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Enzymatic Degradation of Chitosan using *Staphylococcus species* strain TU005 (E) Chitinase

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บทคัดย่อ

งานวิจัยนี้ได้กล่าวถึงการย่อยไคโตซานด้วยเอนไซม์ไคตินเนสซึ่งสกัดจากเชื้อ *Staphylococcus species* สายพันธุ์ TU005 (E) ที่แยกได้จากดินในประเทศไทย การประเมินแอคติวิตี้ของไคตินเนสต่อ colloidal chitin ถูกทำโดยการวัดความขุ่นและการวัดสีที่เปลี่ยนไป สภาวะที่เหมาะสมสำหรับการย่อยด้วยไคตินเนสสรุปได้จากการศึกษาการวิเคราะห์น้ำหนักโมเลกุลที่ลดลงภายใต้การเปลี่ยนแปลงของ pH ความเข้มข้นของสับสเตรทและอุณหภูมิ โครงสร้างของไคโตซานที่ย่อยด้วยเอนไซม์แล้วได้ถูกวิเคราะห์โดยเทคนิค FT-IR EA และ XRD

Abstract

Enzymatic degradation of chitosan via chitinase obtained from *Staphylococcus species* strain TU005 (E) which is isolated from the soil in Thailand is discussed. The activity of chitinase for colloidal chitin is evaluated by means of turbidimetric and colorimetric assays. The optimum condition is proposed for chitinase degradability on the basis of the molecular weight reduction under the variables of pH, substrate concentration and temperature. Structural analyses of the enzymatic-degraded chitosan studied by FT-IR, EA, and XRD are clarified.

Keywords: *Staphylococcus species* strain TU005 (E), Oligochitosan, Turbidimetric assay, Colorimetric assay, Enzymatic degradation.

Introduction

Chitin, (1→4)-linked-2-acetamido-2-deoxy-β-D-glucose, is the second most abundant biopolymer after cellulose. In nature, chitin appears to be a copolymer chain with chitosan unit ((1→4)-linked-2-amino-2-deoxy-β-D-glucose). Chitin-Chitosan is applicable in biomedical, agricultural, pharmaceutical fields owing to its bioactivity, biocompatibility and biodegradability. For the past decades, the researches on chitin-chitosan have received much interest in various aspects such as physical modification, i.e., film and gel crosslinking (Wang, *et al.* 1997; and Chen, *et al.* 1997), fiber extrusion (Tokura, *et al.* 1979; and Rathke, *et al.* 1994), membrane casting (Kanke, *et al.* 1989; and Bonvin, *et al.* 1994), and chemical modification, i.e., N-acylchitosan (Hirano, *et al.* 1989), and carboxymethylchitin (Tokura, *et al.* 1983).

Concerning the structural developments, chitosan faces the problems of solubility and reactivity due to its high molecular weight and strong inter- and/or intra-molecular hydrogen bonding along the main chain. Decreasing of molecular weight of chitosan or chain length will lead to the effective chemical modification for unique derivatives which cannot be achieved in high molecular weight chitosan as evidenced from specific properties (Kendra and Hadwiger, 1984). Theoretically, the reduction of molecular weight can be done by acid or base hydrolysis (Defaye, *et al.* 1989; and Allan, *et al.* 1997), photoirradiation (Ulanska, *et al.* 1992; and Andrady, *et al.* 1996) and enzymatic hydrolysis (Hirano, *et al.* 1989; and Aiba, 1994). Enzymatic system is the most interesting pathway to provide the well-defined low molecular weight and/or oligochitosan under the mild and environmental friendly conditions. Although many enzymes have been reported for the degradation such as lysozyme, chitinase (*Streptomyces griseus*, *Bacillus sp.*, *Bacillus sp.* PI-7S, *Serratia marcescens* QMB 1466, *Aeromonas hydrophila*), chitosanase (*Bacillus sp.* No. 7-M, *Streptomyces griseus* HUT 6037), we still need to explore other novel chitinases and/or chitosanases to achieve an effective pathway.

The present work is, thus, based on the enzymatic degradation of chitin-chitosan by using chitinase produced from bacterial strain isolated from soil in Thailand, *Staphylococcus species* strain TU005 (E). The work is also extended to the structural characterization of the obtained chitosan product.

Experimental

Materials

Chitosan (74.57% deacetylation) was a gift from Assoc. Prof. Suwalee Chandrkrachang, the

Asian Institute of Technology, Thailand. Ammonium sulfate, dipotassium hydrogen orthophosphate, sodium acetate, and sodium hydroxide were the products of UNIVAR, Australia. Polyethylene glycol (MW 6 000), potassium dihydrogen phosphate, sodium carbonate anhydrous, N-acetyl-D-glucosamine were purchased from Fluka Chemical, Switzerland. Glacial acetic acid was from J.T Baker, U.S.A. Magnesium sulfate was from CARLO ERBA, France. Potassium ferricyanide was purchased from BDH Laboratory Supplies, England.

