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Synthesis and characterization of gibberellin–chitosan conjugate for controlled-release applications

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ABSTRACT

Controlled release formulations (CRFs) are promising in improving the efficiency of pesticide and minimizing the spreading of hazardous residues in environment. By coupling with the pesticide covalently, chitosan can be used as a carrier material for the vulnerable ingredient. For the first time, gibberellic acid (GA3), one of plant growth regulators, was attached to chitosan (CS) to form a GA3–CS conjugate via the formation of an amide bound using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide. The novel conjugate was structurally characterized by fourier transform infrared spectroscopy, ultraviolet spectrophotometer, and thermal gravimetric analysis. Effects of pH, temperature, and UV irradiation on the release of this conjugate were investigated. The results showed that the new conjugate had a remarkable modification degree for CS (more than 60%, w/w) and the optimal coupling conditions were defined as: the molar ratio of GA3:EDC/NHS:CS was 1:1.2:1.2, at pH 6.0 for 24 h. The release data showed the novel conjugate protected GA3 against photo- and thermal-degradation effectively and the concentration of GA3 in GA3–CS kept unchangeable about 60 d in different pH conditions. Compared with GA3 technical, the conjugate had better water solubility and stability and have potential applications. The present study also provides a novel preparation method of CRFs comprising a pesticide with long duration, sustained-release performance and good environmental compatibility. This method may be extended to other pesticides that possess a carboxyl group.

1. Introduction

Pesticides, as a common way to improve the quantity and quality of agricultural production, are widely used in agronomic practice to satisfy the demand of food by increasing global population [1]. However, low utilization ratio of conventional formulations caused by loss of pesticides through leaching, degradation and volatilization, etc. accumulates risks to the environment and increases the cost in manpower and energy [2]. For this reason, controlled release formulations (CRFs) are recognized to be advantageous for maintaining an optimum concentration of ingredient over a period of time and thereby requiring fewer applications, providing more effective biological efficacy and minimizing the impact on the non-targets and the environment [3,4].

Suitable carrier materials are vital to CRFs for impacting the drug loading, the effects of releasing, and environmental compatibility [5]. Natural polymers such as pectin, starch, chitosan and cellulose, can be used as adjuvant or drug carriers in which the active ingredient is dissolved, entrapped, encapsulated, adsorbed or chemically attached [6–10]. Most of natural polymers are also desirable for biodegradation and nontoxicity compared with other categories of materials. Some studies also revealed that the biodegradation of natural polymers may accelerate the release of the active ingredient from the CRFs to provide more effective biological effect [11]. Therefore, natural polymers have become promising materials for CRFs.

Chitosan (poly-β(1,4)-2-amino-2-deoxy-D-glusose), a natural polymer derived from chitin which is the main component of crab and shrimp shells, has shown great potential in the development of economical and versatile materials of CRFs [12]. Chitosan has high encapsulation capacity to pesticide and can be easily processed into formulations such as gel and bead [13–15]. However, the kinetics of the physical encapsulation CRFs are not sustained and instead exhibit a burst release of ingredient in the primary period due to its weak physical force between the materials and the ingredient. In addition, the longevity of this kind of CRFs depends on the thickness of coating materials, which may increase the cost and impact the properties of releasing [16]. The majority of a chitosan molecule consists of homogalacturonan domains, which have an abundance of amino groups and hydroxyl groups . The amino group of chitosan can be connected to the carboxyl group of the drug through dehydration to yield chitosan–drug conjugate [9,17]. In this way, the amide linkage can control the release

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of the active ingredient and the polymer backbone can protect the vulnerable agent from rapid degradation and running-off [18]. Despite that the amide bond is relatively stable and hydrolyzed slowly under aqueous environment, it can be decomposed by amidase or amidohydrolases, which are abundant in plant. Therefore, this kind of novel conjugate can be fractured easily and then result in the release of active ingredients faster [19–21]. Considering the chitosan has been used as a fertilizer to increase plant production, and stimulate the immunity of plants, it is a good choice of pesticide carrier for development of CRFs by the advantage of amino groups and hydroxyl groups [11,22,23].

Gibberellins are plant-growth-regulating hormones, which play an essential role in the regulation of plant growth and development, such as seed germination, epidermal cell elongation, leaf expansion and flower development [24–28]. Gibberellin A3 (GA3), known as gibberellic acid, is of high potency in most bioassays among all the gibberellins [29]. The main property of GA3 is its ability to stimulate elongation of plant shoots and induce growth of stems in rosette and dwarfish forms [30]. However, GA3 is less solvent in water and thereby is dissolved by ethanol first in practice. Particularly, GA3 is sensitive to heat and light and can be degraded quickly under neutral and alkaline conditions. In practice, GA3 is repeatedly applied in conventional formulations such as dusts, emulsifiable concentrates, and wettable powders, which results in its low utilization efficiency [24].

