Asia-Pacific Journal of Science and Technology: Volume: 28. Issue: 01. Article ID.: APST-28-01-06. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by the Research and Graduate Studies,
Khon Kaen University, Thailand

Enzymatic determination of the degree of chitosan deacetylation by family 18 chitinase

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Received 4 July 2021
Revised 5 March 2022
Accepted 6 March 2022

Abstract

The degree of chitosan deacetylation is the ratio of D-glucosamine (GlcN) units to the sum of N-acetyl-D-glucosamine (GlcNAc) and GlcN. An enzymatic method was developed to determine the percent degree of chitosan deacetylation (%DD) samples. A completely hydrolyzed chitosan solution was obtained by using crude chitinase from *Bacillus licheniformis* SK-1. The optimum condition was as follows: 20 mU of crude chitinase per milligram of chitosan in 0.1 M acetate buffer (pH 4.5) at 40 °C for 8 h. The %DD was determined by generating a standard calibration curve. The total reducing sugar (TRS) and the %DD of standard chitosan samples (ranging from 43% to 100%) were determined by using nuclear magnetic resonance and colloidal titration methods. The standard calibration curve for determining the %DD was obtained with a linear correlation: %DD = − 21.479 (TRS) + 102.45, r^2 = 0.9933. The enzymatic method showed reproducible results and did not require special instruments.

Keywords: Chitosan, Chitinase, DD, Hydrolysis, N-acetyl-D-glucosamine

1. Introduction

Chitosan is a polysaccharide of β-1,4 linked D-glucosamine (GlcN) residues. It has been widely used in many applications, such as the food and pharmaceutical industries. It is produced from chitin by thermochemical alkaline deacetylation. The percent degree of chitosan deacetylation (%DD) depends on the condition used for preparation and the sources of the chitin raw material. The %DD influences the physical, chemical, and biological properties of chitosan and its derivatives [1,2]. The %DD plays a vital role in chitosan qualities; thus, the %DD needs to be determined.

Many techniques can be used to measure the %DD of chitosan [3-8]. Titration methods are cheap; however, they lack solubility and require samples to be dried, and the mathematical models of calibration curves need to be modified to align the data correctly [3]. Ultraviolet spectrometry is particularly useful. Chitosan has no absorption peak at 200–400 nm, but most of its products present a characteristic peak in this range. This method and Fourier transform infrared spectroscopy data give us a good form of characterization [4,5]. The protein in crude chitosan samples can interfere with the results, which can lead to inaccurate %DD. Infrared spectroscopy can also be used to distinguish between α- and β-chitin. Nuclear magnetic resonance (NMR) spectroscopy (liquid-state ^{1}H NMR or solid-state ^{13}C NMR) is simple, quick, and accurate. Moreover, ^{1}H NMR was selected as the standard method for determining the degree of chitosan deacetylation by the American Standard Test Method Organization [6,7]. In ^{13}C NMR spectroscopy, the sample does not need to be dissolved, but the sample must be of high purity to obtain an appropriate spectrum [8].

Chitinase (EC 3.2.1.14) is an enzyme that catalyzes the hydrolysis of the β-(1,4)-glycosidic linkages between the sugar units in chitin. Chitinases are found in glycoside hydrolase (GH) families 18 and 19. The enzymes in family 18 use the substrate-assisted double displacement mechanism, whereas those in family 19 use the inverting direct displacement mechanism [9]. Chitinases can also hydrolyze chitosan depending on the degree of

acetylation of chitosan. They hydrolyze chitosan using endo mode of activity and different degrees of processivity. The enzymes are specifically hydrolyzed toward the GlcNAc (A) linker (A–A) to produce chitooligosaccharides (COS) [10-12]. Nanjo et al. [13] used enzyme mixtures (hexosaminidase, chitinase, and chitosanase) to determine the degree of chitosan deacetylation. However, this method is time-consuming and costly. In contrast to Nanjo et al., Aiba [14] found that the degree of chitosan deacetylation increases the hydrolysis rate.

The present study aimed to develop a method for determining the degree of chitosan deacetylation. The complete and specific hydrolysis of chitosan by family 18 chitinases was investigated.

2. Materials and methods

2.1 Chitosan samples

Chitosan samples in powder form with DD ranging from 43% to 100% were studied. The colloidal titration method and NMR method were used to determine the standard chitosans. This study used 43%, 74%, 84%, 92%, 95%, and 100% DD for chitosan standard and 10 chitosan samples for the determination of %DD by enzymatic method.

