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A one-pot method for lipase-catalyzed synthesis of chitosan palmitate in mixed lonic liquids and its characterization

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ABSTRACT

Long-chain fatty acid chitosan esters are important chitosan products with attractive application prospects in biological and biomedical applications. A new enzymatic method for synthesis of chitosan esters via transesterification with methyl palmitate has been developed. A reaction system containing a hydrophilic ionic liquid (IL) 1-ethyl-3-methyl imidazoliumacetate and a hydrophobic IL 1-Butyl-3-methylimidazolium tetrafluoroborate enables the transesterification of chitosan with high DS and selectivity in a homogeneous phase. FT-IR and NMR analysis confirmed that the chitosan ester was formed and the lipase selectively catalyzed the acylation of the 6-OH groups of chitosan without protection of its $-NH_2$ groups. The effects of several key reaction factors were investigated and the maximal degree of substitution (DS) value of 0.214 was obtained under the optimum conditions. The X-ray diffraction analysis showed that morphological and crystallographic properties of native chitosan were largely destroyed after modification. Thermogravimetric analysis showed that the thermal stability of chitosan palmitate was reduced. This is the first time that long-chain chitosan esters was biocatalytically synthesized in ILs and the biocatalytic method presented here showed potential application for chitosan modification processes of industrial interest.

1. Introduction

Chitosan, the deacetylated product of chitin, is one of the largest storage of biological alkaline polysaccharides in the nature. It has excellent properties of biocompatibility, biodegradable, hygroscopicity, antimicrobial and fibre into membranous, showing a wide application prospect in medicine, food, textile, daily chemicals, papermaking and other fields [1,2]. However, owning to the strong intramolecular and intermolecular hydrogen bonds, chitosan has a dense crystalline structure and low solubility in ordinary organic solvents, which make it hard to be melted or processed [3].

To improve the physic-chemical properties of chitosan, lots of researches have been focused on its modification. In those researches, hydrophobic modification methods are mainly investigated, including acylation, alkylation, carboxyationl modification and etherification [4]. After modification, both the hydrophobic and hydrophilic properties of chitosan can be changed and the modified chitosan with new functions can be widely applied in medicine [5], surfactant, immobilization support [6], separating materials and other fields [7,8]. When esterification methods were used for chitosan modification, small molecule inorganic and organic acids were usually adopted and the chitosan esters achieved may possess new bioactivities. For example, it has been confirmed that the chitosan derivatives with its 6-OH groups being selectively esterified showed remedial properties of anticoagulation, antiviral, antineoplastic and blood compatibility [9]. Under the conditions that $-NH_2$ groups were protected, chitosan can be esterified with organic acids (benzoic acid, *p*-hydroxybenzoic acid, salicylic acid and sorbic acid) and the esters showed significant effects against the growth of some pathogens, including *Saccharomyces cerevisiae*, *Aspergillus niger*, *Staphylococcus aureus* and *Escherichia coli* [10].

Compared with short-chain chitosan esters, saturated long-chain fatty acid esters of chitosan had selective accumulation property and anticoagulant activities for some cancer cells [11]. Some amphiphilic chitosan products can be used as surfactants for drug controlled release systems, especially for improving the encapsulation efficiency of the drugs with poor solubility in water [12,13]. In the previous researches of chitosan esterification, the commonly used solvents and catalysts were strong organic acids with high toxicity and corrosivity. To achieve selectively acylation of certain hydroxyl groups of chitosan, the protection of $-NH_2$

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groups are necessary, which brings more reagents and made the whole processes tedious. Besides, the esterification of chitosan by long-chain fatty acids was quite rarely reported. Hence, it is necessary to find a simple environmental-friendly approach to synthesize the long-chain fatty acid esters of chitosan.

As a kind of biological catalysts, enzymes have the characteristics of high efficiency and specificity, allowing the reactions to be carried out under mild reaction conditions [14]. Enzymes are also "solvent-designable", the catalytic activity and selectivity of which can be regulated by the reaction solvents [15–19]. The discovery of enzymatic catalysis in organic solvent has greatly extended the scope of enzyme applications [20]. However, the use of traditional organic solvent can cause pollution along with the evaporation [21], and thus closed systems and solvent recycle have become industrial standards for organic solvent-involved industries. It had confirmed that many enzymes can keep activity in ionic liquids (ILs) or water-ionic liquid systems [22]. Enzymes, especially lipases, showed excellent catalytic efficiency, stereoselectivity and improved stability in ILs [23]. In addition, ILs have the advantages of low vapor pressure, high thermal stability and good performance in the aspect of dissolving chitosan [24]. Hence, ILs have attracted great attentions on the modification of chitosan.

In this research we proposed, for the first time, a facile and efficient method for synthesis of long-chain chitosan esters by using the lipase Novozymes 435 in a mixed ionic liquid system and the properties of the ester derivatives of chitosan were also investigated.

