CHAPTER IV
CONJUGATION OF GALLIC ACID ONTO CHITOSAN: AN APPROACH FOR GREEN AND WATER-BASED ANTIOXIDANT

4.1 Abstract

A novel derivative of chitosan, chitosan-gallic acid (chitosan-GA), obtained from a simple conjugating condition with gallic acid using carbodiimide is proposed. The conjugation with gallic acid brings the water solubility or water swelling property to chitosan. The studies on the free radical scavenging of chitosan-GA clarify its significant antioxidant activity on free radicals, i.e. carbon-centered radicals and hydroxyl radicals. Chitosan-GA clearly shows a synergistic antioxidant activity on hydroxyl radical scavenging where iron metals were involved. The work demonstrates how chitosan-GA can be a potential natural-compound-based antioxidant.

Keywords: Chitosan; Gallic acid; Antioxidant; Water soluble; Carbodiimide conjugating agent; Electron paramagnetic resonance (EPR)

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4.2 Introduction

It is well known that free radicals play an important role in metabolic and chemical changes in biological systems, which may result in disease, food deterioration, and aging (Halliwell & Gutteridge, 1989). Antioxidants reduce or retard free radical generation and prevent the oxidation of cellular oxidizable substrates. Antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are representative; however, they are synthetic compounds and have to be used under strict regulations. (Bran, 1975; Whysner, Wang, Zang, Iatropoulos, & Williams, 1994). Vitamins such as C and E are well-known for the major tissue antioxidants in biological systems; however, we have to be aware of their instability and some adverse effects as prooxidants (Mayo, Tan, Sainz, Natarajan, Lopez-Burillo, & Reiter, 2003). Health professionals and consumers welcome the natural antioxidants and are searching for alternative ones; especially those from plant extracts, for example, green tea (Nakagawa & Yokozawa, 2002), rosemary (Sebranek, Sewalt, Robbins, & Houser, 2005), olive oil (Beltrán, Aguilera, Rio, Sanchez, & Martinez, 2005), and so forth. In recent years, polysaccharides, such as sulfated seaweed-based polysaccharide, have been reported as antioxidants. Sulfated polysaccharide from *Porphyra haitanesis* (Zhang, Yu, Li, Zhang, Xu, & Li, 2003), fucoidan from *Fucus vesiculosus*, and fucans from *Padina gymnospora* (Souza, Marques, Dore, Silva, Rocha, & Leite, 2007) shows the scavenging effect on superoxide radical, hydroxyl radical, and lipid peroxide.

Chitin-chitosan (Fig. 1) is the second most naturally abundant copolysaccharide of β-(1-4)-2-acetamido-2-deoxy-β-D-glucose and β-(1-4)-2-amino-2-deoxy-β-D-glucose, next to cellulose, but exists in the shells of crustaceans, insects, and fungi. Similar to the polysaccharide from seaweeds, chitosan has also been reported for its antioxidant ability, in addition to its antibacterial (Kendra & Hadwiger, 1984) and antimutagenetic (Kogan et al., 2004) properties. Chitosan sulfate and low molecular weight chitosan sulfate were found to have radical scavenging activity on the superoxide and hydroxyl radicals (Xing et al., 2005; Xing, Liu, Yu, Guoa, Lia, & Lia, 2005). Huang, Mendis, and Kima (2005) proposed hydroxyethyl chitosan sulfate as a radical scavenger as evidenced from the model

studies of DPPH, hydroxyl, and carbon-centered free radicals. Xie, Xu, and Liu (2001) reported that maleic acid grafted hydroxypropyl chitosan or carboxymethyl chitosan showed radical scavenging activity against hydroxyl radicals. It is important to note that for those cases, the approaches are the functionalization with the hydrophilic terminal groups, such as sulfate (Xing et al., 2005; Xing, Liu, Yu, Guoa, Lia, & Lia, 2005), carboxymethyl, hydroxylpropyl (Xie, Xu, & Liu, 2001), and so forth.

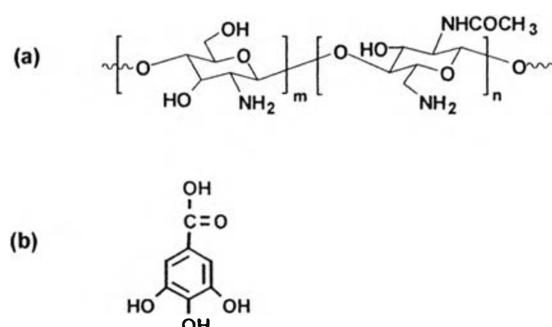


Fig. 1. (a) Chitosan and (b) gallic acid, GA.

As chitosan exhibits strong metal ion chelating ability (Burke, Yilmaz, & Hasirci, 2000) owing to the nitrogen atom, chitosan is a potential preventive antioxidant based on its metal ion deactivation. However, chitosan has some limitations in being a practical antioxidant; especially, (i) its poor solubility due to its inter- and intramolecular hydrogen bond network, and (ii) the lack of on H-atom donor to serve as a good chain breaking antioxidant. As a result, we need to improve the solubility of chitosan in neutral aqueous solution as well as introduce the H-atom donor group if we are to consider the development of a chitosan-based antioxidant.

