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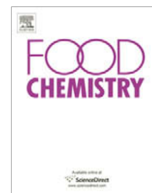

Low molecular weight chitosan prepared with the aid of cellulase, lysozyme and chitinase: Characterisation and antibacterial activity

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ABSTRACT

A continuous set of low molecular weight chitosan (LMWC) products was successfully made for this study by coordinating three enzymes (chitinase, lysozyme and cellulase) and two different deacetylated chitosan substrates (80% and 92%). It was observed that the intrinsic viscosity molecular weight (M_V) and SEC-HPLC-determined M_W distribution of LMWC were directed by both the used enzyme and the degree of chitosan substrate acetylation. LMWC prepared using lysozyme and 92%-deacetylated chitosan had larger M_W and, therefore, possessed higher antibacterial activity, as compared to other combinations. LMWC enzyme-directed properties suggest chitinase is more predictable and flexible to produce the specified M_V of LMWC. LMWC's solubility and antibacterial activity, determined as minimum inhibitory concentration (MIC), against *Escherichia coli* exhibited a negative linear relationship with log M_V. *E. coli* strains showed much higher susceptibility to LMWCs than *Staphylococcus aureus* strains did. Both species also showed intra-species sensitivity diversity toward LMWC.

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1. Introduction

Chitin, the raw material of chitosan composed of *N*-acetyl-glucosamine (NAG), is an offal product of the seafood processing industry. Chitosan, obtained by partial de-*N*-acetylation of chitin, has drawn increasing attention due to its superior solubility and reactivity, in comparison with chitin, which gives the former enhanced anti-microbial, anti-tumour, hypolipidaemic, hypocholesterolaemic, and immuno-stimulation biofunctionalities (Kim & Rajapakse, 2005; Tharanathan & Kittur, 2003). Chitosan's biofunctionalities are highly related to its molecular weight and degree of acetylation. The antibacterial functions of chitosan and its derivatives represents their primary utility in biological applications. Regardless of the source of chitosan, its antibacterial efficacy is influenced by a number of factors, which include degree of polymerisation (Park, Kim, & Lee, 2002; Park, Lee, & Kim, 2004; Tsai, Zhang, & Shieh, 2004), microorganism species (Gerasimenko, Avdienko, Bannikova, Zueva, & Varlamov, 2004; Park, Je, Byun, Moon, & Kim, 2004), and the degree of deacetylation (DD) at which antibacterial activity increases (Tsai, Su, Chen, & Pan, 2002). Low molecular weight chitosan (LMWC), due to its ready solubility in water, is more amenable for a wide variety of biomedical applications. It was reported that LMWC (5–10 kDa) had the highest bactericidal activity against pathogenic bacteria (Jeon, Park, & Kim, 2001), whereas a 20 kDa product prevented progression of diabetes mellitus and showed a higher affinity for lipopolysaccharides than the native chitosan of about 140 kDa (Kondo, Nakatani, Hayashi, & Ito, 2000). Several studies revealed its antimicrobial potential as dependent on degree of acetylation (Jeon et al., 2001). Besides, LMWC of 5–10 kDa has also been shown to hold potential as a DNA delivery system (Richardson, Kolbe, & Duncan, 1999).

Chitosan could be depolymerised into LMWC by physical, chemical or enzymatic methods (Cheng & Li, 2000; Ogawa, Chrispinas, Yoshida, Inoue, & Kariya, 2001). Physical methods (e.g., sonication, shearing) require special equipment, while reactions from chemical hydrolysis using HCl, H_2SO_4, H_2O_2, or HNO_2 are difficult to control, often resulting in inconsistent and over-depolymerised products. Enzymatic depolymerisation, based on either specific or non-specific enzymes, holds specific advantages over the other two methods, as it minimises the cited drawbacks while offering easy control of reactions. Chitosanase, a specific enzyme for chitosanolysis, is expensive, unavailable in bulk and often results in a preferential formation of chitooligomers-monomers due to its specificity. On the other hand, non-specific enzymes

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(e.g., pectinase, papain), which are inexpensive and commercially available, result mainly in the formation of LMWC (Kittur, Kumar, Gowda, & Tharanathan, 2003; Vishu Kumar, Varadaraj, Lalitha, & Tharanathan, 2004). By varying reaction conditions (e.g., reaction medium pH, temperature and time), LMWC of *M*w in the range 9.0 ± 0.5 kDa was obtained by depolymerising chitosan using the non-specific enzyme pronase (Vishu Kumar, Gowda, & Tharanathan, 2004).

It has been demonstrated that lysozyme only recognizes the $NAG_{(3\text{-}5)}$ section along the polymer as a hydrolysing site (Kurita, Kaji, Mori, & Nishiyama, 2000). However, chitinase, a glycosidase (EC 3.2.1.14), can cut the β-1,4-glycosidic bond at the non-reducing end of NAG (Liu, Kao, Tzeng, & Feng, 2003). The production of chitooligosaccharide with an acceptable degree of polymerisation was made possible by selectively breaking down *N*-acetylated chitosan on an acetylated site with chitinases (Aiba, 1994). Chitinases have been used intensively for the purpose of generating fungal protoplasts (Kumari & Panda, 1992) and are an attractive alternative to fungicides and insecticides as environmentally safe bio-control agents in the inhibition of phytopathogens (Lorito, Hayes, Di Pietro, Woo, & Harman, 1994). Cellulase, similar to chitinase, is an endoglycosidase (EC 3.2.1.4) able to cut randomly the β-1,4-glycosidic bond along the chitosan fibre.