Equipments

Quantitative and Qualitative FT-IR spectra were obtained from a VECTOR 3.0 BRUKER spectrometer with 64 scans at a resolution of 4 cm^{-1} using an OPUS 3.0 software. The percent elements were obtained from a YANAKO CHN CORDER MT-3, MT-5 with a combustion temperature of 950°C. The samples were run under He (flowing rate 200 mL/min) and O_2 (flowing rate 20 mL/min). Du Pont TGA 2950 thermogravimetric analyzer, was applied for study the thermal stability of chitosan. Samples (approximately 5-7 mg) were loaded in a platinum pan and heated under a N_2 flowing rate of 20 mL/min with the heating rate 20°C/min from 30°C to 600°C. X-ray diffraction patterns were obtained from a RIGAKU RINT2000 using CuKα(λ = 0.154 nm) as an X-ray source at 40 kV, 30 mA with Ni filter. Samples (0.1-0.2 g) were ground with agate mortar and spread on a glass slide specimen holder to examine 2θ of 5-50°C. Brookfield viscometer RVDV-III, with a small sample adapter (SSA 21/13R) and a water bath, was used to measure the chitosan viscosity before and after treatment with the enzyme. The measurement was operated at 37°C by controlling the temperature in a water bath. Cannon-Ubbelohde viscometer Cole Palmer, U.S.A., capillary no. 50 B582, with a thermostatic bath DT-2 Heto, Denmark was applied to measure the chitosan viscosity in 0.1M sodium acetate / 0.2M acetic acid at 30°C. The chitinase enzyme solution was frozen under liquid nitrogen and lyophilized by a Flexi-Dry FTS, STONE RIDGE, New York, U.S.A.

Culture and media

Staphylococcus species strain TU005 (E) was isolated from soil in Thailand. *Staphylococcus species* strain TU005 (E) was maintained on colloidal chitin agar containing: colloidal chitin, 0.2%; yeast extract, 0.5%; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4.7H_2O$, 0.3 g; KH_2PO_4, 6.0 g; K_2HPO_4, 10.0 g; bacto-agar, 20.0 g; and 1.0 L. Colloidal chitin was prepared by acid hydrolysis of chitin (Shimahara and Takiguchi, 1988). Colloidal chitin liquid medium (CM medium) was used to grow bacteria for enzyme production. Luria-Burtani medium (LB medium) containing bacto-

tryptone 10 g, bacto-yeast extract 5 g, and NaCl 10 g (pH7.2-7.4) was used to generate innocumn. All of the media were sterilized by autoclaving for 20 min of 121°C before use.

Enzyme production

A single colony of *Staphylococcus species* strain TU005 (E) was grown overnight in LB broth medium to produce an innocumn. The obtained cell culture was innoculated at 1:100 dilution into CM medium. The culture was grown in a shaking water bath at 30°C for two days, at 250 rpm. The medium was sampled out to assay for chitinase activity using turbidimetric assay. After the activity reached the maximum point, the culture medium containing crude enzyme was centrifuged at 7 000 rpm for 20 min. The crude enzyme was concentrated to 1/3 of starting volume with PEG. The obtained enzyme was dialyzed in phosphate buffer (10 mM) pH 7.0 and lyophilized to obtain powder chitinase.

Enzyme assay

Turbidimetric assay

Enzyme solution (1 mL) was added to a mixture of 0.5 μg/mL colloidal chitin (0.5 mL) and 0.1 M phosphate buffer pH 7.0 (0.5 mL). After adding enzyme, the transmittance (%T) at 650 nm was measured every 5 min for 1 h.

Colorimetric assay

Chitinase activity was assayed by measuring reducing sugar produced from a mixture of excess colloidal chitin and the amount of enzyme (concentration 10 mg/mL) varying from 10 μL to 50 μL in 0.1 M phosphate buffer pH 7.0 (1.5 mL). The mixture was shaken at 37°C for 30 min. The reaction was terminated by boiling for 10 min. The released amino-sugar was measured by the modified Schales method (Imoto and Yagishita, 1971).

One unit (U) of chitinase activity is defined as the amount of enzyme that liberated 1 μmoL of reducing sugar as N-acetylglucosamine (GlcNAc) equivalent per min.

Enzyme stability

Enzyme stability was evaluated by measuring enzymatic activity after incubating chitinase solution (concentration 0.6 U/mL) for different length of time at 37°C. Chitinase solution was aliquoted and assayed for enzymatic activity using turbidimetric assay every 30 min. The enzyme stability was also studied in acetate buffer pH 4.5 compared to that of phosphate buffer pH 7.0.