2.2 Enzyme activity and stability

Crude chitinases from *Bacillus licheniformis* SK-1 were used to assay the chitinase activity. The reaction mixture consisted of 0.1% w/v colloidal chitin in 0.1 M phosphate buffer (pH 6.0) and incubated at 50 °C for 1 h.

The modified Shales method was used to measure the reducing sugar [15]. One unit of chitinase was defined as the amount of enzyme that liberates 1.0 μmoL of GlcNAc per min. The temperature and pH stability of crude chitinases from *B. licheniformis* SK-1 were studied. The crude enzymes were kept at 40 °C–
50 °C and pH 4–5 for 24 h. The enzyme activity was assayed every 4 h.

2.3 Hydrolysis of chitosan by chitinase

The preceding procedure investigated the action of crude chitinases on chitosan. The reaction mixture consisted of 0.5% w/v chitosan in 0.1 M acetate buffer (pH 4.5) and incubated at 40 °C for 12 h. After boiling for 5 min, the reaction was stopped, and the modified Shales method was used to measure the increase of reducing sugar value. These conditions represented the rate of chitosan hydrolysis. The total reducing sugar (TRS) was calculated.

A standard curve was a plot of the %DD versus a varying amount of reducing sugar from standard chitosan (μmoL/mg) compared with unknown chitosan samples. As shown in Figure 4, the calibration curve determined using linear regression analysis had high reliability (r^2 = 0.9933). The %DD was calculated using a standard curve.

2.4 Colloidal titration assays

The %DD of standard and chitosan samples were obtained by using the method of Terayama [16].

2.5 NMR spectroscopy

Chitosan samples were dissolved in 1% w/v deuterated acetic acid (CD_3COOD) in water. The NMR spectra of chitosan were obtained by using a Bruker BZH 200 at 200 and 450 MHz (^1H, ^{13}C). The %DD was calculated from peak areas of $–CHNH_2$ at 2.68 ppm and $–CH_3$ of the N-acetyl group at 1.55 ppm [17,18].

2.6 Statistical analysis

All experiments were performed in triplicate. The results were analyzed using an analysis of variance with a 95% confidence interval. SPSS (version 18.0) was used for all statistical analyses.

3. Results and discussion

3.1 Enzymatic hydrolysis condition of chitosan

Family 18 chitinases bind with the acetylated unit (A) in subsite -1 for soluble substrate (chitosan) [19]. Furthermore, when chitosan binds to a deacetylated unit (D) in subsite -1, it inhibits the chitinase activity [20]. Chitinases hydrolyze chitosan at specific bonds between A–A, not D–D bonds; conversely, chitosanases cleave A–D and D–D linkages [10,11]. Moreover, Sikorski et al. [21] found that *Serratia marcescens* chitinases had different effects on chitosan hydrolysis. *Bacillus licheniformis* SK-1 chitinase is a family 18 chitinase with DXXDXDXE motif [22, 23]. Chitinase from *B. licheniformis* SK-1 has been studied to recycle chitin waste in nature [24]. The crude enzyme contains high chitinase and chitobiase activities (ranging from 20 kDa to 72 kDa) by activity staining. The crude chitinase of *B. licheniformis* SK-1 was used to produce 2-acetamido-2-deoxy-D-glucose (GlcNAc) and chitobiose. The crude enzyme completely hydrolyzes β-chitin to obtain 75% GlcNAc and 20% chitobiose; 41% GlcNAc yield was achieved from α-chitin. The enzymes hydrolyze soluble chitin, amorphous chitin and crystalline chitin. On the other hand, *B. licheniformis* SK-1 produces a mixture of chitinases with altered amorphous and crystalline forms. The activity on the soluble substrate (chitosan) will freely associate and dissociate from the active site [23]. Moreover, crude chitinases of *B. licheniformis* SK-1 were used to prepare COS from chitosan for pharmaceutical application [24]. In a preliminary study, crude chitinases from *B. licheniformis* SK-1 were studied for enzyme stability. The crude enzymes were stable at 40 °C and pH 4–5 for 24 h, as shown in Figure 1.