Gallic acid (GA, 3,4,5-trihydroxy benzoic acid) (Fig. 1) is a natural phenolic antioxidant extractable from plants, especially green tea (Lu, Nie, Belton, Tang, & Zhao, 2006). It is widely used in food, drugs, and cosmetics to prevent rancidity induced by lipid peroxidation and spoilage. From the structural viewpoint, GA is attractive for conjugating onto chitosan for a novel green antioxidant because of (i) the high reducing potential and low O-H bond dissociation enthalpy of the tri-hydroxyl groups on the benzene ring (Ji, Zhang, & Shen, 2006); (ii) the possibility that the bulky group of the benzene ring of GA obstructs the inter- and

intramolecular hydrogen bond network of chitosan; (iii) the multi-functional hydrophilicity based on the hydroxyl and carboxyl groups; (iv) the carboxylic acid group for conjugation with chitosan; and, (v) it being a natural product. It is also important to note that chitosan is soluble in acid conditions (pKa ~ 5.5), which renders this polymer inapplicable in the neutral aqueous or in the nonderivatized form. It can be expected that the chitosan-conjugated gallic acid may show antioxidant activity and water-solubility, together with the metal chelating ability (Burke, Yilmaz, & Hasirci, 2000; Burke, Yilmaz, Hasirci, & Yilmaz, 2002; Guzman, Saucedo, Revilla, Navarro, & Guibal, 2003; Ngah, Ghani, & Kamari, 2005), biodegradability (Yamamoto & Amaike, 1997), biocompatibility (Richardson, Kolbe, & Duncan, 1999), and bioactivity (Dumitriu, Popa, Cringu, & Stratome, 1989).

The present work focuses on the development of a novel natural-compound-based antioxidant by functionalizing chitosan with gallic acid. Here, the article shows the simple conjugating reaction and the water solubility improvement of chitosan. The article also demonstrates the antioxidant activity of the derivative by using a series of model radical species; i.e. 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, carbon-centered radical and hydroxyl radical.

4.3 Experimental

4.3.1 Materials

Chitosan with a percent degree of deacetylation (%DD) of 95 (M_v = 9.5 $\times 10^5$ Dalton) was provided from Seafresh Chitosan (Lab) Company Limited, Thailand. Gallic acid (GA) and 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) were purchased from Aldrich Chemical Company, Inc., USA, and was used after purification with charcoal. α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (POBN) was purchased from Sigma, USA. Hydrogen peroxide solution (30%), sodium hydroxide solution (1 M), ferrous ammonium sulfate ($Fe(NH_4)_2 \cdot 6H_2O$), and methanol were from Fisher Scientific Co., Fair Lawn, NJ, USA. 2,2 -Azo*bis* (2-amidinopropane)•HCl (AAPH) was from Polysciences, Inc., USA. 1-Ethyl-3-(3 -dimethylaminopropyl) carbodiimide (EDC) and 1,1-Diphenyl-2-picrylhydrazyl

(DPPH) were obtained from TCI, Japan. *N*-hydroxysuccinimide (NHS) was purchased from Wako (Osaka, Japan). Ethanol and acetic acid (AR grade) were from Lab Scan, Co., Ltd, Thailand. All chemicals were used without further purification.

4.3.2 Instruments

Fourier transform infrared spectroscopy (FTIR) was carried out by using a Thermo Nicolet Nexus 670 with 32 scans, 2 cm^{-1} resolution. Elemental analysis was carried out by using a Yanako CHN Corder MT-3, MT-5 with a combustion temperature at 950°C under air atmosphere with O_2 as a combustion gas (flow rate 20 mL/min) and He as a carrier (flow rate 200 mL/min). ^{13}C Cross-polarization magic angle spinning nuclear magnetic resonance (^{13}C CP/MAS NMR) spectra were taken by using a Bruker DPX-300 at 23 ± 1°C. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded at 70°C for the samples dissolved in CD_3COOD/D_2O (2%v/v) using a Bruker UXNMR/XWIN-NMR Avance DPX400. Powder X-ray diffraction (XRD) spectra were recorded by a Rigaku RINT 2000 over 2°-60° 2θ using CuK_α as an X-ray source, operating at 40 kV and 30 mA with a Ni filter. A Dupont thermogravimetric analyzer was applied under a N_2 flow rate of 20 mL/min and a heating rate of 10°C/min from 30°C to 600°C. The solubility was evaluated by a ChemStation 845x. ReV.A., 10.10, USA, UV-visible spectrometer at 500 nm. The radicals scavenging capacity was determined by a Bruker EMX EPR spectroscope with a transverse mode (TM_{110}) cavity and flat cell. The EPR spectrometer settings were: receiver gain, 2.5 × 10^5; microwave power, 10 mW; modulation frequency, 100 kHz; frequency, 9.780 GHz; modulation amplitude, 0.10 G; time constant, 40.96 ms; number of scans, 20; and scan rate 10 G/ 21 s.