Enzymatic hydrolysis of chitosan

Chitosan in 2% acetic aqueous solution (w/w) was prepared. A series of chitosan solution with different concentration (2%, 3% and 4% w/w) was prepared by dissolving in acetic aqueous solution (2% v/v). In each solution, 1 μL enzyme (18 mU/μL) solution was added and kept at 37°C. The enzyme activity was evaluated from the decrease of the chitosan solution viscosity. The condition that gave

the higher reduction in viscosity was selected and chitosan was reprecipitated with 1 M NaOH. The yellowish precipitant was collected by centrifugation at 15 000 rpm for 5 min.

Results and Discussion

Preparation of chitinase enzyme

Colloidal chitin was prepared and added to the culture medium for the *Staphylococcus sp.* to induce chitinase production. The highest activity of enzyme was achieved at 1.5-2 days cultivation Figure 1, after that the enzyme activity starts decreasing. This may due to depletion of nutrition, death of bacteria or degradation of the enzyme by some proteinases.

Generally, precipitation by $(NH_4)_2SO_4$ is the practical way to partially purify proteins. In our experiments, precipitation by $(NH_4)_2SO_4$ was found to cause a decrease in enzymatic activity of the crude enzyme. Therefore, the enzyme was partially purified by dialysis and concentrated by PEG to remove water and small molecules. After the enzyme was concentrated by PEG for 3 h, the activity was retained for 80-90% of its original activity. The enzyme was dialyzed and prepared into powder form by lyophilization. The total activity was found to be approximately 46% of the initial activity after lyophilization process, determined by turbidimetric assay Table I.

Figure 2(a) shows the calibration curve for chitinase degradation using standard N-acetylglucosamine determined by colorimetric assay. The amount of N-acetylglucosamine unit produced by chitinase 1 µL was measured as shown in Figure 2(b) to find the activity at 18 mU/mL.

Enzyme stability

The enzyme stability is an important factor determining the period of time in which an enzyme can function in a reaction. As shown in Figure 3, in either 0.1 M phosphate buffer or 0.2 M acetate buffer, chitinase activity decreases gradually to 50% of its initial activity after 2 h. There were only 10% of the activity left after 3-4 h.

However, in the preparation of low molecular weight chitosan, we incubated chitosan solution for 4 days with an additional enzyme every 24 h to ensure the complete digestion of chitin unit. The enzyme continued the activity over 2 h as observed from the viscosity reduction of the chitosan material. Comparing the activity of chitinase in the case of incubation in buffer and chitosan solution, it was clarified that the degradability of chitinase was prolonged by the existence of chitosan in the system.

Table 1 Activity of enzyme obtained from each preparation step (Mekkriengkrai, *et al.*)

Preparation step	Turbidimetric assay			
	Total volume (mL or mg)	Activity ($\Delta\%T_{650}$/min/mL)	Total activity ($\Delta\%T_{650}$/min)	% yield
Crude enzyme	2756	0.9	190	100
PEG absorbed enzyme	50	2.9	167	88
Lyophilized enzyme	360	0.3 [a]	87	46

[a] $\Delta\%T_{650}$/min.mg.