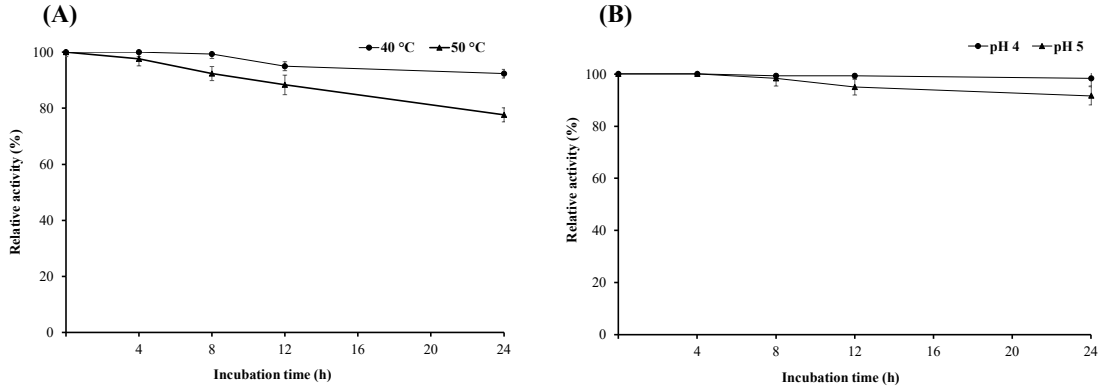

Figure 1 Temperature (A) and pH stability (B) of crude chitinases from *B. licheniformis* SK-1.

Chitosan was used as a substrate in a homogeneous reaction, and hydrolyzed sites were regulated. About 20%–45% of the degree of N-acetylated chitosan was used to study the specific hydrolysis of chitinase. In addition, bacterial chitinases were used to hydrolyze the different degrees of chitosan deacetylation [13,14,25-28]. The enzymatic reaction requires complete chitosan hydrolysis by crude chitinases. The enzyme reaction was carried out using the standard procedure with the exception of 1.5 mL of 0.5% w/v chitosan solution and 20 mU of enzyme. At different time intervals, aliquots of 0.1 mL were taken and analyzed for free GlcNAc. TRS was calculated using the weight of chitosan samples. Three chitosan samples were hydrolyzed using varying amounts of enzymes. In this study, 0%, 26%, and 57% of acetylated chitosan, of which 100%, 74%, and 43% DD were measured by using the colloidal titration method and NMR method, were used to represent the varying N-acetyl contents of chitosan. The amount of varying GlcNAc released was measured by using the colorimetric method. The TRS produced from chitosan samples is shown in Figure 2. We found that hydrolytic products were completely hydrolyzed with an enzyme containing 20 mU/mg of chitosan. In Figure 3, the TRS per milligram of chitosan was measured to determine the time course of hydrolysis of the chitosan samples. Within 8 h, the enzyme hydrolyzed 8%, 26%, and 57% acetylated chitosan corresponding to 92%, 74%, and 43% DD, respectively. This result suggested that an enzymatic reaction time of 8 h was sufficient for complete chitosan hydrolysis.

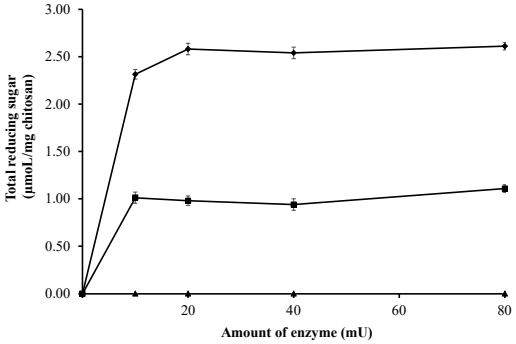

Figure 2 Amount of crude chitinases from *B. licheniformis* SK-1 (mU) to completely hydrolyze partially acetylated chitosan. 43% (◆), 74% (■), and 100% DD (▲). The value represents mean ± SD (n=5).

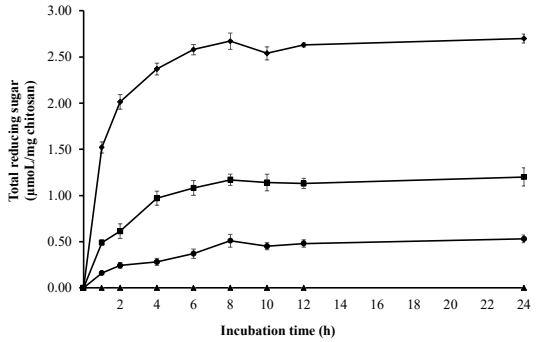

Figure 3 Time course of crude chitinase hydrolysis of partially acetylated chitosan. 43% (◆), 74% (■), 92% (●), and 100% DD (▲). The value represents mean ± SD (n=5).

