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Controlled acetylation of water-soluble glucomannan from *Bletilla striata*

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ABSTRACT

Glucomannans from *Bletilla striata* (bletillan) were used as excipient for controlled deliveries of drugs, genes and tissue engineering. In the present study, a controlled acetylation method was developed to improve water solubility of bletillan 70 (BT) firstly, by reacting with acetic anhydride (AA) in N,N-dimethylformamide solvent. The preparation parameters, such as reaction temperature, reaction time and molar ratio of BT/AA, were optimized based on degrees of acetyl group in addition. IR and ^{1}H NMR spectra were applied to elucidate the reaction process and substitution pattern, which indicated that the acetylation took place at C-6 and C-2 of the hexose units in a ratio of 2:1, with DS up to 0.83. Relative viscosity analysis revealed that the resulted products had improved water solubilities. This novel method is simple, economic and easily controlled.

1. Introduction

The tubers of *Bletilla striata* have been widely used in East Asian countries to treat alimentary canal mucosal damage, ulcer, bleeding, bruises and burns (Chinese pharmacopia, 2010). Water-soluble glucomannan is abundant in the tubers of *B. striata* (bletillan), which was responsible for its medicinal function (Diao et al., 2008). Its backbone structure is composed of $\beta 1 \rightarrow 4$ linked mannose and glucose with molar ratio of 3:1, having 1.7 branched hexose residue at C-2 of mannose and two acetyl substitution every 12 hexose units. The molecular weight is about 180 kDa or 135 kDa (Tomoda, Nakatsuka, Tamai, & Nagata, 1973; Tomoda, Nakatsuka, & Satoh, 1974; Wang et al., 2006). Since it is biocompatible and biodegradable, bletillan could be applied as excipient for controlled delivery of drugs, genes and tissue engineering (Qiu, Wang, Li, Han, & Zhu, 2007; Dong et al., 2009; Feng et al., 2003). Like many other glucomannans, such as Konjac glucomannan (KGM) (Ratcliffe, Williams, Viebke, & Meadows, 2005), bletillan has poor water solubility due to the strong interchain interactions resulting from the extensive hydrogen bonding (Dave, Sheth, McCarthy, Ratto, & Kaplan, 1998). Therefore, physical or chemical modifications were necessary to improve water solubility before it can be widely used.

Chemical modifications of glucomannan, including esterification, formation of ether and graft copolymerization, have been applied to reduce the hydrogen bonding and improve the properties of KGM (Chen, Zong, and Li, 2006; Lu & Zhang, 2002; Lu, Zhang, & Xiao, 2004; Xia et al., 2010). Suitable acetylation of glucomannan could effectively reduce the hydrogen bonding, which was supposed to be one of the efficient methods to improve its water solubility like acemannan from aloe (Bozzi, Perrin, Austin, & Vera, 2007). Acetylation of KGM was extensively studied in food processing in regard to gelation and water solubility of acetylated KGM with different degrees of substitution (DS). KGM with low DS (0.05–0.23) was synthesized by using acetic anhydride in the presence of pyridine as catalyst, and the water solubility was improved gradually by the introduction of acetyl groups within the DS range (Gao & Nishinari, 2004a,2004b). However, high DS (1.8) KGM showed water resistance as reported (Koroskenyi & McCarthy, 2001). Substitution pattern of acetyl group could also affect their physical properties too, as it is known for acetylated cellulose (Heinze, Liebert, & Koschella, 2006, chaps. 4 and 9). Therefore, it was of great interest to develop straight acetylation method with controlled DS and substitution pattern. There is a report about synthesis of bletillan sulfate (Chen et al., 2007), but little is known about bletillan acetylation and its effects on water solubility.

In the present study, a simple acetylation method was developed to improve water solubility of bletillan 70 (BT) for the first time, by using acetic anhydride (AA) in N,N-dimethylformamide (DMF) as reaction solvent. The reaction temperature, reaction time and molar ratio of BT/AA were optimized based on DS of acetyl groups. IR and ^{1}H NMR spectra were applied to elucidate the reaction process and substitution pattern. Relative viscosity of the resulted acetylated BT (ABT) was analyzed to evaluate its water solubility.