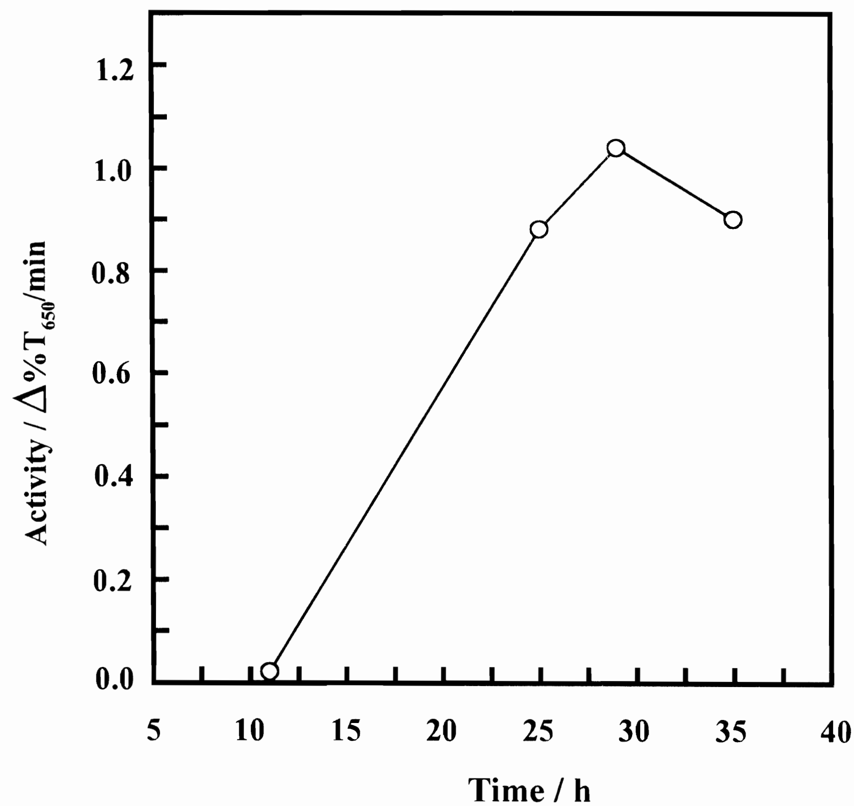

Figure 1 Induction of chitinase production in *Staphylococcus species* strain TU005 (E) grown in colloidal chitin minimum media. (Mekkriengkrai, *et al.*)

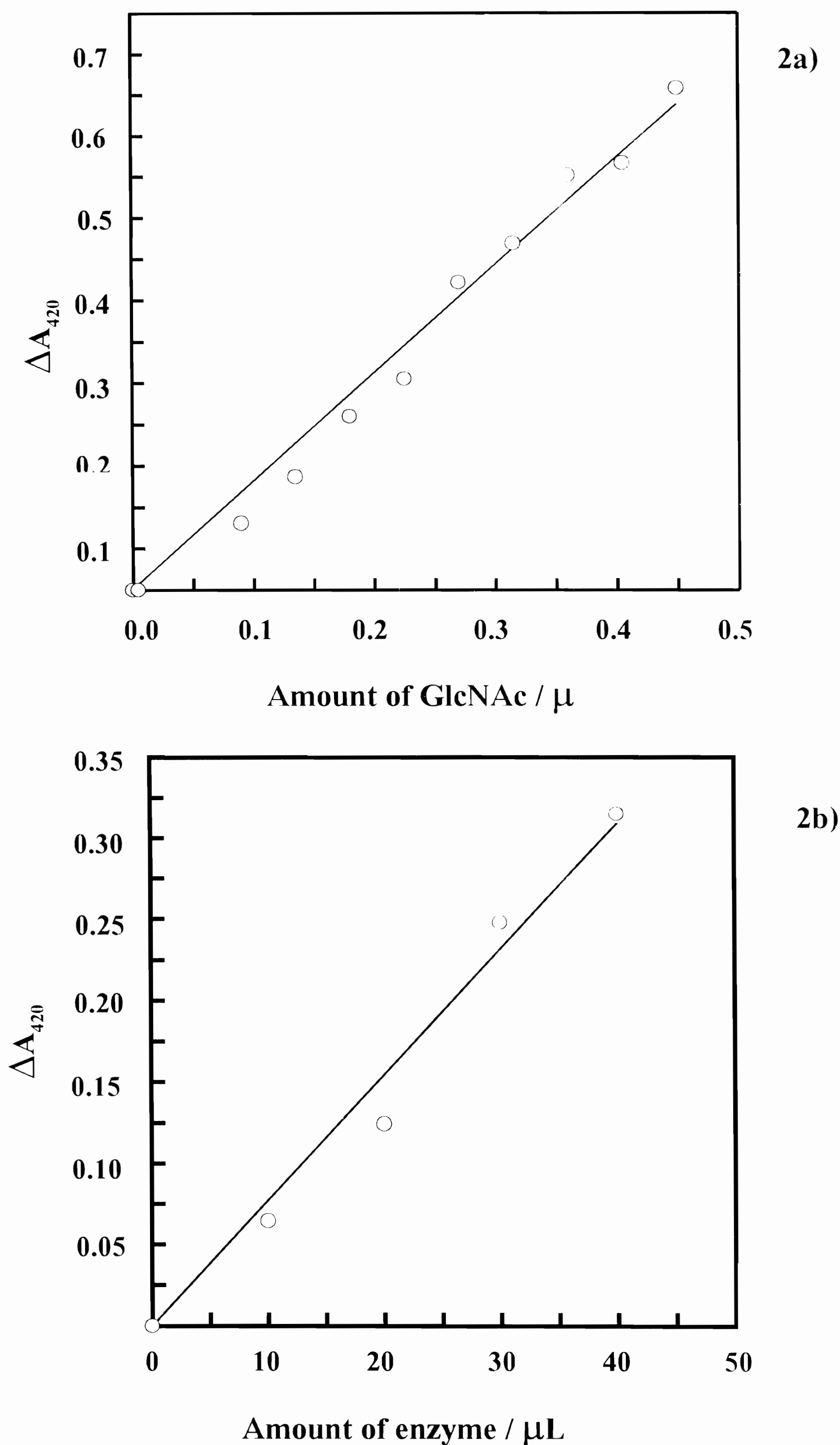

Figure 2 Enzyme activity by colorimetric assay (modified Schales method) (a) standard curve of N-acetylglucosamine and (b) degradation of colloidal chitin by enzyme. (Mekkriengkrai, *et al.*)

