Acid-base evaluation of chitosan-ferulic acid conjugate by a free radical grafting method

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Abstract. Chitosan-ferulic acid conjugate has been synthesized based on the free radical-mediated grafting reaction. This study investigated the application of potentiometric titration to evaluate the free radical grafting method of ferulic acid on chitosan. The synthesis was done by hydrogen peroxide/ascorbic acid induced free radical reaction to conjugate ferulic acid with chitosan. The conjugate product of ferulic acid-chitosan was characterized by FTIR, UV-Vis spectrophotometry, potentiometric titration, and SEM images. Evaluation of the grafting ratio of the ferulic acid-g-chitosan was determined by Folin–Ciocalteu method. The chitosan-ferulic acid conjugate gave 84% in yield with total phenolic showed the value was 92.53 mg per gram of dry polymers and the conjugate product only soluble at pH around 6. The antioxidant activity of chitosan was increased after functionalized with ferulic acid.

Keywords: Ferulic acid, chitosan, potentiometric titration, free radical grafting, antioxidant activity

1. Introduction
Chitosan (Cs) is a partially composed of D-glucosamine and N-acetyl-D-glucosamine linked by β(1-4) bond which is obtained by deacetylation of chitin with alkaline treatment [1]. Chitin mainly comes from the exoskeleton of shrimp, prawn, lobster, and insects. Chitosan contains an amino (-NH₂) and hydroxyl (-OH) groups in D-glucose backbone, which has a wide range of applications in tissue engineering [2], pharmaceutical [3-4], food preservation [5-6], cosmetics [7], and agriculture [8], due to its nontoxicity, biodegradability and biocompatibility. However, the utilization of chitosan is limited by its poor solubility in neutral or alkaline media, since chitosan is only soluble in acidic condition. Chemical modification to the chitosan functional groups improves its physicochemical and biological properties. Chitosan can be modified by many chemical reactions, such as carboxymethylation, esterification, alkylation, N-quarternization, and graft copolymerization. In order to introduce certain functional molecules into chitosan, graft copolymerization reaction has been most frequently used [9]. Grafting some phenolic compounds such as eugenol [10], gallic acid [11], epigallocatechin gallate [12] onto chitosan remarkably increased its biological activities.
Phenolic compounds are plants secondary metabolites which have precious biological activities such as anticancer, antioxidant and antimicrobial [13-14]. Ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid) is a natural phenolic acid which commonly isolated from Grain [15]. Because of its low toxicity and has many physiological functions, including anti-inflammatory, antioxidant, anticancer [16], and antimicrobial activity [17], this bioactive compound has been extensively used in pharmaceutical, food, and cosmetic industries. Ferulic acid contains phenolic, methoxy and alkene conjugated to carboxylic acid groups which readily oxidized in the high temperature. The phenolic molecules covalently grafted onto polymers (i.e., cellulose and chitosan) expand their stabilities, physicochemical properties and biological activities (e.g., antioxidant, antimicrobial, antifungal and antitumor) [18-19].

Several studies reported that chitosan-phenolic compounds conjugates formed complex had good solubility in water [20]. Due to the instability of ferulic acid in the high temperature, free radical grafting was preferable than another grafting method. This study aimed to produce chitosan-ferulic acid conjugate by a simple modification with the eco-friendly approach. Ferulic acid was grafted onto chitosan by free radical grafting with hydrogen peroxide/ascorbic acid. The synthesized product was evaluated by using total phenolic contents with Folin-Ciocalteu reagent, potentiometric titration, UV-Vis spectrophotometry, and Fourier-transform infrared (FT-IR) to confirm the conjugation.

2. Materials and Methods

2.1. Materials
Ferulic acid (Sigma-Aldrich), chitosan from Indonesian crab shells (66.5% degree of deacetylation), hydrogen peroxide (Merck), ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl radical (Merck, Darmstadt, Germany), Folin-Ciocalteu reagent (Sigma-Aldrich), acetic acid (99%w/w) (Merck), sodium hydroxide (Merck), ethanol (Merck), and hydrochloric acid (37% w/w) (Merck) were used without any purification. The synthesis products were identified by infrared spectrophotometer (FTIR, Shimadzu-8201PC) over a wavenumber range of 400–4000 cm$^{-1}$ on KBr pellet, UV-Vis spectrophotometer (Dynamica Scientific Halo RB-10), pH meter (Thermo Scientific Orion Star A214), and Scanning Electron Microscopy (Hitachi SU3500).