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2. Experimental

2.1. Materials

Bletillan used in this study was isolated from the tubers of *B. striata* and processed by controlled acid hydrolysis (Zhang, 2010). The tubers of *B. striata* were collected from Guizhou province in southwest China in October, 2008. Briefly, dry materials (500 g) were extracted with distilled water (7 L × 2) under reflux for 1.5 h, two volumes ethanol was then added to the aqueous solution and precipitated to give crude bletillan (90 g). The crude bletillan solution (2.0%, w/w) was then hydrolyzed by using 1.0 M HCl at 85 °C for 210 min, 1 M NaOH was added dropwise to adjust pH to 6–7, two volumes of ethanol was then added and followed by filtration, the precipitate was washed with ethanol three times and dried overnight at 55 °C to give final product BT, the purity of BT was greater than 95% by HPGPC/ELSD analysis. The average molecular weight of BT was 73 kDa, with molar ratio of mannose to glucose as 2.88:1 and DS of acetyl groups as 0.11, which was applied throughout the present study. Acetic anhydride (higher than 98.5%), DMF and other chemical reagents used in the study were all of analytical grade (higher than 98.0%) and supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Synthesis of glucomannan acetates

Acetylation of BT with AA was performed in DMF. BT (1.0 g, 0.0062 mol) was dried under vacuum at 55 °C overnight before use. The pretreated sample and 37.5 ml DMF were added into a round-bottom three-neck flask equipped with a mechanical stirrer. After stirring for 2 h under 100 °C to swell the polysaccharide particles, 0.15 ml (0.0185 mol) AA was added dropwise into the flask to start the reaction. At different time intervals (2–12 h), sample solutions was collected and cooled under water bath filled with ice water, 1 M NaOH was added dropwise to adjust pH to 6–7, 75 ml ethanol was then added and followed by filtration, the precipitate was washed with ethanol three times and dried overnight at 55 °C under vacuum to give final ABT products. ABT1–ABT4 were thus prepared at 2 h, 6 h, 8 h and 12 h reaction time respectively. Different process conditions, such as different BT/AA molar ratios (1:1, 1:2, 1:3), reaction temperatures (80 °C, 100 °C, 110 °C, 120 °C) and times (within 20 h) were conducted with same procedure to find optimal conditions.

Another two acetylation methods were also studied by using anhydrous sodium acetate (Zeng & Qian, 2006) or pyridine (Long, Rheo, Shinsaku, Tokuzo, & Katsuyoshi, 2002) as catalyst as reported. In anhydrous sodium acetate scheme, BT (1.0 g) was dried under vacuum at 55 °C overnight before use. Anhydrous sodium acetate (0.1 g) and AA (15 ml) were added into a round-bottom three-neck flask equipped with a mechanical stirrer. After stirring for 2 h under 60 °C to swell the reagent, the temperature was set at 40 °C, BT (1.0 g) was added into the flask to start the reaction. At different time intervals (2 h, 6 h, 8 h, 12 h), 3 ml sample solution was collected and precipitated with 9 ml alcohol, filtered off, washed with alcohol three times and dried at 55 °C under vacuum. In pyridine scheme, 1.0 g BT and 6.3 ml AA were added into a round-bottom three-neck flask equipped with a mechanical stirrer. After stirring for 1 h at room temperature, 0.55 ml pyridine was added dropwise into the flask to start the reaction, At different time intervals (2 h, 6 h, 8 h, 12 h), 1.5 ml sample solution was collected and precipitated in 5 ml alcohol, washed with ethanol three times and dried overnight at 55 °C under vacuum.