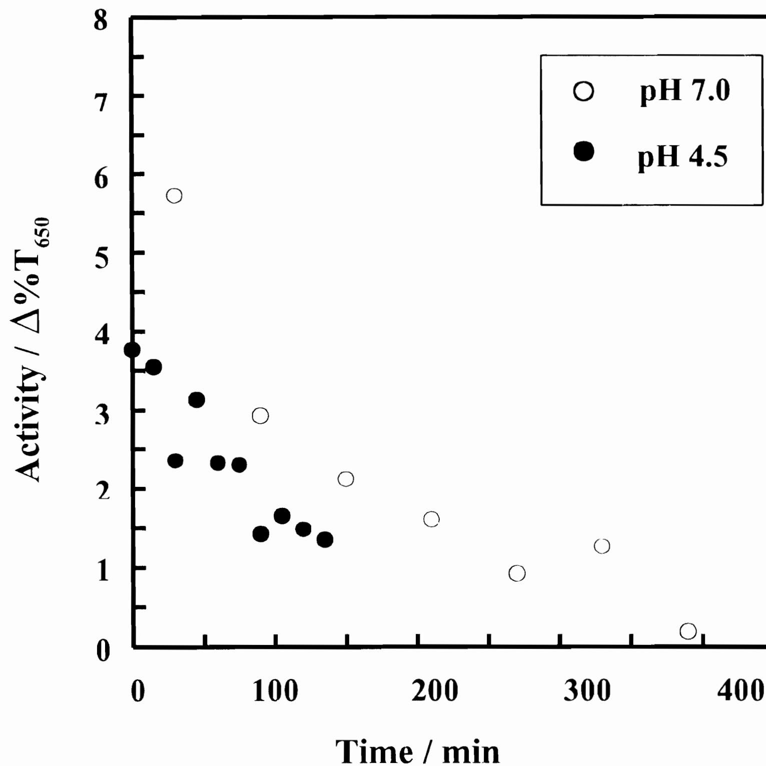

Figure 3 Enzyme stability by turbidimetric assay at 37°C with excess colloidal chitin: ○ phosphate buffer (0.1 M) pH 7; and ● acetate buffer (0.2 M) pH 4.5. (Mekkriengkrai, *et al.*)

Enzymatic hydrolysis of chitosan

The effect of substrate concentration on chitinase is studied. The probability of enzyme-substrate formation at low substrate concentration is lower than that of high substrate concentration. Figure 4 shows that at 2% chitosan concentration (d), the decrease in viscosity of chitosan is not as high as that of 3-4% concentration (a, b, and c). In the present work, we chose 4% concentration to prepare the low molecular weight chitosan.

Characterization of oligochitosan

Molecular Weight and Chemical Structure of Oligochitosan

After the enzyme treatment for 24 h, the molecular weight of chitosan was decreased rapidly Figure 5. The enzymatic degradation proceeded gradually from the second day and at the forth day the molecular weight reduced to 40% of the initial and reaches the equilibrium point.

The product after enzymatic degradation was quantitatively analyzed using amide I and II at 1655 and 1550 cm^{-1}, according to Sannan, *et al.* (1978), to evaluate the change in chemical structure. As shown in Figure 6, the decrease of chitin unit is significant as soon as the chitinase was applied to the system. The result implied that the enzymatic degradation with chitinase remove chitin unit from chitosan chain.