3.2 Determination of the degree of chitosan deacetylation

In this study, chitosan samples with 43% to 100% DD were studied. Ohtakara et al. [28] found that the %DD of chitosan samples ranged from 58% to 96% DD depending on the crustacean species and the preparation method. The %DD and TRS from various degrees of N-acetylation of chitosan (µmoL/mg) are shown in Figure 4.

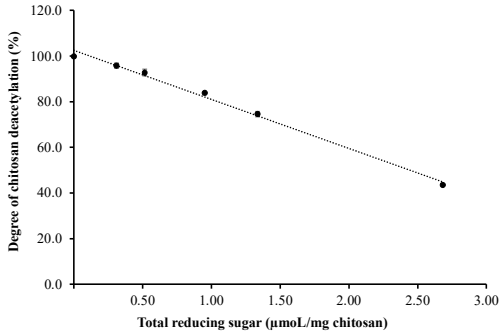

Figure 4 Standard curve for the determination of the %DD of chitosan. The value represents mean ± SD (n=5).

The chitosan samples were determined using a calibration curve of %DD, as shown in Table 1. This curve was used to compare the colloidal titration and NMR methods. The enzymatic method produced repeatable results. The percent DD of chitosan samples determined by colorimetric assays and the NMR method was in agreement. In addition, Lavertu et al. [17] validated that the %DD of chitosan samples ranged from 48% to 100% DD by using ^{1}H NMR. The method is simple, rapid, and precise, but it needs specific instruments. On the other hand, the colloidal titration method produced repeatable results. The method was unsuitable for extremely deacetylated chitosan samples and showed inaccurate results. The accelerator technique showed repeatability and accuracy. This method can be used to simplify the determination of the %DD of chitosan for characterization. The increase of reducing sugar was used to obtain calibration curves. The %DD of chitosan

samples can be determined using these calibration curves. The results demonstrated that the method gave better or comparable results to other currently available methods.

Table 1 Degree of chitosan deacetylation samples with varying N-acetyl content.

Chitosan samples	TRS (μmoL/mg)	%DD			
		Enzymatic method[I]	Colloidal titration method[I]	NMR method[II]	
				^{1}H NMR	^{13}C NMR
CS01	2.68 ± 0.01^{a}	44.67 ± 0.00^{j}	43.52 ± 0.41^{j}	42.90	46.30
CS02	0.33 ± 0.02^{i}	95.29 ± 0.44^{ab}	96.25 ± 1.11^{a}	91.60	94.14
CS03	0.72 ± 0.02^{g}	87.41 ± 0.50^{d}	89.48 ± 2.22^{d}	88.00	89.90
CS04	0.96 ± 0.02^{f}	81.97 ± 0.14^{e}	83.50 ± 0.15^{e}	90.00	84.80
CS05	1.35 ± 0.02^{c}	73.31 ± 0.07^{h}	74.12 ± 1.20^{gh}	75.00	80.71
CS06	0.51 ± 0.02^{h}	91.78 ± 0.40^{c}	93.66 ± 1.47^{bc}	91.50	92.30
CS07	1.33 ± 0.02^{c}	73.74 ± 0.07^{gh}	72.62 ± 0.45^{h}	73.52	75.26
CS08	1.06 ± 0.03^{e}	79.61 ± 0.38^{f}	81.64 ± 0.22^{ef}	81.80	83.57
CS09	1.22 ± 0.03^{d}	76.03 ± 0.50^{g}	74.37 ± 0.51^{gh}	77.10	77.62
CS10	2.24 ± 0.01^{b}	54.41 ± 0.26^{i}	58.31 ± 0.35^{i}	57.10	57.87

The data show the mean ± SD derived from three replicates. Means in the same column followed by a different superscript letter are significantly different ($p \leq 0.05$).[I] The results from three experiments (n=3).[II] The results from two experiment (n=2).

4. Conclusion

The enzymatic method showed reproducible results and did not require special instruments. These results were consistent with the colloidal titration method and NMR method. The method will be useful for determining the degree of chitosan deacetylation.

5. Acknowledgements

The authors thank Prof. Suwabun Chirachanchai, Ph.D., Petrochemical College, Chulalongkorn University, Bangkok, Thailand, for providing chitosan samples with various N-acetyl content.

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