2.3. Determination of degree of substitution

The DS of acetylated BT products was determined by UV spectrophotometric method, using β-D-glucose pentaacetate as reference compound (Yin et al., 2010). Acetyl content (W) was calculated according to the following equation:

$$W\% = \frac{m_{\mathrm{Ac}}}{m_{\mathrm{Ac}} + m} \times 100$$

where m_{Ac} was mass of acetyl group in gram, m was the mass of the sample in gram. As molar mass of acetyl group was 43 g/mol, the DS was calculated as follows:

$$\mathrm{DS} = \frac{162 \times W}{4300 - (43 - 1) \times W}$$

2.4. Fourier transform infrared spectroscopy

Fourier transform infrared spectra of dried BT and ABT samples were measured on a Nicolet 6700 FTIR spectrometer in the range of 400–4000 cm^{-1}.

2.5. ^{1}H NMR spectroscopy of perpropionyl derivates of ABT acetates

Perpropionyl derivates of BT acetates were synthesized for ^{1}H NMR analysis of DS (Hussain, Liebert, & Heinze, 2004). ABT1–ABT4 (0.10 g) were suspended in 5 ml pyridine respectively, then treated with 10 ml propionic anhydride in the presence of 0.5 g of 4-dimethyl aminopyridine for 20 h at 80 °C. The reaction solutions were precipitated in 100 ml ethanol, filtered off, washed and dried at 60 °C under vacuum to obtain perpropionyl derivates (ABT1p–4p).

^{1}H NMR spectra of ABT1p–4p (in $\mathrm{CDCl_3}$) were recorded with Bruker Avance 400 spectrometer. $\mathrm{DS_{acyl}}$ was calculated according to the following equation (Zhao, Li, Zhong, & Jian, 2009):

$$\mathrm{DS}_{\mathrm{acyl}(n)} = \frac{7 I_{\mathrm{H(acyl)}}}{3 I_{\mathrm{H(AGU)}}}$$

where $\mathrm{DS_{acyl}}$ was the DS of acetyl or propionyl groups, $I_{\mathrm{H(acyl)}}$ was the peak area of methyl protons of acetyl (at δ 2.00 for C-2, δ 2.10 for C-6) or propionyl (at δ 1.07 for C-2 and C-3, δ 1.16 for C-6), n (2, 3, 6) was the location of the acyl, $I_{\mathrm{H(AGU)}}$ was the peak area of anhydroglucose unit (AGU) protons (at δ 3.52–5.35).

2.6. Relative viscosity determination

Relative viscosities (η_{r}) were measured for BT/ABT samples to evaluate water solubilities of different DS products (Chinese pharmacopia, 2010). BT or ABT1–4 powder (0.2 g) was dispersed in water (1.0 wt%) and heated at 60 °C until the samples were dissolved totally. Viscosity measurements were undertaken at 25 °C using a capillary Ubbelohde viscometer with a diameter of 50 mm. Eluting time between two test lines of water (t_0) or sample solution (t) in the apparatus was tested, while t_0 was measured to be 103 s. Relative viscosity was calculated as follows:

$$\eta_{\mathrm{r}} = \frac{t}{t_0}$$

3. Results and discussion

3.1. Selection of reaction system

Different acetylation methods of BT were tested initially using two different base catalysts. Taking anhydrous sodium acetate as catalyst, BT could be acetylated quickly by acetic anhydride. DS of ABT arose at first 3 h and the product kept water soluble, and then turned to insoluble after 4 h due to high DS. Different reaction conditions were tested, such as reaction temperatures, reaction

Table 1
Effects of reaction temperature and time on the DS of ABT in DMF with molar ratio of BT/AA set at 1:3.

Temperature (°C)	Reaction time (h)	DS
80	2	0.1374
	4	0.1568
	6	0.1964
	10	0.2353
	20	0.4548
100	2	0.2107
	4	0.2701
	6	0.4770
	8	0.7229
	12	0.8287
110	2	0.2404
	4	0.3868
	6	0.6879
	8	0.7882
	10	0.8036
120	1	0.4560
	2	0.6745
	3	0.7135
	4	0.7787

times and material ratios. However, the reaction could not be well controlled, and water solubility of resulted products could not be improved. Taking into account that BT could not well dispense with acetic anhydride, another acetylation method was tested by applying pyridine as catalyst. The similar results were observed as that in anhydrous sodium acetate. Using strong bases, such as NaOH, BT colloid formed and no reaction were observed.