2.2. Synthesis of Ferulic Acid Grafted-Chitosan
Free radical grafting method of Chitosan-Ferulic acid was conducted following to Curcio et al. (2009) [21], with some modification. Chitosan (0.25 g) was dissolved in 25 mL of acetic acid solution (2% v/v) in a 100 mL flask tube. In the ice bath, 0.027 g of ascorbic acid in 1 mL hydrogen peroxide 1M was added slowly into chitosan solution. 0.2708 g of ferulic acid in 5 mL ethanol was dropped into the flask, and the mixture was continuous stirring under atmospheric condition at room temperature for 24 h. NaOH 2 M was added into the mixture and pH was adjusted to 10, followed by filtration. The residue was washed with absolute ethanol three times to give a yellow solid with 84% in yield.

2.3. Total phenolic compounds evaluation
The quantification of the total phenolic group on chitosan-ferulic acid conjugate by Folin-Ciocalteu procedure according to the literature with some modifications [22]. Chitosan-ferulic acid conjugates (25 mg) was dissolved in acetate buffer pH 5.6 up to 25 mL in a volumetric flask. Folin-Ciocalteu reagent (0.5 mL) was supplemented to the sample solution (0.5 mL) and was mixed thoroughly, followed by addition of 1.5 mL of sodium carbonate (20%w/v), 7.5 mL distilled water and allow to stand for 2 h. The concentration of phenolic content was measured at wavelength 765 nm with the chitosan solution under the same conditions as a blank. The concentration of total phenolic groups in the chitosan conjugate product was represented as ferulic acid equivalent concentrations. The calibration curves of ferulic acid were made by plotting the concentrations to 20, 45, 60, 80, 100, 125 and 150 ppm versus absorbance. Total phenolic of ferulic acid-chitosan was expressed as milligrams of ferulic acid per gram conjugate product.
2.4. Potentiometric titration experiments
The degree of substitution of chitosan was estimated by potentiometric titration adapted from Kong (2012) [23]. The weighed chitosan-ferulic acid conjugate (50 mg) was dissolved in 10 mL of aquadest and adjusted to below pH 2 with 0.1 M hydrochloric acid, and NaOH solution (0.1M) was used as the titrant. The figure was obtained based on the sodium hydroxide volume against the corresponding pH and first derivatives of electric potential difference ∆E(mV)/∆V(mL) value recorded.

2.5. Antioxidant activity test by DPPH method
The antioxidant activities of chitosan-ferulic acid conjugate were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Briefly, the stock solution (1000 ppm) was prepared by dissolving a conjugate product in acetate buffer pH 5.6. The series of concentrations of the conjugate product (50, 100, 150, 200, 250, and 500 ppm) were made by adjusted the stock solution with acetate buffer pH 5.6 and methanolic DPPH (0.2 mM). The mixtures were kept in the dark condition for 30 min at room temperature to perform radical scavenging reaction. The same portion of absolute methanol and acetate buffer pH 6 used a blank. The absorbance was determined at 517nm, and the antioxidant activities were defined as inhibition of DPPH radicals calculated as follows:

\[
Inhibition \, (\%) = \left( \frac{A_0 - A_1}{A_0} \right) \times 100\%
\]

where \(A_0\) is the absorbance of the standard (mixture methanolic DPPH and acetate buffer pH 6), and \(A_1\) is the absorbance of the sample only. The IC\(_{50}\) value denoted as the concentration of chitosan-ferulic acid conjugate that inhibited 50% DPPH radicals [24].

3. Results and Discussion
3.1. Synthesis of Chitosan-Ferulic Acid Conjugate
Chitosan-ferulic acid conjugate was successfully grafted by free radical method using hydrogen peroxide/ascorbic acid. The reaction was carried out temperature for 24 h at ambient with the mass ratio of chitosan, and ferulic acid was 1:1 (w/w). The reaction mechanism divided into two steps, first is the chitosan initiated by the radical reaction and the second step is the grafting of the ferulic acid into chitosan. The reaction started by oxidation of ascorbic acid by hydrogen peroxide in cold temperature to form ascorbate radical (Asc\(^{\cdot}\)) instead of hydroxyl radical (OH\(^{\cdot}\)). The existence of ascorbate radicals abstracts hydrogen atom of chitosan chain to produce dehydroascorbic acid and chitosan radicals [25]. The grafting reaction was occurred by added ferulic acid into chitosan radicals to yield conjugate product. Afterward, pH of the reaction mixture was adjusted into alkaline (pH) condition to dissolve unreacted ferulic acid and to precipitate the conjugate product. The yellow solid product was washed with absolute ethanol to eliminate residue of ferulic acid. This method is simple to produce conjugate of chitosan-ferulic acid without dialysis membrane, by changing the pH condition, to precipitate the desired compounds. The radical reaction can be performed in lower temperatures to prevent the ferulic acid oxidation. Moreover, this reaction does not generate toxic side products.