This study was intended to use these three enzymes to produce a series of LMWC. Due to the different catalytical behaviours of these enzymes in reaction with chitosan, a series of LMWC products with a more complete molecular weight spectrum could possibly be created by hydrolysing adjusted-DD chitosan through flexibly manipulating cellulase, chitinase, and lysozyme. Such a study should provide more complete information, in order to understand how LMWC exerts its antibacterial efficacy and how this is affected by DD, M_W, bacteria species, and solubility during antibacterial action. Furthermore, the complete molecular weight spectrum can also provide a more precise range of molecular size based on specific biofunctionality need.

2. Materials and methods

2.1. Materials

N-acetyl-D-glucosamine, tri-*N*-acetylchitotriose, and hexa-*N*-acetyl-chitohexaose were purchased from Sigma (St. Louis, MO). Lysozyme (EC 3.2.1.17) and cellulase (EC 3.2.1.4, produced by *Trichoderma reesei* (ATCC 26921) were purchased from Sigma C2730. A crude chitinase enzyme was prepared by inducing *Trichoderma harzianum* (BCRC 30821) into a chitin-containing medium. This research also made use of Difco culture media (BD, Franklin Lakes, NJ). Chemicals and solvents were all analytical grade and purchased from Merck (Darmstadt, Germany). Chitin was purchased from Sigma and chitosans with DDs of 92 and 80, determined by Fourier Transform infrared (FTIR) spectroscopy, were purchased from a local company.

2.2. Microorganisms

Microorganisms used in this study were all purchased from the Bioresource Collection and Research Centre of the Food Industry Research and Development Institute (FIRDI, Taiwan). The five strains of bacteria used for testing the antibacterial activity of LMWC included three strains of *S. aureus* (BCRC 10451, 10780, and 10781) and two strains of *E. coli* (BCRC 10314 and 10675). *T. harzianum* (BCRC 30821) was used to prepare crude chitinase. *E. coli* was cultured in nutrient broth medium at 37 °C, and *S. aureus* was cultured in trypticase soy broth medium at 37 °C.

2.3. Preparation of crude chitinase enzyme complex and chitinolytic activity assay

Chitinase was prepared according to Felse and Panda (2000). The seed culture medium contained (in g/l): dextrose, 10; $(NH_4)_2SO_4$, 4.2; NaH_2PO_4, 6.9; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.3; peptone, 1.0; citric acid monohydrate, 10.5; and urea, 0.3. The medium was inoculated with *T. harzianum* spores (approximately 5×10^5 spores per ml) from a 120-h-old slant. The chitinases induction medium had the following composition (in g/l): colloidal chitin 10; $(NH_4)_2SO_4$, 4.2; NaH_2PO_4, 6.9; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.3; Tween 80, 0.2; $FeSO_4 \cdot 7H_2O$, 0.005; $MnSO_4$, 0.0016; $ZnSO_4$, 0.0014; $CaCl_2 \cdot 2H_2O$, 0.002; pH 5.0. The induction medium was inoculated with 5% (v/v) 48-h-old seed culture and cultured for 5 days at 30 °C to obtain an optimal chitinase yield.

The chitinolytic activities of enzymes used in this study were assessed by measuring enzyme-released reducing sugar in the hydrolysing reaction through the dinitrosalicylic acid (DNS) method (Ilyina, Tikhonov, Albulov, & Varlamov, 2000). An enzymatic hydrolysing reaction was performed by mixing 1% colloidal chitin solution (pH 4.0) with properly diluted enzyme solutions and reacting in a 42 °C water bath for 1 h. Enzymes were then inactivated by heating at 100 °C for 10 min. Supernatant of reaction mixture obtained after a centrifugation at 10,000*g* for 10 min was mixed with a 2-fold volume of DNS reagent and boiled for 15 min, followed by a quick cool down in water. The result was read at OD_{590}, using a Biomate-3 spectrophotometer (Thermo Spectronic, Rochester, NY). One unit of enzyme activity represented one μmole equivalent of NAG produced per hour.

2.4. Preparation of enzymatic chitosan hydrolysates

Two hundred millilitres of 2.2% chitosan (DD80 or 92), dissolved in 100 mM sodium acetate buffer (pH 4.0), was mixed with 20 ml crude chitinases, cellulase, or lysozyme at 100 U/ml. This mixture was then incubated at 42 °C. An aliquot (10 ml) of the mixture was withdrawn at the necessary time and then boiled for 10 min to stop the enzymatic reaction. The chitosan hydrolysate prepared from a specified condition is designated as DD#_enz_time, where # represents the value of DD, enz represents the used enzyme, and time represents the hydrolysing period. For example, an LMWC product produced by hydrolysing DD92 chitosan with chitinase for 24 h would be designated as DD92_chit_24 h. Chitosan hydrolysate samples were kept at −20 °C until use.