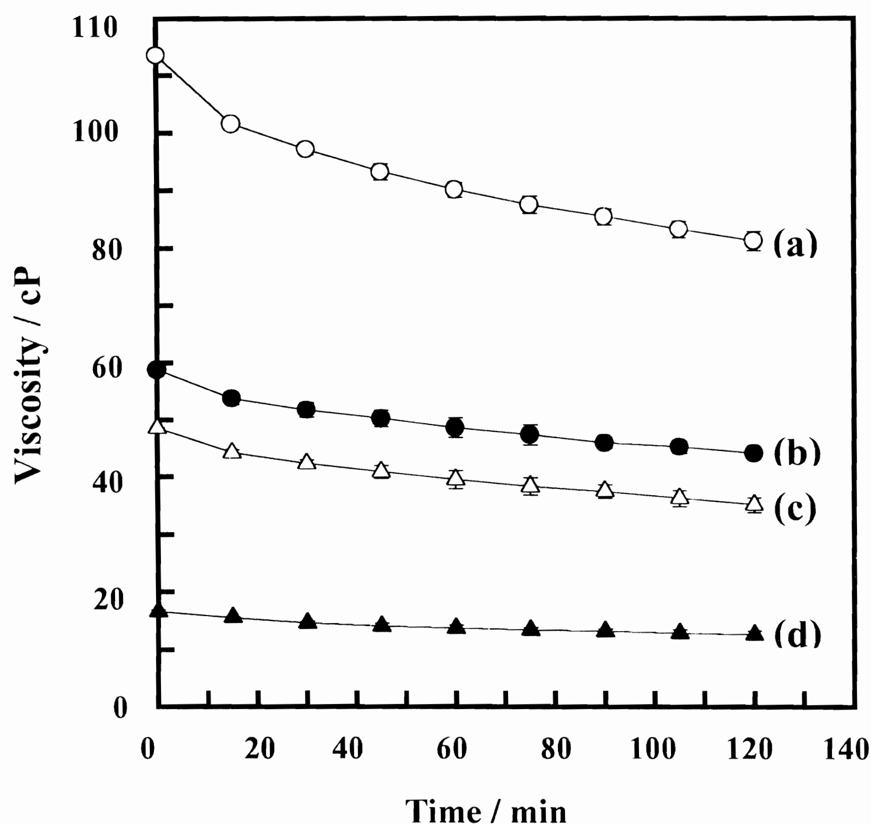

Figure 4 Viscosity reduction of chitosan solution when treated with chitinase 18 mU/mL of chitinase was used to digest by Brookfield viscometer (a) 4% chitosan, (b) 3.5% chitosan, (c) 3% chitosan, and (d) 2% chitosan, at 37°C. (Mekkriengkrai, *et al.*)

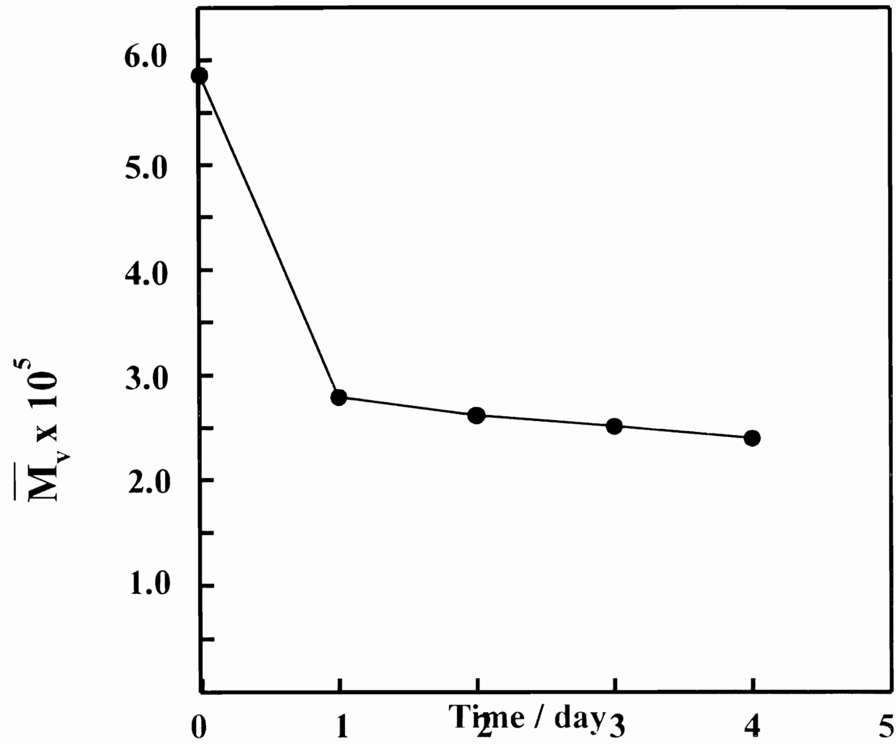

Figure 5 Intrinsic viscosity of chitosan after treatment with enzyme, 18 mU/mL for 1, 2, 3, and 4 days by Ubbelohde viscometer. (Mekkriengkrai, *et al.*)

