOH at 80 °C) as compared with the use of KOH.[2,11] Hence, it is necessary to investigate which alkaline solvent performs more appropriately on β-chitin from jumbo squid pens.

While using the Kurita method,[12] NaOH was first diluted to targeted concentrations by dissolving it in distilled water. Chitin was then added to the NaOH solution at a ratio of 1:20. Four treatment factors, including NaOH concentration (40%, 50%), temperature (60, 90 °C), time (2, 4, 6 h), and treatment step (1, 2, 3 times) were investigated. The typical deacetylation conditions for α-chitin from shrimp or squilla shells were using 40–50% NaOH at 80–100 °C for 6–12 h.[2] Since the squid pens from the species of *Dosidicus gigas* are much smaller and thinner than other ones frequently used (*Loligo* or *Illex pens*), the mild treatment conditions could be applied. The multiple treatment steps at same treatment time were evaluated for the possible prevention of chitosan degradation

during deacetylation.[2] After NaOH treatment under given conditions, samples were washed with distilled water to reach neutral pH, and the residue that remained was washed with MeOH and acetone. Samples were then dried at 50 °C in a drying oven (Precision Scientific Inc., USA) for 24 h.

In the Broussignac method,[11] KOH (50% w/w) was dissolved in a mixture of 96% EtOH (25% w/w) and monoethylene glycol (25% w/w) solution. Chitin was then added to the solution at a ratio of 1:20. According to the previous study, the Broussignac process required minimal treatment conditions of 90 °C for 2 h in 1% HOAc[2] to obtain soluble chitosan. Therefore, three treatment factors including temperature (90, 120 °C), time (2, 4, 6 h), and treatment step (1, 2, 3 times) were considered. After the treatment, the same washing and drying procedures as those used in the Kurita method were applied.

The Taguchi experimental design with orthogonal arrays was applied for each method (Table 6). By using orthogonal arrays, it was expected that the optimal treatment conditions for obtaining chitosan with high DDA and desirable MM values can be identified with minimal treatment combinations (9 in this study) in each method. The factors that contributed most to achieve desirable results and their levels could then be identified through the Taguchi design.

3.3. Depolymerization of chitosan

The HMM and LMM chitosan prepared from the deacetylation study were depolymerized by cellulase or lysozyme, known to have different susceptibilities based on the properties of chitosan. The chitosan samples were dissolved in 5% HOAc solution at a ratio of 2:100 (chitosan:solvent). Cellulase or lysozyme was added into the solution at 1% (w/w) and allowed to react for up to 7 h. A 20-mL aliquot was taken out hourly and boiled for 10 min to stop the enzymatic reaction. NaOH was then added into the solution to reach a final pH 11 for precipitation. The precipitated samples were washed with distilled water to remove other residues and dried in an oven at 42 °C for 24 h. The MM and DDA of the depolymerized chitosan were measured at each sampling time.

3.4. Measurement of degree of deacetylation (DDA)

The DDA was measured by using the colloidal titration method.[6] A 50 mg sample of deacetylated chitosan (0.5%, w/w) was dissolved in 10 mL of 5% (v/v) HOAc solution, and then 1 g of solution was transferred into a flask and diluted up to 30 mL with distilled water. After adding 100 μL of Toluidine Blue indicator, the solution was titrated with 1/400 potassium polyvinyl sulfate (PPVS) till the solution color changed from blue to violet. The DDA was calculated as:

$$\mathrm{DDA}(\%) = (X/161)/(X/161) + (Y/203) \quad (1)$$

$$X = \frac{1}{400} \times \frac{1}{1000} \times F \times 161 \times V \quad (2)$$

$$Y = 0.5 \times \frac{1}{100} - X \quad (3)$$

where X was the weight of the D-glucosamine residue, g; F was the factor of 1/400 PPVS; V was the volume of consumed PPVS, mL; Y was the weight of *N*-acetyl-D-glucosamine residue, g; and 161 and 203 in (Eq. 1) were the molecular weight of D-glucosamine and *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose), respectively.