4.3.3 Synthesis of chitosan conjugated with gallic acid, **3**

Chitosan (1 wt./vol.-%) was dissolved in acetic acid (2 vol.-%) and stirred overnight to obtain a chitosan solution and was re-precipitated in sodium hydroxide (1 wt./vol.-%) solution to obtain fine particles. The product obtained was thoroughly washed with water several times until neutral and was kept in ethanol. Gallic acid (GA, 0.5104 g, 3 moles equivalent to Chitosan) was dissolved in ethanol

(20 mL) and 1-Ethyl-3-(3 -dimethylaminopropyl) carbodiimide (EDC, 1.7253 g, 1 mole equivalent to GA) was reacted with GA to obtain **1.** *N*-hydroxysuccinimide (NHS, 1.0358 g, 1 mole equivalent to GA) was further added. The reaction was stirred in an ice bath for 1 h in ethanol to obtain **2.**

The solution **2** was gradually added into the chitosan suspension in ethanol (0.1631 g, 1 mole) and stirred in an ice bath for 30 min. The reaction was carried out heterogeneously at room temperature for 24 h. The solution obtained was centrifuged and washed thoroughly with ethanol several times to obtain chitosan conjugated with gallic acid (Chitosan-GA), **3c** (Fig. 1). Similarly, **3a, 3b,** and **3d** were prepared by varying the Chitosan: GA: EDC: NHS ratios (1:1:1:1 for **3a**; 1:2:2:2 for **3b**; and 1:4:4:4 for **3d**), respectively.

$(C_{27}H_{23}O_{16}N)_{0.92}(C_{21}H_{21}O_{12}N)_{0.08}$: Calcd. C 54.04, H 3.84, N 1.62, O 40.50; Found for **3a** (DS ~ 15.49%) C 38.83, H 6.64, N 7.50, O 47.03; Found for **3b** (DS ~ 15.52%) C 38.34, H 6.62, N 7.39, O 47.65; Found for **3c** (DS ~ 15.62%) C 36.93, H 6.70, N 7.07, O 49.30; Found for **3d** (DS ~ 15.51%) C 37.59, H 6.69, N 7.25, O 48.47.

IR (KBr) for **3**: 1640 (amide I), 1580 (amide II), 1730 (C=O ester), 3464 (OH), and 895 (pyranose ring).

^{13}C NMR for **3c:** δ = 160.2 (C=O ester), 140 (aromatic), 104.6 (C-1 of pyranose ring) 75.5-82.5 (C-3 to C-5 of pyranose ring), 57.3-61.1 (C-2 and C-6 of pyranose ring).

^{1}H NMR (2% CD_3COOD in D_2O) for **3**: δ = 7.6 (2H, s, H-a), 2.5 (3H, s, H-Ac), 5.3 (1H, d, H-1), 3.0 (1H, d, H-2), 3.9-4.5 (5H, m, H-3 to H-6 of pyranose ring).

4.3.4 DPPH radical (DPPH$^•$) scavenging capacity

A 250 μL sample of each concentration of GA (20, 40, 48, 60, and 80 μM), **3c,** and Chitosan (480, 970, 1450, 1940, 2900, 4000, and 4850 μM, respectively) was mixed with the methanolic DPPH$^•$ solution (200 μM, 750 μL). The reactions were carried out at room temperature under subdued light and left for 30

min before collecting the EPR spectra. The relative percent of DPPH$^{\bullet}$ scavenging capacity was calculated as in Eq. 1:

$$\%RC = (h_0 - h_c)/ h_0 \times 100 \quad (1),$$

where h_c and h_0 are the peak heights of the EPR spectrum with and without antioxidant, respectively.

4.3.5 Carbon-centered radical (R$^{\bullet}$) scavenging capacity

An amount of **3c** solution (4mM; 2, 3, 6, 15, 30 μL) or GA (5 mM; 1, 2, 5, 10, 20 μL) was mixed with AAPH (300 mM, 25 μL) and POBN (500 mM, 25 μL). PBS pH 7.4 was added to adjust the volume of the mixture to 500 μL. The EPR spectrum of POBN/R$^{\bullet}$ spin adducts was measured. The relative percent of R$^{\bullet}$ scavenging capacity was calculated as in Eq. 1.

4.3.6 Hydroxyl radical (HO$^{\bullet}$) scavenging capacity

GA or **3c** (10 mM) were prepared and each sample (2.5, 5, 10, 25, 50, 75, 100, 150, or 250 μL) was taken to mix with H_2O_2 (250 μM, 20 μL), DMPO (1 M, 5 μL), and Fe^{2+} ((Fe $(NH_4)_2 \cdot 6H_2O$) solutions (100 μM, 10 μL). PBS pH 6.5 was used to adjust the total volume to 500 μL. The EPR spectrum of DMPO/HO$^{\bullet}$ spin adducts was measured. The relative percent of HO$^{\bullet}$ quenching capacity was calculated as in Eq. 1.

4.4 Results and Discussion

4.4.1 Conjugation of chitosan with GA and its water swelling property

As GA, EDC, and NSH are soluble in ethanol and the re-precipitated chitosan was dispersed well in ethanol, the reaction mixture was then carried out heterogeneously in ethanol at ambient temperature. As shown the mechanism in Fig. 2, after EDC and NHS initiated the reactive ester species on GA, the nucleophilic reaction possibly occurs at C-2, C-6, including C-3. The un-reacted species and the

by-products **4**, as well as NHS, were removed by washing thoroughly with a good solvent (i.e. ethanol) several times. The suspension obtained was centrifuged to collect the wet powder particulate product. Here, it is important to note that when the product was combined with water, a rather clear solution was observed (Fig. 3(A)). To confirm the water solubility, the transmittance of **3a-3d** aqueous solution (1.2 mg mL^{-1}) at 500 nm was studied. Compounds **3c** and **3d** show the transmittance above 80%, whereas **3a** and **3b** are only 4% and 8% (Fig. 3(B)). It was also found