2.5. The assay of degree of LMWC polymerization

Chitosan hydrolysates were sampled at 3, 6, 9, 24, 48, 72, 96, 120, 144, 168 h and measured for viscosity. The M_V of LMWC was determined based on its intrinsic viscosity, obtained in 0.25 M HAc/0.25 M NaAc at 25 °C, according to the equation of Mark-Houwink-Sakurada (MHS) (Kasaai, Arul, & Charlet, 2000) stated as:

$[\eta] = 1.49 \times 10^{-4} M_V^{0.79}$ Due to viscometry limitations, the measurable range for M_V reaches only about 6 kDa.

2.6. SEC-HPLC

The M_W profiles of LMWC samples were determined by a size-exclusion column, TSKgel G3000PW (7.5 × 300 mm, Tosoh Co., Tokyo, Japan). The HPLC system was composed of an isocratic pump (SpectraSeries P100; ThermoQuest, San Jose, CA) and a differential refractive index detector (Shodex RI-101; Showa Denko Co., Tokyo, Japan). The mobile phase was composed of a 100 mM sodium acetate buffer (pH 4.0) at a flow rate of 1.0 ml/min. The monomer,

trimer, and hexamer of *N*-acetyl-D-glucosamine and dextran with 10 kDa were standard markers.

2.7. Solubility assay

The solubility of each LMWC sample was defined as the water-soluble solid content (w/v) in a neutral solution. The LMWC sample was neutralised with 200 mM phosphate buffer at a volume ratio of 1:1. The insoluble solid of the mixture was precipitated by centrifugation at 6000*g* for 30 min and washed three times with distilled water to remove solvent residue. Total solids and insoluble solids were dried in an oven at 60 °C until a constant weight was obtained. Solubility (g/l) was calculated as:

(total solid dried weight – insoluble solid dried weight)/sample volume.

2.8. Minimal inhibition concentration (MIC) test

One hundred microlitres of each 16-hour-old, $\sim 5 \times 10^6$ cfu/ml tested microorganism was mixed with 100 μl sterilised chitosan hydrolysate samples, which were 2-fold serially diluted in advance with a corresponding medium buffered with 100 mM phosphate buffer (pH 7.0) to the designated concentration. A corresponding mixture containing no tested strain was used as a control. The mixture was then applied to each well of a 96-well microplate and incubated for 48 h at a temperature suited for each microorganism. Growth of the tested microorganism was monitored at OD_{590} every 6 h for 48 h by an ELISA reader (Biolog, Hayward, CA). All treatments were conducted five times. MIC was ultimately defined as the lowest concentration of the sample required to inhibit bacterial growth for over 48 h beyond that of the control sample.

2.9. 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 test, paired *t*-test, multiple regression, and Pearson correlation analysis using SAS Version 8.01 (SAS Institute, Cary, NC).

3. Results and discussion

According to the catalytic behaviours of lysozyme and chitinase, as mentioned in the introduction section, in hydrolysing chitosan, the chitosan with higher DD will be digested into larger fragments. Kurita et al. (2000) suggested the best DD for chitosan to be hydrolysed by lysozyme to be about 56%, and that chitosan with DDs of 11% and 97% (having, respectively, too many or too few continuous NAG) cannot be hydrolysed by lysozyme. Therefore, in order to create a continuous M_V spectrum for LMWC products, this study chose to use chitosans of DD80 and DD92.The expected result was observed in Fig. 1A, which indicated the relationship between LMWC's M_V and the hydrolysing time interval. The M_V of LMWC was determined in weak acid solution by intrinsic viscosity. Due to viscometry limitations, the measurable range for M_V reaches only about 6 kDa. Apparently, the M_V of LMWC from each of the combinations decreased steeply during the first 3 h (from 323.7 kDa (DD92) and 371.5 kDa (DD80) to 15–38 kDa) and approached stable sizes by 24 h. LMWC products collected at a hydrolysing time of 96 h, representing the near completion of reaction, produce molecular sizes (from largest to smallest) as: DD92_lys (22.2 kDa), DD80_lys (13.9 kDa), DD92_chit (11.2 kDa), DD80_chit (8.3 kDa), DD80_cel (<6 kDa), and DD92_cel (<6 kDa). By performing the paired *t*-test (Fig. 1A), the products prepared from DD80 were significantly smaller than that from DD92 in their M_V at