The reaction took place more readily by comparison with KGM, partly due to lower molecular weight of BT. After trials, low DS of ABT was obtained in DMF at 80 °C, and relative viscosity of the products declined obviously. Finally, DMF was selected as the reaction solvent for further experiments.

3.2. *Determination of the optimal conditions by degrees of substitution analysis*

DS of ABT was measured to monitor the reaction process conveniently. Table 1 showed the effect of reaction temperature and time with molar ratio of BT/AA set at 1:3. At 80 °C, DS of ABT reached 0.24 after 10 h. The reaction moved smoothly at 100 °C and 110 °C, with DS of ABT ranging between 0.21 and 0.83 within 12 h and water-soluble; after 24 h reaction, ABT dissolved in glycol instead of water. The reaction went fast at initial 4 h at 120 °C, and ABT turned to water-insoluble after 6 h; GPC analysis also revealed the molecular weight of ABT products had slight degradation at 120 °C after 6 h (data not shown). Taking into account of stability, safety and the reaction speed, the optimal reaction temperature was 100 °C.

Table 2 showed the effect of molar ratio of BT/AA at 100 °C in DMF solvent. For BT/AA molar ratio of 1:1 or 1:2, DS of ABT changed slightly from 0.129 (at 2 h) to 0.377 (at 12 h). Reaction speed kept steady as for BT/AA mole ratio of 1:3, with DS of ABT ranging from 0.214 (at 2 h) to 0.829 (at 12 h). The reaction ran too fast for higher BT/AA molar ratio at 1:5 and the resulted product after 8 h could not dissolve in water (data not shown). BT/AA molar ratio was thus set at 1:3.

Table 2
Effects of molar ratio of BT/AA on the DS of ABT in DMF at 100 °C.

Reaction time (h)	DS		
	1:1	1:2	1:3
2	0.1293	0.1711	0.2136
4	0.2157	0.2234	0.2914
6	0.2545	0.2498	0.4781
8	0.2638	0.2854	0.7203
10	0.2738	0.3405	0.7894
12	0.3098	0.3774	0.8287

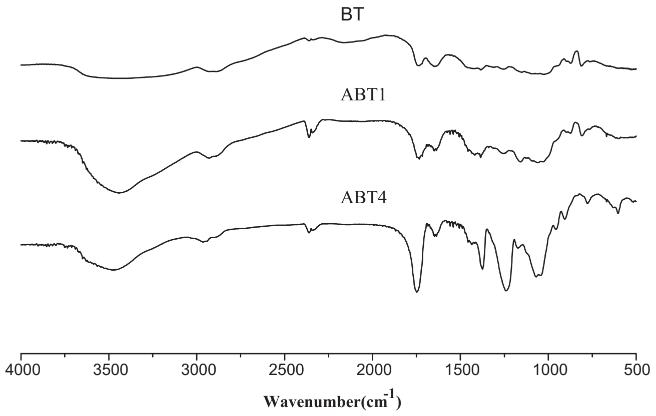

Fig. 1. FT-IR spectra of BT, ABT1 and ABT4.

The optimal condition for preparing ABT was thus determined. Four representative products, ABT1–ABT4 with various DS between 0.2 and 0.8 were synthesized accordingly.

3.3. *FT-IR analysis*

FT-IR spectra of BT, ABT1 and ABT4 were shown in Fig. 1 and Table 3. The absorption at 1742 cm^{-1} (Lin et al., 2010), which was assigned to stretching vibration of the C—O in carbonyl groups, was remarkably increased with an increase of DS values of ABT. Concomitant with the increase of the carbonyl stretching vibration, the absorptions at 1245 and 1060 cm^{-1} assigned to stretching vibration of the C—O—C in carbonyl groups, was also increased with an increase of DS values of ABT. So did as for the absorption at 1377 cm^{-1} which was assigned to the CH_3 symmetric deformation vibration. These results indicated that acetylation of BT proceeded and degree of acetylation was enhanced with increase of substitution degree.