The aim of the study is to prepare a novel gibberellic acid–chitosan (GA3–CS) conjugate and evaluate its controlled-release characters in vitro. By utilizing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS), chitosan was reacted with GA3 to form conjugate and the parameters such as reactant ratio, pH were studied and optimized. The conjugate was characterized by Fourier transform infrared spectroscopy (FT-IR), ultraviolet spectrophotometer (UV–vis), and thermal gravimetric analysis (TGA). Release of this conjugate was also evaluated in different conditions, with varying pH, temperature, and UV irradiation.

2. Methods and materials

2.1. Chemicals and reagents

Chitosan (3000 Da, degree of deacetylation is 90%) was purchased from Dalian Glycobio Co. Ltd. (Liaoning, China). GA3 was purchased from Jiangsu Fengyuan Bioengineer Co. Ltd. (Jiangsu, China). All other reagents were purchased from Baker (Phillipsburg, NJ, USA) and were of analysis grade or better. Milli-Q water obtained in the laboratory using a Milli-Q water purification system (Millipore, Billerica, MA, USA) was used in all experiments. The 0.1 N phosphate buffer solutions (PBS) with different pH values were prepared by mixing 0.1 mol L^{-1} NaH_2PO_4 solution with 0.1 mol L^{-1} Na_2HPO_4 solution.

2.2. Synthesis of GA3–CS

The covalent attachment of GA3 to chitosan was achieved via the formation of amide bonds between carboxylic acid moieties of GA3 and amine groups of chitosan. Briefly, GA3 (2.75 g), EDC (1.15 g) and NHS (0.69 g) were dissolved in 20 mL H_2O, stirring for 30 min. Then chitosan (1.35 g) dispersed in 10 mL of H_2O was added dropwise into the reaction mixture under vigorous stirring. The whole solution was adjusted to pH 6 with 1 N HCl and kept at 4 °C for 24 h. Finally, the solution was precipitated and washed sufficiently with acetone, and the residue was filtered and dried at 60 °C in oven.

2.3. Characterization of GA3–CS

The product was sufficiently dialyzed against H_2O using a dialysis membrane (MWCO: 1 kDa, Spectrum Laboratories, Laguna Hills, CA) for 3 days (water was changed every 12 h), and then vacuum dried at 313 K for characterization.

2.3.1. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra, recorded on Jasco FT-IR 5300 spectrophotometer, were used to compare the structures of GA3, chitosan and GA3–CS. Samples were prepared as KBr pellet and scanned against a blank KBr pellet background at wave number ranging from 4000 to 650 cm^{-1} with resolution of 4.0 cm^{-1}.

2.3.2. Ultraviolet spectrophotometer (UV–vis)

UV–vis experiments were performed with a UV-2100S spectrophotometer (Shimadzu, Japan) to compare the difference in UV absorption of GA3, chitosan and GA3–CS.

2.3.3. Thermal gravimetric analysis (TGA)

TGA was carried out with a SDT Q600 (TA Instruments–Waters LLC, USA) analyzer from 25 to 600 °C with heat rate of 10 °C/min.

2.4. Determination of modification degree and GA3 content in GA3–CS

The HPLC method was referenced from Kirti Bhalla with minor revise [31]. Briefly, the mobile phase consisted of eluent (A) acetonitrile, (B) water with 0.1% H_3PO_4 (30:70 v/v). Analytical C_{18} ODS column (250 mm × 4.6 mm, 5 μm; DIKMA, USA) was equipped with a guard column (4 mm × 3 mm) and pre-equilibrated with the mobile phase for about 40 min before analysis. The injection volume was 20 μL. Detection wavelength was 210 nm and flow rate was kept at 1.0 mL min^{-1}.

The degree of modification was calculated by measuring the unreacted GA3 in the final reaction solution. 0.5 mL reaction solution was dissolved in 100 mL H_2O, and 20 μL of the diluent was subsequently injected into the HPLC system following by filtered with a 0.45 μm membrane filter. The degree of modification was determined according to the following equation: $D = (T - R)/T^*100\%$ where D is the degree of modification, T is the total amount of GA3 and R represents the remaining GA3 in the final reaction solution.

2.5. Evaluation of controlled-release properties

Release of GA3 from GA3–CS with equivalent GA3 concentration of 200 mg L^{-1} was carried out in PBS buffer with different pH (5, 7 and 9) and temperature (25, 35 and 45 °C) to study the hydrolysis property of this conjugate. The effect of UV irradiation (high-pressure mercury lamp, the light intensity was 700 μW/cm^2 detected using ZDZ – 1 UV irradiation photometer) on the release of GA3 was also investigated, and the temperature kept at 25 °C. GA3 technical sample with equivalent concentration of CS in the same conditions was set as control. Equal known aliquot of sample was suspended in equal volume of preheated buffer solution at each different condition and sealed in dialysis tubing (1000 MWCO). The dialysis tube was placed in PBS and shaken in a shaking water bath at each different condition, and then sampled at predetermined intervals. Each sample was repeated in triplicate and determined by HPLC (LC-20ATvp, Shimadzu, Japan) to monitor the released amount of GA3 as above. Each sample was assayed in six replicates, and all the assays were carried out at ambient temperature.