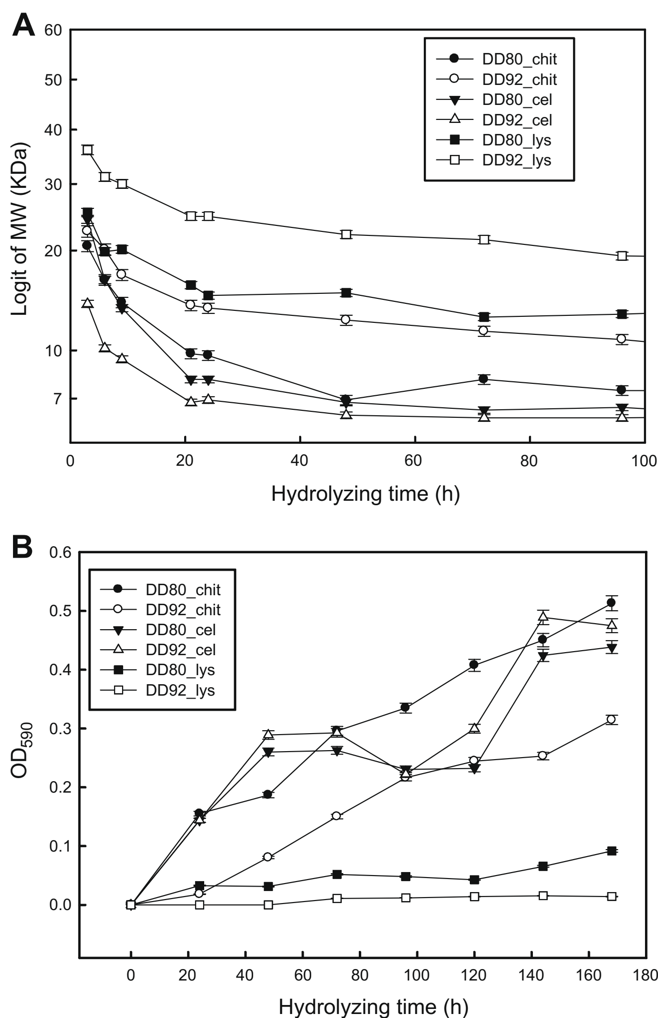

Fig. 1. The decrease in molecular weight of chitosan hydrolysates (A) and DNS-reacted reducing sugar released (B) in the enzymatical hydrolysis of chitosan. Chitosan with DD of 80 (solid symbol) and 92% (open symbol) were hydrolysed using one of three enzymes (chitinase [circle], cellulase [triangle], and lysozyme [rectangle]) to create six chitosan hydrolysate sample groups. To better highlight y-scale values, M_V (kDa) was transformed using a logit calculation. The paired *t*-test was performed to analyse the DD effect (80% and 92%) on the LMWC's M_V prepared using lysozyme, chitinase, and cellulase, and the significance level of difference (*p*) for each enzyme was 0.001, 0.036, and 0.025, respectively.

corresponding hydrolysing time for lysozyme ($p = 0.001$) and chitinase ($p = 0.036$), except cellulase. Chitinase and lysozyme can recognize NAG and $(NAG)_{3-5}$, respectively, which also helps explain the smaller molecular size of LMWC_chit as compared with LMWC_lys, due to the fact that NAG sites can be found more easily, in theory, than $(NAG)_{3-5}$ sites along the chitosan molecule. Besides, both the chitinase and cellulase used in this study were mixtures of endo- and exoenzymes. Fast degradation during the early hydrolysis stage was attributed to endoenzyme activity. Exoenzymes modified the initial LMWC product into smaller fragments by liberating dimer and monomer NAG or AG flanked on the reducing end of chitosan. Taking another perspective, LMWC_chit demonstrates better flexibility in producing a variable M_W range as compared with LMWC_lys because the former can be further modified by exo-chitinase.

Interestingly, the DD conversely affected the M_V profile of LMWC when produced with cellulase, by which the LMWC made from DD80 was significant larger than DD92 ($p = 0.025$). This phenomenon could be attributed to the higher solubility of chitosan_92. Since it is well-known that higher DD of chitosan is

more soluble in weak acid solution, the lower solubility of chitosan_DD80 could slightly limit its accessibility to cellulase and result in a slower reaction.

In order to further investigate the stability of product molecular sizes in relation to hydrolysing time, the amount of reducing sugar was monitored using the DNS method. Fig. 1B shows how the reducing sugar of DD92_chit, DD92_lys, and DD80_lys gradually stopped increasing at 96 h of hydrolysing time, and the other combinations, DD80_chit, DD80_cel, and DD92_cel, kept releasing reducing sugar afterward along the hydrolysing time and became smaller in molecular size. The extensive degradation of LMWCs in DD80_cel and DD92_cel reaction was attributed to the nonspecific digesting behaviour of cellulase, which led the digestion of chitosan to be more complete. A similar result, although the released reducing sugar was not as much, was observed from LMWC_DD80_chit, which could be due to the chitosan_DD80 provided plenty of NAG cutting sites to chitinase, especially exo-chitinase.