3.4. *^{1}H NMR analysis of perpropionyl derivates of ABT*

Four perpropionyl derivates of ABTs were synthesized to characterize their acetylation position (Iwata, Azuma, Okamura, Muramoto, & Chun, 1992). For example, ABT4p had acetate groups at C-6 (at δ 2.09) and C-2 (at δ 2.00), and no acetate groups at C-3 (at δ 1.92) was observed (Fig. 2). Furthermore, the DS of acetyl and propionyl groups could be calculated from peak areas of their ^{1}H NMR signals, respectively (Table 3). As for ABT4p, it had a distribution of acetate groups to be 0.53 at C-6, 0.30 at C-2 with total DS of acetyl to be 0.83. The DS of the propionyl of ABT4p was 2.11, the total DS of acetyl and propionyl added up to 2.94 which indicated

Table 3
DS of acetylation (Ac), propionylation (Pr) of ABT1p–ABT4p measured by ^{1}H NMR analysis.

Derivate	DS of Ac			DS of Pr	DS in total
	C6	C2	Total		
ABT1p	0.13	0.08	0.21	2.76	2.97
ABT2p	0.32	0.15	0.47	2.56	3.03
ABT3p	0.46	0.25	0.72	2.35	3.07
ABT4p	0.53	0.30	0.83	2.11	2.94

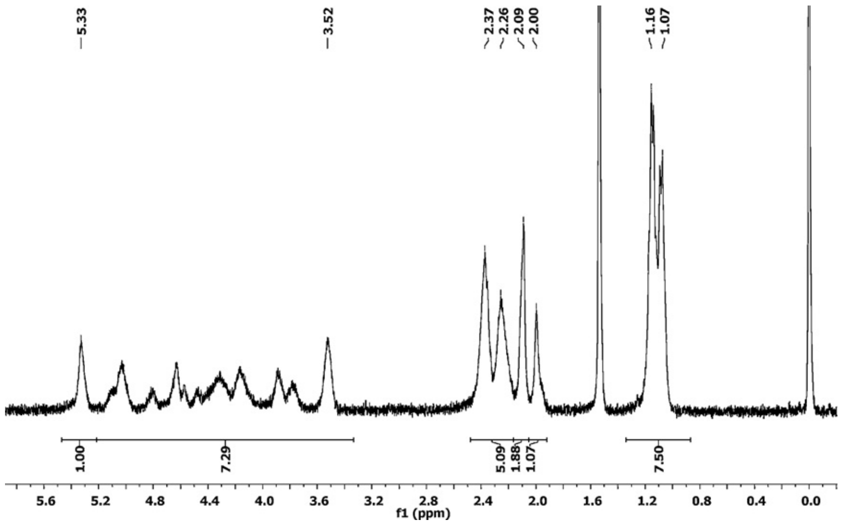

Fig. 2. ^{1}H NMR spectrum of ABT4p (400 MHz, in $CDCl_3$).