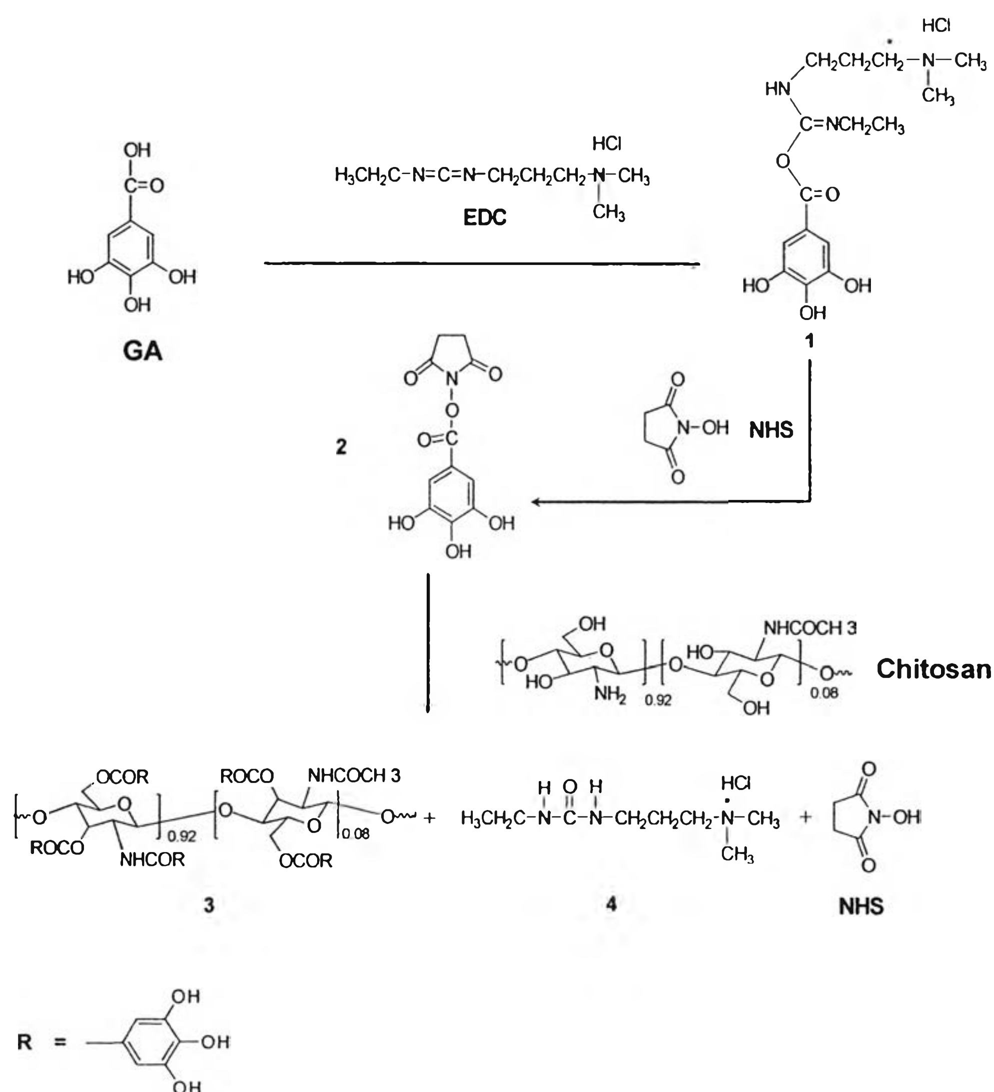


Fig. 2. Reaction pathway of chitosan conjugated with GA via conjugating agents.

that when the samples were freeze-dried, **3c** and **3d** became partially dissolved or swelled in water; whereas **3a** and **3b** were difficult to dissolve or even swell in water. The difficulty in re-dissolving **3a** and **3b** implied the recovery of the H-bond along the chitosan chain after freeze-drying.

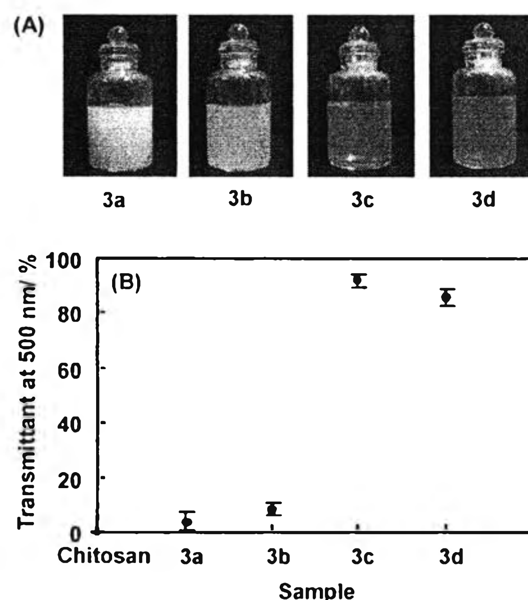


Fig. 3. Appearances (A) and transmittance at 500 nm of **3a**, **3b**, **3c**, and **3d** aqueous solutions (1.2 mg ml^{-1}) (B). Results are mean ±SD (n=3).