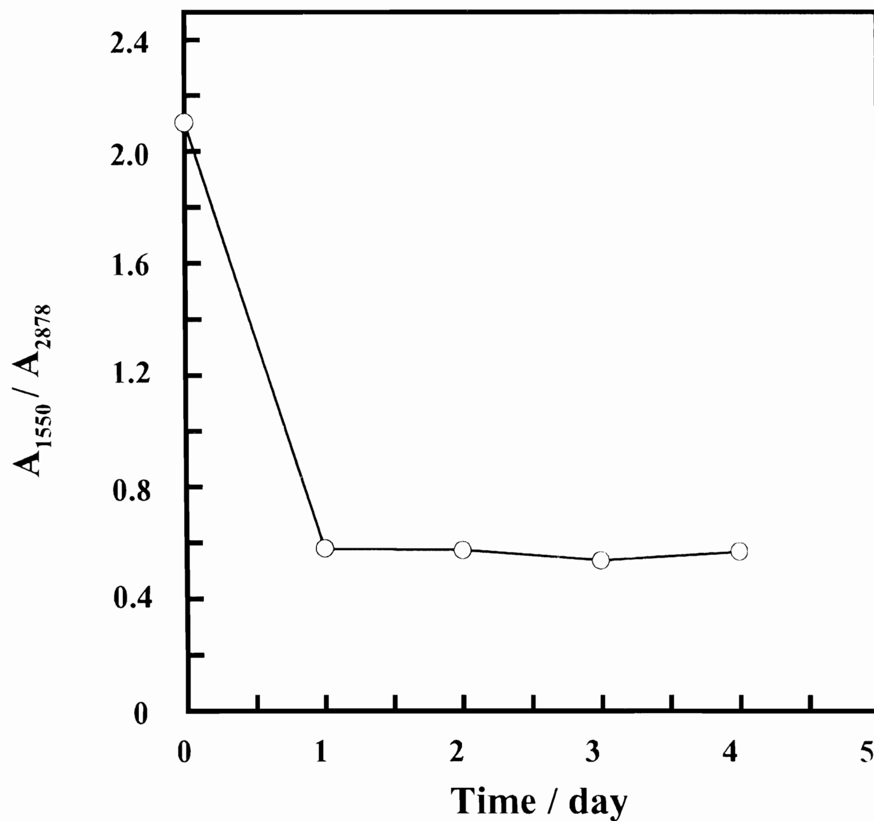

Figure 6 The quantitative analysis of the obtained chitosan, comparing the amide II band to C-H band (A_{1550}/A_{2878}) by FT-IR. (Mekkriengkrai, *et al.*)

Elemental analysis was applied to confirm the changing in degree of deacetylation of the product. In the case of 75% of degree of deacetylation, the chitosan should give C/N ratio at 5.58. Enzymatic degradation will make C/N ratio be 5.14 when chitin unit is completely degraded by chitinase. In the present work, we observed C/N ratio at each degradation time (1-4 days) Figure 7. It was found that C/N was in the range of 5.42-5.32, which implied the partial hydrolysis of chitin unit.

In order to evaluate the thermal stability after the degradation, we applied TGA. The result revealed that the obtained chitosan remained the degradation temperature at 305-308°C as similar to that of starting material. This suggested that enzymatic degradation did not effect to the intra and intermolecular hydrogen bonding.

The packing structure was also studied by powder x-ray diffraction. Figure 8 shows the XRD patterns of chitosan before and after enzymatic degradation in each step. It was clearly defined that the peak at 10°C and 20°C was broadened and the peak at 10°C was hardly seen when the degradation proceeded for 4 days. This implied that the enzymatic degradation by chitinase changed the packing structure.