The reaction product was insoluble in acidic and alkaline condition but fully dissolved at pH around 5-6 (Figure 1). The solubility of the product might be influenced by both chitosan and ferulic acid properties. In acidic condition, chitosan is soluble, but ferulic acid is insoluble. Opposite in the alkaline condition, ferulic acid is soluble, but chitosan is insoluble. The conjugate product only soluble in the pH 5-6 might due to the grafted reaction occurred not in the functional group which responded to the solubility such as amino (-NH\(_2\)) in chitosan and phenolic (Ph-OH) or carboxylic acid (-COOH) in ferulic acid.
Figure 1. The solubility of ferulic acid grafted-chitosan in acidic (a), pH 5-6 (b), and alkaline condition (c).

3.2. Characterization of Chitosan-Ferulic acid Conjugates

The conjugate product of free radicals grafted method between chitosan and ferulic acid was evaluated by using Fourier transform infrared, UV-vis spectrophotometry, potentiometric titration, and total phenolic compounds with Folin-Ciocalteu method to determine the degree of substitution.

The Folin-Ciocalteu phenol reagent can be used to estimate the amount of ferulic acid conjugate onto chitosan chain. Ferulic acid as a phenolic compound reacted with phosphomolybdic and phosphotungstic acids existing in the Folin-Ciocalteu reagent to form complex redox mixture. The ferulic acid content in the conjugate product was 92.53 mg/g of conjugate product. It has been reported that radically initiated grafting gallic acid- and catechin-chitosan were 7 and 4 mg/g of the dry product, respectively [21].

Figure 2. The proposed schematic reaction of ferulic acid grafted into chitosan.

The Fourier transform infrared spectra of chitosan, ferulic acid and conjugate product were shown in Figure 3. Infrared spectra of chitosan exhibited broad peaks at around 3460 cm\(^{-1}\) (-O-H st) and 1656 cm\(^{-1}\) (C=O amide of residual chitin groups). Ferulic acid showed the appearance of sharp peaks at 3434 cm (-O-H st), alkenes characteristic band around 1690 cm\(^{-1}\). FTIR spectra of the conjugate product showed shifting of C=O stretching of \(\alpha,\beta\)-unsaturated carboxylic acid of ferulic acid at 1690 cm\(^{-1}\) to aliphatic carboxylic acid...
at 1700 cm$^{-1}$. The absence of a peak at 941 cm$^{-1}$ (C=C-H), overtone at 1822 cm$^{-1}$ and no peak at 1660 cm$^{-1}$ (C=C st) [26] indicate that the alkene group of ferulic acid was converted into a single bond (Figure 2). It implies that covalent bond between ferulic acid and chitosan were formed. Moreover, no peak at 1730 cm$^{-1}$ in the conjugate products, implying no ester bond formed between carboxyl groups of ferulic acid and hydroxyl groups of chitosan, which has been reported by previous researcher [21, 27].

![FTIR spectra of Chitosan-ferulic acid conjugate (a), chitosan (b) and ferulic acid (c).](image)

**Figure 3.** FTIR spectra of Chitosan-ferulic acid conjugate (a), chitosan (b) and ferulic acid (c).

To confirm that grafting between ferulic acid and chitosan was done by comparing UV spectra of chitosan, ferulic acid and chitosan-ferulic acid conjugate (Figure 4) over 190 - 400 nm of wavelength. Chitosan in 2% acetic acid (v/v) revealed no absorption peak between 230 to 400 nm. Ferulic acid in ethanol solution showed a maximum absorption peak around 320 nm. The UV-vis spectra of chitosan-ferulic acid conjugates gave hypsochromic shifting from ferulic acid spectra with maximum absorption around 210 nm. The hypsochromic shifting of maximum absorption band from 320 to 210 nm might due to the breakdown of the alkene group from conjugate carboxylic acid. This spectra supports the FTIR data that insertion of ferulic acid into chitosan was done.