4.4.2 Structural characterization of Chitosan-GA

The conjugation of GA on chitosan is possible at either C-2, to obtain the amide linkage, or C-3 and C-6 to obtain the ester linkage (Paquette, 1995). Compound **3** shows significant peaks at 1730 and 1640 cm^{-1}, implying the ester and amide linkages between chitosan and GA (Fig. 4). Here, to quantify how significant esterification or imidization was, the FT-IR curve fitting was carried out using the integral ratio between the peaks at 1730 cm^{-1} (ester) or 1640 cm^{-1} (amide), and the internal peak at 895 cm^{-1} referred to the C-O-C of the pyranose ring (Fig. 5(A)).

It was found that the ratios of A_{1640}/A_{895} and A_{1730}/A_{895} increased when compared to chitosan (Fig. 5(B)). Fig. 5(C) shows the differences in the integral ratio of A_{1640}/A_{895} and A_{1730}/A_{895} after subtracting with the amide and ester peaks of chitosan.

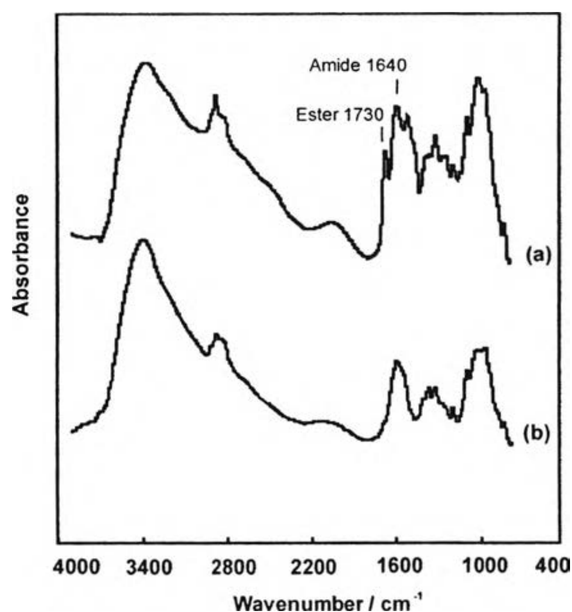


Fig. 4. FT-IR spectra of (a) **3** and (b) chitosan.

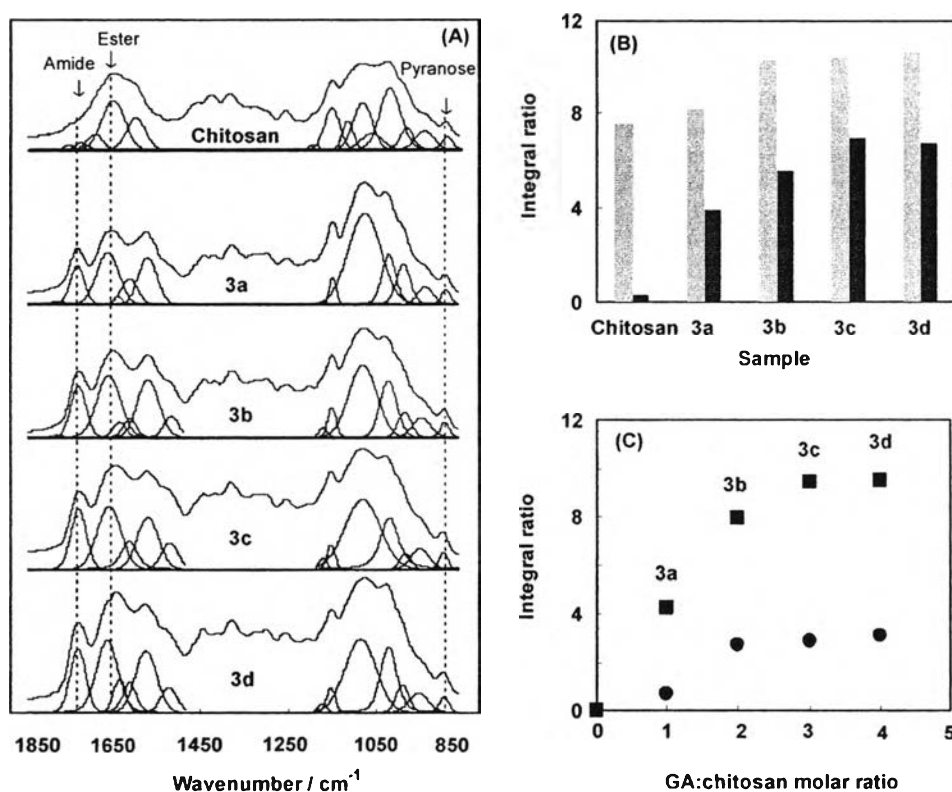


Fig. 5. (A) FT-IR spectra and curve fitting results of chitosan, **3a**, **3b**, **3c**, and **3d**; (B) integral ratio of (▪) $A_{1640/895}$ (amide) and (▪) $A_{1730/895}$ (ester) obtained from (A); and, (C) the subtraction of amide (•) and ester (▪) integral ratio of **3a**, **3b**, **3c**, and **3d** with chitosan (from (B)).