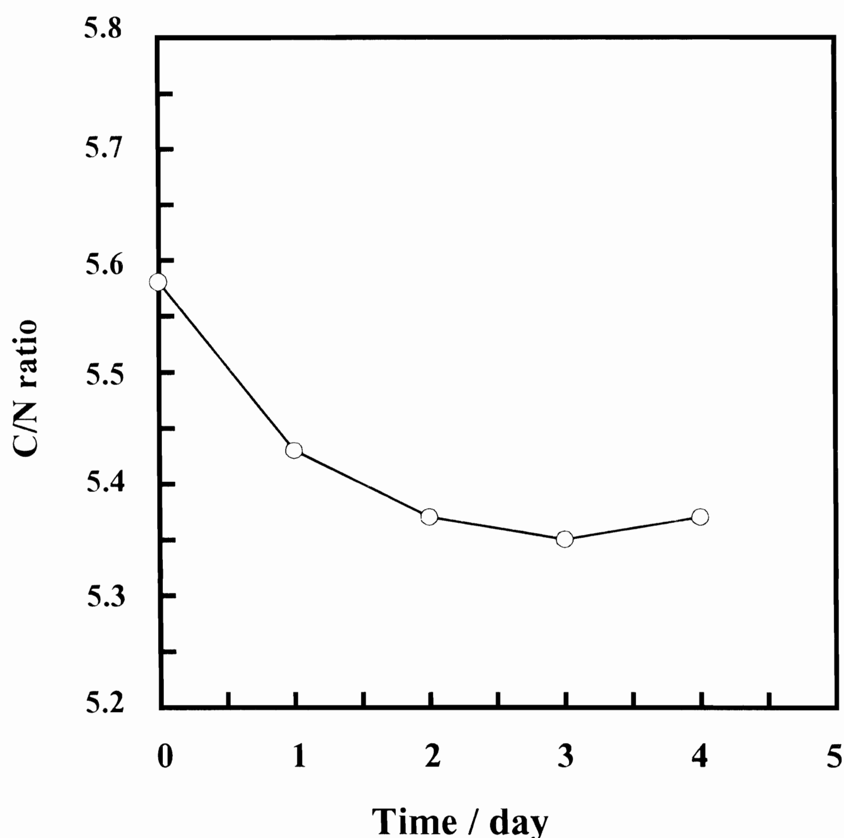

Figure 7 C/N ratios of chitosan after treatment with enzyme, 18 mU/mL for 1, 2, 3, and 4 days by elemental analysis. (Mekkriengkrai, *et al.*)

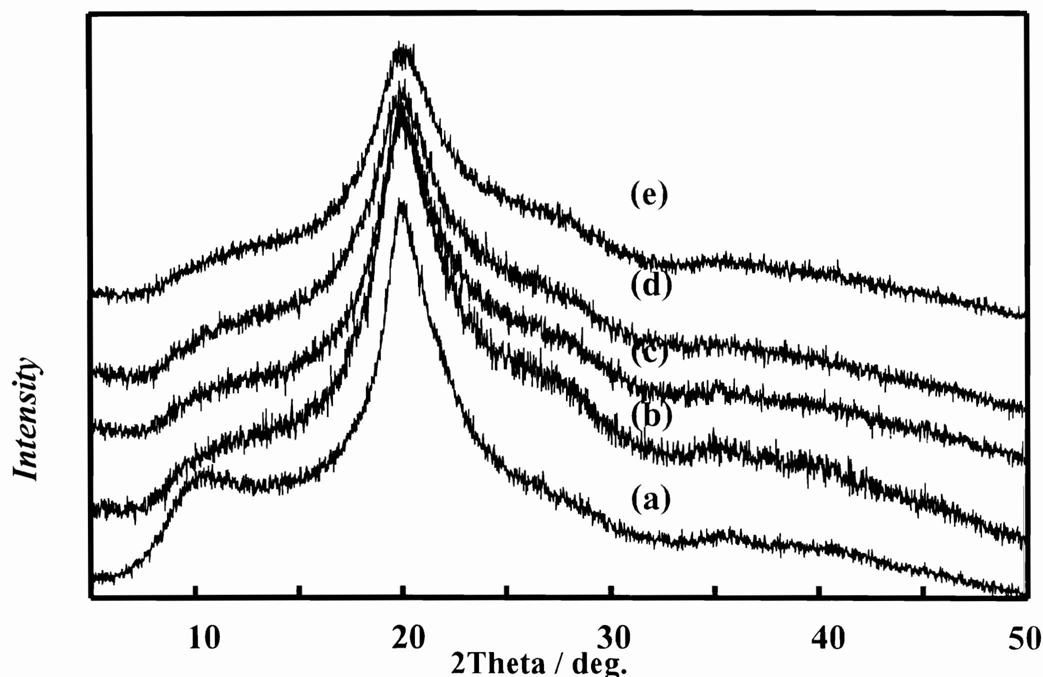

Figure 8 XRD patterns of **(a)** reprecipitated undigested chitosan with 1 M NaOH, **(b)** reprecipitated chitosan after enzyme treatment of 18 mU/mL for 1 day, **(c)** reprecipitated chitosan after enzyme treatment for 2 days with an addition of 18 mU/mL of enzyme at the end of the first day, **(d)** reprecipitated chitosan after enzyme treatment for 3 days with an addition of 18 mU/mL of enzyme at the end of the second day; and **(e)** reprecipitated chitosan after enzyme treatment for 4 days with an addition of 18 mU/mL of enzyme at the end of the third day. (Mekkriengkrai, *et al.*)

Conclusions

The present work has demonstrated that chitosan can be degraded easily and effectively by using *Staphylococcus species* strain TU005 (E). The enzymatic degradation showed that chitosan was degraded mainly at chitin unit as observed from the FT-IR and EA. The optimum condition was found to be at 4% chitosan concentration in acetic acid aqueous solution (2% v/v) with chitinase 18 mU/mL at 37°C for a day. The molecular weight of chitosan was reduced to 2/5 as comparing to the molecular weight of the original sample. The low molecular weight chitosan showed the changing in structural packing to be less crystallinity but retained the thermal stability.

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