LMWC is generally known to possess many specific biological functions, especially antibacterial functions, that are dependent on its size. Specifying an exact size range for LMWC, however, has proven difficult. Several researchers reported that the M_W of LMWC, created using only one kind of enzyme, can be managed by controlling hydrolysing time (Vishu Kumar, Varadaraj, Gowda, & Tharanathan, 2007). Apparently, this does not represent a very practical commercial method for producing a particular molecular size range of LMWC products. Our results suggest that creating a series of LMWC products with fairly continuous nominal M_W is

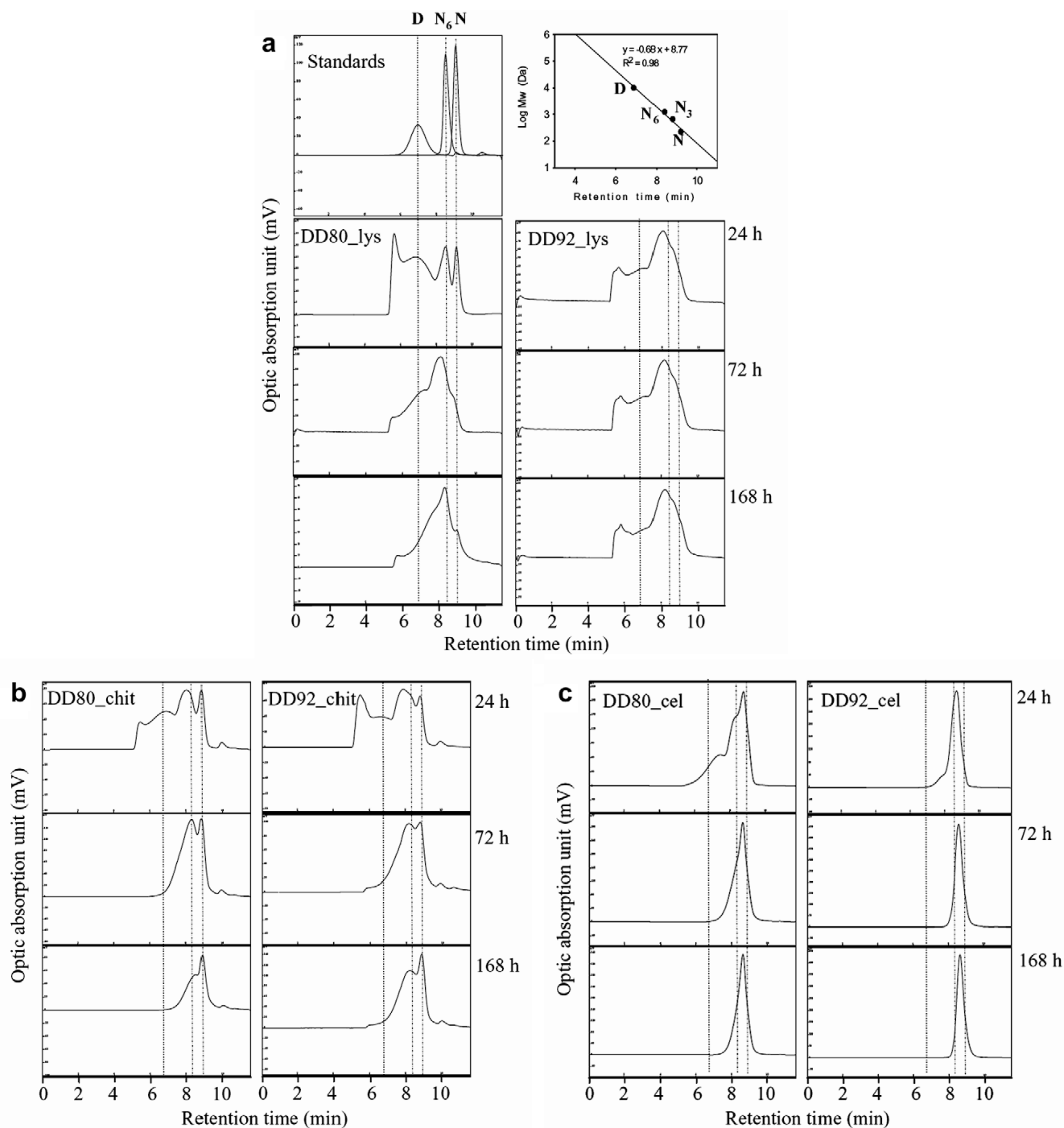

Fig. 2. SEC-HPLC profiles of standard marker, LMWC_lys (A), LMWC_chit (B), and LMWC_cel (C), sampled at hydrolysing times of 24, 72, and 168 h. Standard markers: D, dextran (~10 kDa); N, NAG monomer; N_6, NAG. hexamer.

possible by adjusting chitosan DD by associating it with a suitable target-specified enzyme.