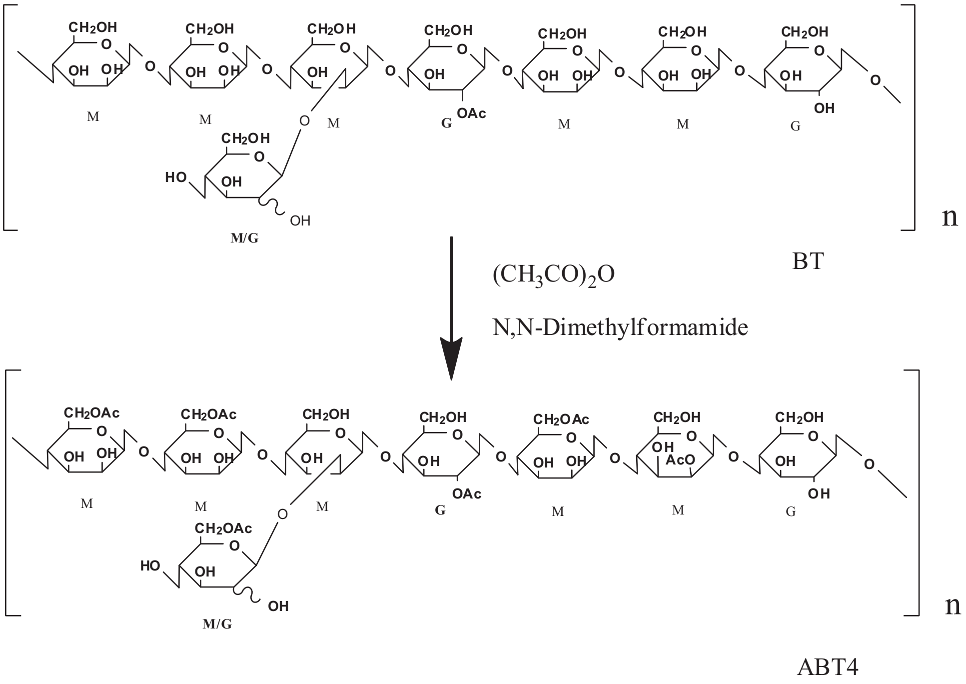

Fig. 3. Proposed structure and reaction mechanism of ABT4 (M = mannose, G = glucose, Ac = $COCH_3$).

the full perpropionylation of ABT4p. Other three ABTs also showed similar substitution pattern as that of ABT4, and the DS results of acetyl groups were incorporated with that measured by UV spectrophotometric method. Taking all information together, proposed structure and reaction mechanism of ABT4p could be represented in Fig. 3.

Typically, for acetylation of polysaccharide with acetic anhydride, hydroxyl at C-6 reacted first due to less steric hindrance, followed by C-2 and finally by C-3 for electronic differences (Heinze et al., 2006, chaps. 4 and 9). However, regioselectivity with a preferred acetylation at C-2 could be obtained by transesterification of a carboxylic acid vinyl ester with the hydroxyl groups in the presence of a low molecular salt as in case of starch (Dicke, 2004). In this study, more acetyl groups located at C-6 than at C-2, which was in agreement with relative acetylation activity of different hydroxyl groups in typical.

3.5. *Relative viscosity and degree of substitution*

Table 4 showed the degree of substitution and the relative viscosities of the BT and ABT sample solutions (1.0 wt%). The relative viscosity decreased apparently from 4.44 (for BT) to 2.00 (for ABT2)

Table 4
Properties of the relative viscosities (η_r) and DS of BT and ABT1–ABT4 solutions (1.0%).

Sample	DS	η_r
BT	0.11	4.44
ABT1	0.21	2.57
ABT2	0.47	2.00
ABT3	0.72	2.30
ABT4	0.83	2.50

accompany with increasing DS (from 0.11 to 0.47) initially, and then increased slowly to 2.50 for ABT4 with DS 0.83. As for ABT2, the relative viscosity (2.0 wt%) was measured to be 3.6, which indicated more than 2 fold increase in water solubility by comparing to that of BT. The results indicated that suitable acetylation could reduce the hydrogen bonding in BT with DS lower than 0.83. Higher DS would result in water insolubility as observed in trials.

4. Conclusions

A convenient method for controlled acetylation of glucomannan from *B. striata* was established in this study. BT acetates were synthesized with acetic anhydride in DMF solvent. The optimal preparation conditions were determined. Their structures were characterized by infrared spectra and ^{1}H NMR analysis of perpropionyl derivates, which revealed that the acetyl groups mainly existed at C-6 and C-2 of the hexose units with a ratio of about 2:1. Relative viscosity analysis indicated that water solubilities of ABTs were improved within DS range. The optimal process of water-soluble ABTs with characteristic substitution pattern was thus successfully developed.

Conflict of interest

All authors have no conflict of interest.

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