The higher integral ratios of **3c** and **3d** demonstrated the higher GA moiety on chitosan, resulting in water solubility improvement, which relates to Fig. 3(A). From these results, it can be concluded that the gallate group of GA was successfully introduced onto chitosan via amide and ester linkage on account of the significant increase in the integral ratio.

For ^{13}C CP/MAS NMR, **3** showed the aromatic carbon of the gallate group at 140 ppm (C=C) and at 160.2 ppm (C=O) implying successful conjugation. Although **3**, especially **3c-3d**, was water soluble after being freshly prepared, the

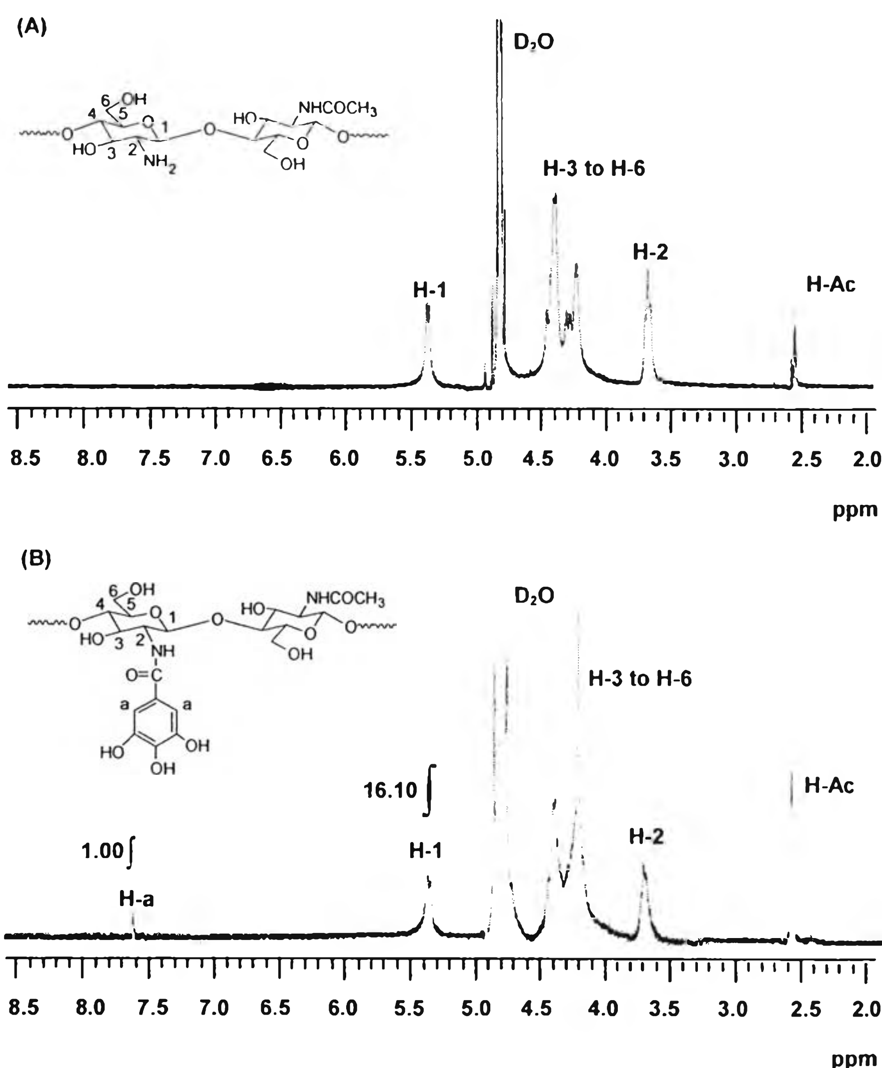


Fig. 6. ^{1}H-NMR spectra of (A) chitosan and (B) **3c** in CD_3COOD/D_2O at 70°C.

belonging to the phenyl protons as compared to chitosan (Fig. 6(A)). This confirms the successful conjugation with GA. As the chitosan peaks are at δ= 2.5 (H-Ac), 5.3

freeze-drying step initiated the packing structure, resulting in the partial dissolvation in water. An attempt to qualitatively and quantitatively analyze the conjugation of GA even though the compound was partially dissolved in CD_3COOD/D_2O (2% v/v), was done by ^{1}H-NMR at 70°C. Compound **3c** shows a new peak at 7.6 ppm (Fig. 6(B)) (H-1), 3.0 (H-2), and 3.9-4.5 ppm (H-3 to H-6 of pyranose ring), the degree of substitution was then calculated from the integration of H-a (benzene ring of GA) and the internal standard peak of H-1 (pyranose ring). The substitution degrees of GA for **3a, 3b, 3c,** and **3d** were 2.77, 2.99, 3.10, and 3.05 ppm, respectively. The low substitution degree might come from the fact that the compounds were only partially dissolved in the solvents.

The substitution degree was also evaluated by elemental analysis. The ideal substitution of GA (100%) on C-2, C-3, and C-6 (Fig. 2) of the chitosan chain brings the C/N ratio to be 33.4. For **3c**, the C/N ratio was 5.22, implying a degree of substitution of 15.6%. In the cases of **3a**, **3b**, and **3d**, the C/N ratios were 5.18, 5.19, and 5.18 and the %DS was 15.49, 15.52, and 15.51, respectively. The combustion of **3** was incomplete, as evidenced from a certain amount of ash after carrying out the elemental analysis; however, we consider the %DS obtained here to be a guideline for the successful conjugation of GA on chitosan.