LMWC products produced from different enzymes, even though they share a similar M_V, may have significantly different molecular size distribution. The M_W profiles of LMWCs were further analysed by SEC-HPLC and the results were shown in Fig. 2. A molecular weight standard curve (Fig. 2A) was constructed by plotting log M_W of standards, NAG (221 Da), NAG_3 (627 Da), NAG_6 (1236 Da), and dextran (10 kDa), against retention time, which demonstrated a high linear relationship ($r^2 = 0.98$). DD92_lys exhibited the best stability in molecular size distribution, with largest molecule of ∼50 kDa, from 24- to 168-h LMWC_lys products (Fig. 2A). The DD80_lys (Fig. 2B) and DD92_chit (Fig. 2C) approached stablility in 72 h, with M_W principally scattering between 1236 Da (NAG_6) and 10 kDa (dextran). Besides, the major form of sugars continuously released during hydrolysing process was NAG for LMWC_chit but $(NAG)_{2\text{-}5}$ for LMWC_cel (Fig. 2C). Actually, many workers have observed similar results of releasing $(NAG)_{2\text{-}5}$ in the degradation of chitosan by cellulases obtained from various microbial sources, including *Bacillus cereus*, *Trichoderma viride*, *Streptomyces griseus*, etc. (Xia, Liu, & Liu, 2008). Chitosan_DD92_cel was almost converted into $(NAG)_{2\text{-}5}$ in 72 h and exhibited a better digestibility to cellulase than chitosan_DD80, which can also explain the faster degradation of DD92_cel than DD80_cel found in Fig. 1A.

In this study, for each of the three enzymes, the same amount of chitinolytic activity unit (100 U/ml), defined with colloidal chitin as substrate, was applied in each hydrolysing reaction. However, cellulase displayed a stronger catalysing capability than lysozyme and chitinase, here indicating its nonspecific cutting behaviour instead of its high chitinolytic activity, and LMWC_cel product profile almost centred at a peak close to $(NAG)_{2\text{-}5}$, which was far off 10 kDa. In contrast, the profiles of LMWC_chit and LMWC_lys were distributed wider somewhere around dextran. The result suggested that to produce an LMWC_cel product with the specified M_W by controlling hydrolysing time was not as easy, as compared with LMWC_lys and, especially, LMWC_chit.

The effective dose of chitosan for antibacterial activity is known to be influenced by DD, degree of polymerisation (DP), solubility and the microorganism species. Generally, higher DD and DP provide enhanced antibacterial efficacy. However, several studies on the relationship between effectiveness and M_W have made widely conflicting statements, with one suggesting LMWC effective only at an M_W larger than 10 kDa and another reporting LMWC effective at M_W less than 1 kDa. Most Gram positive strains of bacteria (especially *S. aureus*) were found to be more resistant to chitosan than Gram negative strains (Chung et al., 2004) (e.g., *E. coli*). However, some studies disagree with this result (No, Young Park, Ho Lee, & Meyers, 2002). Such varying results may arise from one or more of the following: different enzymes used to produce LMWC; microorganism strains tested; and methods used to determine M_W, among others. To resolve these disputes, LMWC products with continuous M_V prepared with three catalytically different enzymes were used in antibacterial activity tests in this study. Table 1 shows *E. coli* to be much more susceptible to LMWC than *S. aureus* in each set of LMWC products. With regard to specific bacteria species, it has previously been demonstrated that Gram-negative bacteria are more susceptible to chitosan than Gram-positive bacteria due to the higher negatively-charged cell wall surface (Chung et al., 2004). These results help to explain different levels of sensitivity to LMWC between bacteria species found in this study, with *E. coli* (BCRC 10314) and *S. aureus* (BCRC 10780), respectively, the most and the least sensitive. As found in several similar studies, LMWC of higher DD (DD92) and larger M_W is more effective than LMWC of lower DD (DD80) and smaller M_W.

In this study, the two *E. coli* strains showed a more complete MIC spectrum to various LMWC, in comparison with the three tested *S. aureus* strains, and were used for follow-on tests to reveal the effects of M_V, substrate's DD, enzyme source, and solubility on LMWC antibacterial activity. The paired *t*-test result (data not shown) revealed a significant difference ($p < 0.005$) between two *E. coli* strains, BCRC10314 and BCRC10675, in their sensitivity to the corresponding LMWC samples. In order to inspect the effect of M_V on MIC value, a continuous log M_V spectrum, constructed by using LMWC_chit and LMWC_lys, was plotted against both *E. coli* strains; here DD and enzyme effects were ignored. The result (Fig. 3) revealed LMWC was more effective against BCRC 10314 and with a higher relationship ($r^2 = 0.77$) between its log M_V and the resulting MIC than to BCRC 10675 ($r^2 = 0.54$). The imperfect relationship may arise from the unique property of LMWC product directed by the specified substrate's DD and enzyme.

To realise the effects of M_V, substrate's DD, and enzyme source on LMWC's antibacterial activity, multiple regression analysis was performed against MIC toward two *E. coli* strains separately.

Table 1
The MIC values of two *E. coli* strains and three *S. aureus* strains as compared against LMWC prepared from two chitosan DDs (80% and 92%) in an enzymatic process using chitinase (Chit), cellulase (Cel), and lysozyme (Lys). LMWC products were collected at several different points in time.