3.5. Determination of intrinsic viscosity and viscosity-average molecular mass

The intrinsic viscosity of chitosan was determined by using a Ubbelohde Dilution Viscometer (Cannon instrument Co., USA) that has a capillary size of 0.58 mm in a water bath at 25 °C. Viscosity-average molecular mass (MM) was then calculated from the measured intrinsic viscosity. Solutions used for measuring the MM of the chitosan samples from deacetylation and depolymerization treatments were different. This is mainly because the chitosan samples obtained from the deacetylation and depolymerization experiments had different DDA values, thus requiring different strengths of acid to completely dissolve and ensure accurate MM measurements. Our preliminary studies (data not shown) evaluated three types of acid solutions that are commonly used for measuring the MM in chitosan: 0.1 M HOAc, 0.1 M HOAc/0.2 M

Table 6
L9 standard orthogonal array of different contributing factors with different levels applied in the Kurita [A] and the Broussignac [B] methods[11,12]

Experimental run	Contributing factors (*i*)			
	Concentration (%) A	Temperature (°C) B	Time (h) C	Multiple-steps D
[A]—*Kurita method using NaOH as reagent*				
1	1 (40)	1 (60)	1 (2)	1
2	1 (40)	2 (90)	2 (4)	2
3	1 (40)	2 (90)	3 (6)	3
4	2 (50)	1 (60)	2 (4)	3
5	2 (50)	2 (90)	3 (6)	1
6	2 (50)	1 (60)	1 (2)	2
7	1 (40)	1 (60)	3 (6)	2
8	2 (50)	2 (90)	1 (2)	3
9	1 (40)	2 (90)	2 (4)	1

Experimental run	Contributing factors (*i*)		
	Temperature (°C) B	Time (h) C	Multiple-steps D
[B]—*Broussignac method using KOH as reagent*			
1	1 (120)	1 (2)	1
2	1 (120)	2 (4)	2
3	1 (120)	3 (6)	3
4	2 (90)	1 (2)	2
5	2 (90)	2 (4)	3
6	2 (90)	3 (6)	1
7	1 (120)	1 (2)	3
8	2 (90)	2 (4)	1
9	1 (120)	3 (6)	2

NaCl, and 0.3 M HOAc/0.2 M NaOAc, and found that 0.1 M HOAc is able to dissolve all the chitosan samples. The 0.1 M HOAc was thus chosen for measuring the MM of all chitosan samples in the deacetylation study. The same test was carried out for identifying the solution in measuring the MM of depolymerized chitosan by focusing on finding a more precise measurement of MM using a commercial chitosan with known MM as an indicator. The 0.1 M HOAc/0.2 M NaCl solution provided the closest MM value to that reported on the commercial chitosan.

Four different concentrations of chitosan in a range of 0.05–0.1% were used for measuring the viscosity of the samples. The intrinsic viscosity was measured by the intercept between the Huggins (reduced viscosity, $\frac{\eta_{sp}}{c} \sim C$) and Kraemer (relative viscosity, $\frac{\ln[\eta]_{rel}}{c} \sim C$) plots when the concentration was 0.[16] Relative viscosity, reduced viscosity, and intrinsic viscosity were determined as:

$$\eta_{rel} = \frac{t}{t_0} \tag{4}$$

$$\eta_{sp} = \eta_{rel} - 1, \eta_{red} = \frac{\eta_{sp}}{c} \tag{5}$$

$$[\eta] = \left(\frac{\eta_{sp}}{c}\right)_{c=0} = \left(\frac{\ln[\eta]_{rel}}{c}/C\right)_{c=0} \tag{6}$$

where t was the flow time measured for the sample solution at a given time t (s); t_0 (s) was the flow time of the solution (0.1 M HOAc and 0.1 M HOAc/0.2 M NaCl) without chitosan sample; C was the concentration of chitosan samples in diluted solution (g/mL),; and $[\eta]$ was intrinsic viscosity, mL/g.

The viscosity-average molecular mass (MM) of chitosan was calculated by the Mark–Houwink–Sakurada (MHS) (Eq. 7):

$$[\eta] = K(\mathrm{MM})^a \tag{7}$$

where K and a were the constants, $K = 1.81 \times 10^{-3}$, $a = 0.93$,[11] and $[\eta]$ was the intrinsic viscosity obtained from the Huggins and Kraemer plots.

3.6. Chitosan hydrolyzing activity assay

The extent of hydrolyzing activity in chitosan was measured by investigating the relative viscosity and the reducing ends. One percent (w/w) of chitosan solution was prepared in 100 mM NaOAc buffer at pH 4.5. Cellulase or lysozyme was added into the solution in a ratio of 1:100 (w/w), respectively, and the reactions were allowed to continue for 210 min in a 40 °C water bath to compare the hydrolyzing activity between the two enzymes.