The titration was performed by dissolving chitosan, ferulic acid and the conjugate product with water and adjusted to pH 2 by hydrochloric acid 0.1 M. The excess of HCl was titrated by using NaOH 0.1 M. The titration curve was plotted by volume NaOH (mL) against pH and first derivatives versus $\Delta E$ (mV)/$\Delta V$ (mL). The typical titration curves of chitosan (Figure 5) showed two inflection points as amino and hydroxyl groups which both contribute to acid-base balance. The first inflection point of chitosan at pH 4.81 was represented as hydroxyl (-OH) and hydroxide (-O$^-$) groups balance, whereas the second inflection point at pH 7.15 characterized as the balance of ammonium (-NH$_3^+$) and amino (-NH$_2$) groups. The characteristic potentiometric titration curves of ferulic acid are shown in Figure 6. The curves exhibited three inflection points (pH 3.87, 7.27, and 10.64) which might correspond to acid-base balance of phenolic, $\alpha,\beta$-unsaturated and carboxylic acid groups of ferulic acid. The potentiometric titration curves (Figure 7) of the conjugate product of ferulic acid and chitosan appeared only two inflection points (5.65 and 11.56). The reduced of total inflection points of the product from the total inflection points of ferulic acid due to one of the functional groups of ferulic acid was break down and converted to another functional group which was
not contributed to acidity. The potentiometric titration curves reinforced the FTIR spectra that unsaturated group of ferulic acid might be changed into a single bond by free radical method.

**Figure 4.** UV-vis spectra of Chitosan-ferulic acid conjugate (a), ferulic acid (b), chitosan (c).

**Figure 5.** The titration curves of chitosan.

**Figure 6.** The titration curves of ferulic acid.

**Figure 7.** The titration curves of chitosan-ferulic acid conjugate product.
The surface morphology images (Figure 8) were carried out by using scanning electron microscope with voltage acceleration 2 kV and a working distance of 6.5 to 6.8 mm. Grafting reaction of chitosan and ferulic acid by using radical method provided the significant change of morphology between chitosan as reactant (a) and the conjugate of chitosan-ferulic acid product (b). The morphology of chitosan exhibited as a big rolled sheet, whereas the conjugate product appeared the flake-like morphology in the smaller size.

![Figure 8](image)

(a) (b)

**Figure 8.** The morphology images of chitosan (a) and chitosan-ferulic acid conjugate product (b)

### 3.3. Antioxidant activity of Chitosan-Ferulic acid Conjugates

Radical scavenging capacities of chitosan-ferulic acid conjugates were evaluated by DPPH test with the decrease in absorbance of yellow-colored compound at 517 nm. Reduction of diphenyl-picrylhydrazine radicals is depended on the hydrogen-donating ability of the conjugate product which might be contributed by ferulic acid content in the chitosan chain. The antioxidant activity of ferulic acid is caused by proton transfer from the phenolic and unsaturated carboxylic acid group to DPPH radicals. The DPPH scavenging activities of chitosan-ferulic acid conjugate are shown in Figure 9. The increasing concentration of conjugate product from 0 to 0.6 mg/mL, showed an increased trend of scavenging activity up to 71.19±1.97% at 0.6 mg/mL with $IC_{50} = 0.386$ mg/mL. This conjugate product has a better antioxidant activity compared with the plain chitosan (24.86% and at 0.2 mg/mL). It means that solubility and antioxidant activity of chitosan were improved by conjugating with ferulic acid. This product has a potential to be used in pharmaceutical, biomaterial and food industries.

![Figure 9](image)

**Figure 9.** Antioxidant activity of chitosan-ferulic acid conjugate by DPPH method
4. Conclusion
In the present study, chitosan-ferulic acid conjugate was successfully synthesized by using free radical method with hydrogen peroxide/ascorbic acid. The conjugate product was confirmed by FTIR, UV-Vis and potentiometric titration. It suggested that the grafting reaction happened between hydroxyl group of chitosan and α,β-unsaturated carboxylic acid groups of ferulic acid. Furthermore, the product was improved in solubility and radicals scavenging activities against DPPH compared with chitosan. In addition, simple preparation of chitosan-ferulic acid conjugate by free radical and precipitate the product by changing pH condition could be further explored in many applications area.

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