DD	Enzyme	Processing time (h)	M_V (kDa)	Solubility (gL^{-1})	*E. coli*[A] (BCRC 10314)	*E. coli* (BCRC 10675)	*S. aureus* (BCRC 10451)	*S. aureus* (BCRC 10780)	*S. aureus* (BCRC 10781)
92%	Initial	0	323.7						
	Chit	24	13.5	1.54	50 ± 0.0[a]	50 ± 0.0[a]	480 ± 178.9[ae]	1600 ± 0.0[a]	800 ± 0.0[a]
		72	11.2	1.86	60 ± 11.2[b]	80 ± 13.7[be]	1120 ± 219.1[b]	NI	1520 ± 178.9[b]
		168	8.5	5.39	100 ± 0.0[c]	120 ± 22.4[c]	NI	NI	NI
	Cel	24	6.9	7.73	120 ± 22.4[d]	180 ± 22.4[d]	NI	NI	NI
		72	<6	>10.0	NI[B]	NI	NI	NI	NI
	Lys	24	24.8	0.16	40 ± 13.7[a]	60 ± 11.2[e]	ND[C]	NI	ND
		72	22.2	0.79	50 ± 0.0[a]	70 ± 13.7[ef]	800 ± 0.0[c]	NI	880 ± 178.9[a]
		168	12.0	1.70	70 ± 13.7[be]	90 ± 11.2[f]	NI	NI	NI
80%	Initial		371.5						
	Chit	24	9.6	3.35	70 ± 13.7[be]	100 ± 0.0[g]	880 ± 178.9[c]	NI	NI
		72	8.3	5.48	100 ± 0.0[c]	140 ± 27.4[cd]	1600 ± 0.0[d]	NI	NI
		168	<6	>10.0	NI	NI	NI	NI	NI
	Cel	24	8.1	6.23	120 ± 22.4[d]	200 ± 0.0[h]	NI	NI	NI
		72	<6	>10.0	NI	NI	NI	NI	NI
	Lys	24	14.8	0.83	50 ± 0.0[a]	100 ± 0.0[g]	400 ± 0.0[e]	800 ± 0.0[b]	720 ± 178.9[a]
		72	13.9	1.20	70 ± 13.7[be]	120 ± 22.4[c]	880 ± 178.9[c]	1040 ± 219.1[c]	880 ± 178.9[a]
		168	10.1	2.80	90 ± 11.2[e]	180 ± 22.4[d]	NI	NI	NI

[A] MIC ($\mu g\ ml^{-1}$). Values are mean ± standard deviation. Means in the same column followed by different letters are significantly different ($p < 0.05$).
[B] NI: No inhibition, MIC > 1600 $\mu g\ ml^{-1}$
[C] ND: Not determined owing to low solubility.

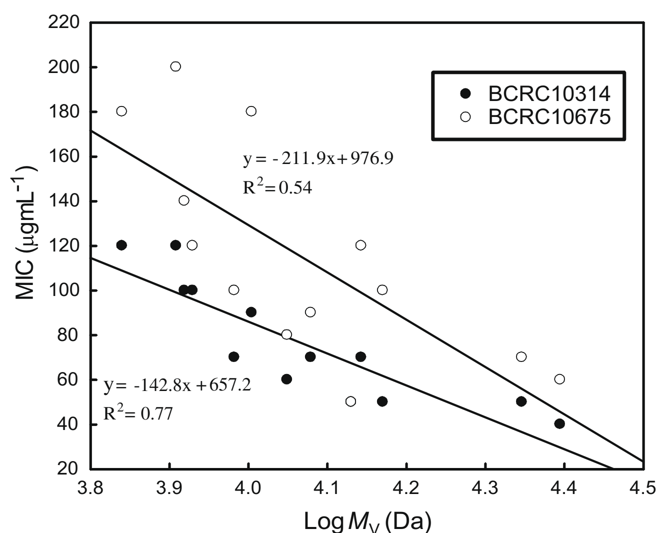

Fig. 3. The relationship between log M_V and the MIC of LMWC against two *E. coli* strains, BCRC10314 and BCRC10675.

Cellulase was excluded in this test because of only limited MIC data available. According to the statistical result (Table 2), the influence of substrate's DD on LMWC's antibacterial activity, exhibited as the standardised partial regression coefficient (b), could be ignored against BCRC10314 strain ($b = 0.06$, $p = 0.54$) and BCRC10675 ($b = 0.15$, $p = 0.1$). Furthermore, the influence of enzyme source on MIC of LMWC against both strains ($b = 0.25$, $p = 0.03$ and 0.48, $P = 0.01$, respectively) was found significant but not as strong as M_V, which showed a much higher regression coefficient to MIC against both *E. coli* strains ($b = -0.83$ and -0.85, $p < 0.001$). Although chitinase and lysozyme share similar chitinolytic behaviour, regarding antibacterial properties, the result showed unignorable differences between them in producing LMWC. LMWC_chit may have better homogeneous properties than LMWC_lys because the NAG residues flanked on the reducing ends are more likely to be removed by exo-chitinase instead of lysozyme. This may be partially explained by performing Pearson correlation analysis between log M_V and the resulted MIC value according to the enzyme source. The results in Table 3 demonstrated a significant correlation existed between the log M_V and MIC against BCRC 10314 and BCRC 10675, where LMWC-chit (−0.77, −0.72) could be better predicted than LMWC_lys (−0.63, −0.60).