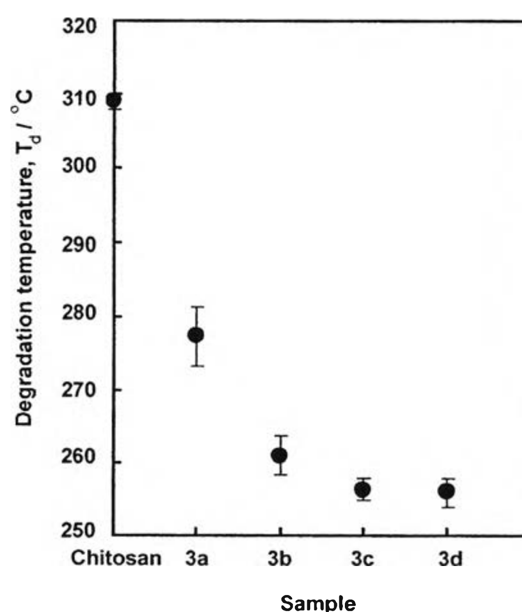


Fig. 7. Degradation temperature of chitosan and **3a** – **3d**. Results are mean ±SD (n=3).

4.4.3 Thermal stability and morphological studies

As chitosan is under strong inter- and intramolecular hydrogen bonds, chitosan does not exhibit thermal-plasticity, but gives the degradation before it melts at 290-310°C (T_d). In general, the introduction of the functional group obstructs the chain packing, resulting in the decrease of T_d. Here, all derivatives (**3a-3d**) show T_d in the range of 250-270°C which is lower than that of chitosan (Fig. 7). This implies the loose of packing structure of chitosan after conjugating with GA.

The morphological studies also reflect the looseness in chain packing after introducing GA onto the chitosan chains. The XRD pattern of chitosan gives the peaks at 9°, 19°, and 22° 2θ (Fig. 8(a)), whereas that of **3c** shows the major peak at 25° 2θ (Fig. 8(b)). The consequent increase of amorphous phase confirms the successful conjugation of gallate group onto chitosan.

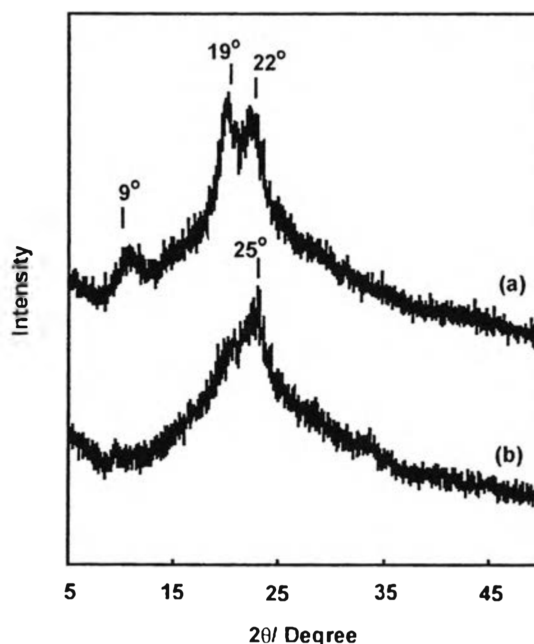


Fig. 8. Morphology study by XRD patterns of (a) chitosan and (b) **3c.**

4.4.4 DPPH• scavenging capacity

It is important to clarify whether chitosan shows radical scavenging ability via the function of gallic acid or not. The DPPH• is a relatively stable free radical because it can be easily detected by EPR; thus, it is useful and practical for

the evaluation of antioxidative potential (Williams, Cuvelier, & Berset, 1995). The DPPH$^{\bullet}$ radical scavenging activity was investigated from the activity of Chitosan-GA to reduce the radicals, as shown in Eq. 2.

$$\text{DPPH}^{\bullet} + \text{Chitosan-GA} \longrightarrow \text{DPPH-H} + \text{Chitosan-GA}^{\bullet} \qquad (2)$$

The antioxidant capacity was evaluated by plotting the concentrations of antioxidant against the percent of free radical scavenging capacity. Fig. 9 shows the comparative studies on the reducing capacity of chitosan, **3c**, and GA. In the case of chitosan (Fig. 9(A)), no DPPH$^{\bullet}$ reduction is observed even though its concentration was as high as

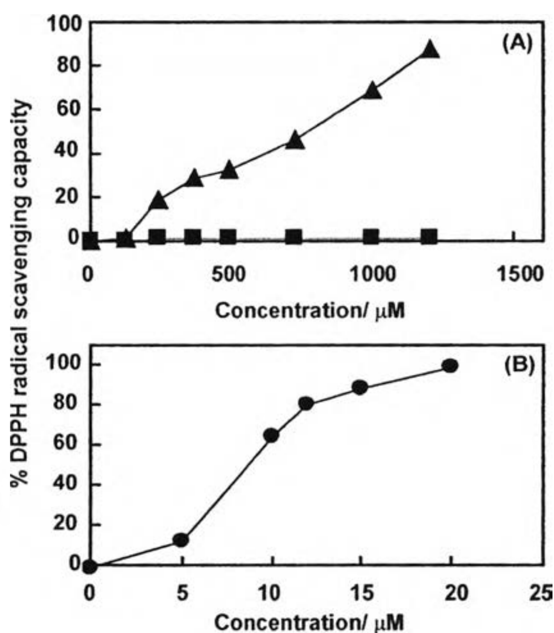


Fig. 9. DPPH radical scavenging capacity of (A) **3c** (▲), and chitosan (■), and (B) GA (●).