The viscosity of the chitosan solutions at different reaction times with the enzymes were measured using a Brookfield DV-III + viscometer (Brookfield Inc., USA). Changes in the viscosity were expressed as the relative viscosity with respect to the control, enzyme-free chitosan solution.[26]

$$\text{Relative viscosity}(\%) = [(\text{Initial viscosity(cp)} - \text{Reduced viscosity(cp)}) / \text{Initial viscosity(cp)}] * 100$$

The absorbance of the reducing ends of chitosan was measured using the method of Shales and Shales[39] with some modifications.[26] Samples (3 mL) were extracted from the solution at different reaction times, boiled for 10 min to inactivate the enzymes, and reacted with 4 mL of a solution of 0.5 g/L potassium ferricyanide dissolved in 0.5 M Na_2CO_3. Mixtures were then boiled for 15 min for inducing color change by the amount of reducing ends. The samples were cooled and then centrifuged to remove the precipitated chitosan. Distilled water (1 mL) was added to 2 mL of supernatant and the absorbance of the solution was measured at 420 nm in a UV spectrophotometer (Shimadzu UV 160U).

3.7. Experimental design and data analysis

The Taguchi design method was used in the deacetylation study to identify optimal deacetylation conditions. The orthogonal arrays used in the Taguchi design had nine treatment trials for both the Kurtia and Broussignac methods as shown in Table 6. Each experimental run represented one trial. This array was designed for determining (1) the contribution of individual treatment factor, and (2) the level of each factor. The Taguchi design offered a simple and systematic approach to optimize the experiments and significantly reduced the number of treatment combinations when multiple factors were considered.[40]

In the Taguchi design, two parameters were applied to optimize the treatment conditions. The first parameter (K_{ij}) was the average value of each measured functional parameter in level j (j = 1, 2, 3) of each factor i (i = A, B, C, D) and expressed as

$$K_{ij} = \frac{1}{N_i}\sum_{u=1}^{N_i} y_{i,j}$$

where i represented the factor A, B, C, and D; j represented the level 1, 2, and 3; N_i is the number of trials for each factor, and $y_{i,j}$ is the measured values of factor i at level j. This parameter could explain how targeted parameters were changed in different levels of each treatment factor.

The second parameter, R_i, was the difference between the highest and lowest values of K_{ij}, and determined the most contributed factor among all factors. R_i was calculated as

$$R_i = (K_{ij})_{max} - (K_{ij})_{min}$$

where $(K_{ij})_{max}$ and $(K_{ij})_{min}$ indicated the highest and lowest values of the measured parameter in each factor, respectively. As R_i values increased among different factors, the factor which showed the highest value was the most contributed factor in determining the characteristics of the samples. Hence, by using the Taguchi design, the levels in each factor and the most contributed factor could be identified by using only nine treatment combinations.

The deacetylation study was conducted in triplicate, and each study was considered as a block. A one-way ANOVA was carried out to determine the significant differences among different factors and their levels, and the Tukey test was done for multiple comparisons in the Taguchi design method (SAS 9.2, SAS Institute, Inc., USA).

A completely randomized 2 × 2 × 8 factorial design was applied in the depolymerization study with two replications. Three independent factors were the type of enzymes (cellulase and lysozyme), the initial MM of chitosan (HMM and LMM), and depolymerized time (0–7 h). Interactions among the three factors were determined. PROC GLM was applied to determine the significant difference (P <0.05) among treatment factors using the SAS program (SAS 9.2, SAS Institute, Inc., USA).

4. Conclusions

Under tested treatment temperature and time, the NaOH deacetylation of β-chitin obtained from the jumbo squid pens resulted in β-chitosan with a wide range of degree of deacetylation, but little change in molecular mass, while the KOH treatment produced β-chitosan with a high degree of deacetylation (>93%) and a huge amount of polymer degradation at 120 °C. When using NaOH, the deacetylation time and temperature were ranked the first- and second-most contributing factors affecting molecular mass and degree of deacetylation, respectively. In the KOH deacetylation, the temperature was the most contributing factor that impacted both the molecular mass and the degree of deacetylation of chitosan. Chitosan could be depolymerized by cellulase and lysozyme, in

which cellulase had higher susceptibility on chitosan, resulted in a higher and faster chitosan degradation than that of lysozyme. Further studies to investigate the antimicrobial and antioxidant functions of β-chitosan from jumbo squid pens are underway.

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