Good chitosan solubility is of the greatest concern, as low solubility limits applicability of this product in the food sector. Partially hydrolysed chitosan was found to have a better antibacterial efficacy than chitosan submitted to extended hydrolysis (Uchida, Izume, Ohtakara, Uchida, Izume, & Ohtakara, 1989), although it is less soluble at neutral pH. Actually, it has been established that enzymatically-hydrolysed chitosan with a M_W between 5 and 27 kDa demonstrates adequate antibacterial efficacy, due to improved solubility over its unaltered form (Gerasimenko et al., 2004). Furthermore, chitosan with an M_W greater than 30 kDa cannot be used as an antibacterial agent, due to poor solubility in aqueous solutions at a neutral pH (Sekiguchi et al., 1994). The data shown in Table 1 indicated similar results that M_V less than 6 kDa showed no antibacterial activity and M_V higher than ~25 kDa had relatively low solubility. Fig. 4 shows that solubility decreased linearly with log M_V for LMWC_lys ($r^2 = 0.73$), and, especially, for LMWC_chit ($r^2 = 0.90$). Also, at a similar M_V, LMWC_chit showed better solubility as compared with LMWC_lys. Such results may be attributed to the more uniform and higher DD of LMWC_chit, which, may also enhance its antibacterial activity.

Table 2
Multiple regression analysis for the factors in preparing LMWC, including M_V, enzyme source, and chitosan's DD, against the resultant MIC toward two tested *E. coli* strains.

MIC	Standardized partial regression coefficients (b)			
Strain	log M_V	Enzyme	DD	Multiple r^{2} [b]
BCRC10314	−0.83 ($p < 0.001$)[a]	0.25 ($p = 0.03$)	0.06 ($p = 0.54$)	0.650 ($p < 0.001$)
BCRC10675	0.85 ($p < 0.001$)	0.48 ($p < 0.01$)	0.15 ($p = 0.1$)	0.606 ($p < 0.001$)

[a] Significance level.
[b] Correlation coefficient.

Table 3
Pearson correlation analysis between log M_V of LMWC, prepared from chitinase and lysozyme, and the resultant MIC.

	MIC	
	BCRC10314	BCRC10675
Log M_V		
LMWC_chit	$r_p^2 = -0.77$	$r_p^2 = -0.72$
LMWC_lys	$r_p^2 = -0.63$	$r_p^2 = -0.60$

r_p^2, Pearson correlation coefficient ($p < 0.001$).

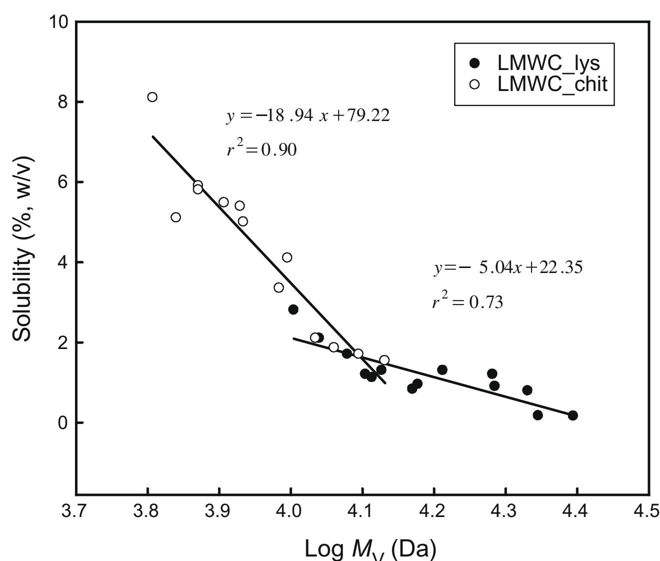

Fig. 4. The relationship between the log M_V and solubility of LMWC prepared using chitinase and lysozyme.

4. Conclusion

The results reported here demonstrated that a broad and more complete M_V spectrum of LMWC can be obtained by coordinating these three enzymes, chitinase, lysozyme and cellulase, and chitosan substrate DDs. The LMWC prepared from chitosan_DD92 has larger M_V and, therefore, possess higher antibacterial activity as compared to chitosan_80. In this study, using chitosan_DD92 as the example, the ideal enzyme to produce a given molecular size range of LMWC is suggested as: lysozyme: $M_V > 12$ kDa, chitinase: $M_V > 8.5$ kDa, and cellulase: $M_V < 6.9$ kDa. According to obtained results, we can conclude that chitinase is more predictable and flexible to produce LMWC exhibiting a specified molecular weight. Besides, in various applications, chitinase is the better choice with which to produce LMWC if inhibition of Gram-negative bacteria

and high solubility represent primary concerns. Compared to chitinase, lysozyme-catalysed LMWC, with a higher range of $M_{V,}$ is less soluble but more effective against both *E. coli* and *S. aureus*. Therefore, lysozyme may be considered first if low MIC or Gram-positive bacteria inhibition represent primary concerns. Cellulase-catalysed chitosan hydrolysate, although highly soluble, lost its antibacterial activity due to extensive hydrolysis. With improved understanding regarding the strong relationships between chitosan biofunctionalities and its molecular weight and acetylation degree, the technique proposed in this paper should provide more flexible utilisation of chitosan as a biomaterial.

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