2. Materials and methods

2.1. Biological and chemical materials

Chitosan (With 95% degree of deacelation) was obtained from Aladdin Industrial Corperation (Shanghai, China) and dried at 50 °C for 24 h before use. [EMIM]Ac (purity >99%) and [BMIM][BF_4] (purity >99%) were purchased from Shanghai Chengjie Chemical co., LTD. The enzyme Novozyme 435 (Candida antarctica B lipase immobilized on a macroporous acrylic resin, 30U/mg) were supplied by Novozymes A/S(Bagsvaerd, Denmark). All other chemicals were of highest purity available and supplied by commercial sources.

2.2. General experimental procedures

0.326 g dried chitosan was added into 5 g mixed ionic liquids of [EMIM]Ac and [BMIM][BF_4] in a 50 ml three-neck round flask. The mixture was stirred and heated at 110 °C under the protection of pure nitrogen for about 2 h. Methyl palmitate was added to the solution with certain molar ratio of glucosamine unite. A certain amount of Novozyme 435 was also added into the solution after the reaction mixture was cooled to the required reaction temperature. The reaction was stopped by removing the immobilized lipases with 200 mesh filter cloth. The resulting products were precipitated, washed by 200 ml anhydrous ethanol, and then separated by centrifugation at 8000 rpm/min for 5 min. The residue was extracted by acetone for 12 h and dried under 50 °C for 24 h in a constant temperature oven.

2.3. Structural characterization

The FT-IR spectra of the native chitosan and chitosan ester were acquired using KBr discs on a Vector 33 spectrometer (Bruker, Germany) in the range of 400–4000 cm^{-1}. ^{13}C NMR spectras were obtained by using a Bruker 600 MHz (Bruker Corp., Germany). Each spectrum was recorded at 30 °C. The delay time was 10 s, and the acquisition time was 2 s. XRD patterns were performed on a D8 ADVANCE X-ray diffractometer (Bruker,Germany). The diffraction patterns were operated at 40 mA and 40 kV by using Cu Kαradiation(λ = 1.54 Å). Scattering angle (2θ) was varied from 5° to 60°, and the step width was 0.02° with scanning speed of 19.2 s per step. TGA experiments were performed on a TA Instruments Q500 thermo Gravimetric analyzer (TGA Q500, TA, USA). No more than ten milligram of product was used for each experiment. All TGA runs were conducted under nitrogen flow, and temperature was increased to 600 °C at a constant heating rate of 10 °C/min.

2.4. Analysis methods

0.05 g dried chitosan ester was accurately weighted and added into erlenmeyer flask which contianed 25 ml of 0.05 mol/L NaOH solution. The solution was mixed by magnetic stirring at 80 °C for 1 h 2–3 drops of phenolphthalein indicator was added until the solution was cooled to room temperature. The solution was titrated against 0.05 mol/L HCl solution and the consumption volume V_1 of the HCl solution was recorded. All experiments were performed in triplicates. The reaciton rate (W) and degree of substitution (DS) value is measured by the following formula:

$$W = \frac{(V_0 - V_1) \times 10^{-3} \times C \times 239}{M} \times 100\%$$

$$DS = \frac{163 \times W}{100 \times 239 - (239 - 1) \times W}$$

Where 239 is the molecular mass of methyl palmitate acyl donor; V_0 is the volume of 0.05 mol/L HCl solution consumed when titrating the blank; V_1 is the volume of 0.05 mol/L HCl solution consumed when titrating the sample; C is the molarity of standard HCl solution; M is the exact weight of the dry sample analyzed; 163 is the molecular mass of glucosamine unite.

3. Result and discussion

3.1. The enzymatic esterification of chitosan in mixed ILs

To form a homogeneous catalytic reaction, a suitable solvent should be selected to well dissolve the substrates. The main ionic liquids that can dissolve chitosan were found to be several hydrophilic ILs, such as imidazoles and glycine ionic liquids. In particular, 1-ethyl-3-methyl imidazolium acetate ([EMIM]Ac), a hydrophilic imidazole IL, showed a great capability for dissolving chitosan, much better than carboxylic acid imidazole and other glycine ILs [25]. However, these ILs with high polarities are poor reaction media for an enzymatic reaction, which are found to be capable of breaking hydrogen bonds of an enzyme active cite [26]. It has been observed that enzymes are most active in ILs that have hydrophobic and non-coordinating anions such as BF_4^- and PF_6^- [27].

Herein, we adopted a binary-solvent medium for Novozyme 435 catalyzed transesterification by taking into account both the substrate solubility and enzyme activity. In this medium, [EMIM]Ac (a typical hydrophilic IL with high chitosan-dissolving capability) and [BMIM][BF4] (a hydrophobic one that can well maintain enzyme activities) were mixed with different mass ratios to form a homogeneous phase [26,27]. Fig. 1 showed that in each pure IL, the DS value of the reaction was disappointed low. In a mixture of [EMIM]Ac and [BMIM][BF_4] with their mass ratio varied from 9:1 to 6:4, the DS value increased rapidly and the maximum DS value of 0.105 was found with the mass ratio of the two ILs being of 6:4. Further increase in [BMIM][BF_4] content, however, resulted in a decrease in DS of the reaction.The results indicated that the lipases may