1200 μM. The DPPH$^{\bullet}$ scavenging capacity of **3c** (Fig 9(A)) increases as the concentration increases and is up to 87.3% when the concentration reached 1200 μM. The EC_{50} value, which expresses the antioxidant concentration to reduce the radicals by 50%, is a good indicator to quantify the antioxidant capacity. As shown in Fig. 9, the EC_{50} in scavenging the DPPH$^{\bullet}$ of **3c** (Fig. 9(A)) is 740 μM. Although the value is

90 times less than that of pure GA (EC_{50} = 8 μM) (Fig. 9(B)), it implies the function of Chitosan-GA as a polymer antioxidant.

4.4.5 Carbon-centered radical ($R^•$) scavenging capacity

Carbon-centered radical, $R^•$, is one of the representative oxidized products in lipid membranes and lipoproteins (Venkataraman, Schafer, & Buettner, 2004). In this study, we determined the ability of Chitosan-GA to scavenge $R^•$ being induced by AAPH (Eq. 3). It is important to point out that the EPR signal of POBN/$R^•$ is related to the antioxidant potential of chitosan-GA (Eqs. 4 and 5):

$$\text{R-N=N-R} \longrightarrow 2R^• + N_2 \quad (3)$$

$$R^• + \text{chitosan-GA} \longrightarrow RH + \text{chitosan-GA}^• \quad (4)$$

$$R^• + \text{POBN} \longrightarrow \text{POBN}/R^• \quad (5)$$

Fig. 10 demonstrates that the EC_{50} in scavenging $R^•$ of **3c** is 110 μM, while GA is 10 μM. This indicates that GA on chitosan functions in donating an H-atom as well as free GA. Huang, Mendis, and Kima (2005) reported a $R^•$ scavenging capacity of 60% when using chitosan sulfate was about 0.125 mg·mL^{-1}. In our case, as the same 60%

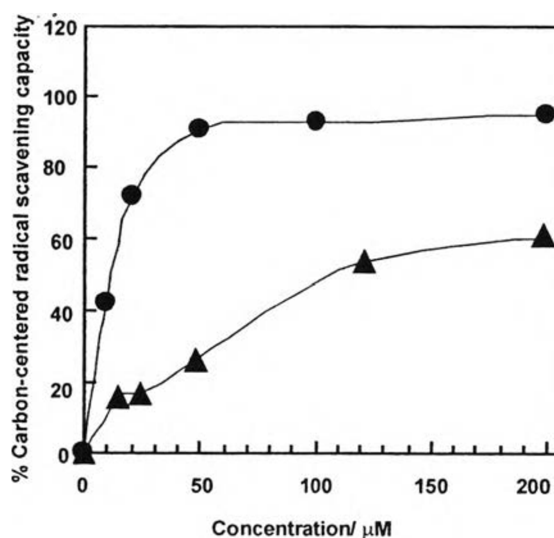
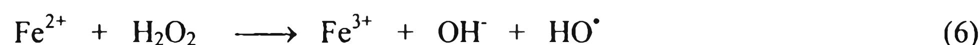


Fig. 10. Scavenging capacity of GA (●) and **3c** (▲) on carbon-centered radical.

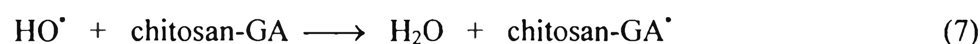
needs **3c** for 200 μM (0.038 mg·mL^{-1}), this implies an effective R$^{\bullet}$ scavenging of chitosan.

4.4.6 Hydroxyl radical (HO$^{\bullet}$) scavenging capacity

The hydroxyl radical (HO$^{\bullet}$) is a highly oxidizing product related to the Fenton and Haber Weiss reactions, which involves the initiation of biological oxidations; for example DNA, protein, and lipid oxidations (Haber & Weiss, 1934). The loosely bound iron is well known to be an important component of biological free radical oxidation as it is a key factor to produce HO$^{\bullet}$, as seen in Eq. 6:



$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO^{\bullet} \quad (6)$$



$$HO^{\bullet} + \text{chitosan-GA} \longrightarrow H_2O + \text{chitosan-GA}^{\bullet} \quad (7)$$

$$HO^{\bullet} + \text{DMPO} \longrightarrow \text{DMPO/HO}^{\bullet} \quad (8)$$

In this work, hydroxyl radicals were generated via the reaction of Fe^{2+}/H_2O_2 according to the Fenton reaction (Eq. 6). The antioxidant capacity of chitosan-GA was determined by the ability of chitosan-GA to stabilize HO$^{\bullet}$ via Eq. 7. The HO$^{\bullet}$ remaining was detected relating to the amount of DMPO/HO$^{\bullet}$ (Eq. 8). The ability of **3c** in scavenging HO$^{\bullet}$ is plotted against its concentrations (Fig. 11). The EC_{50} in

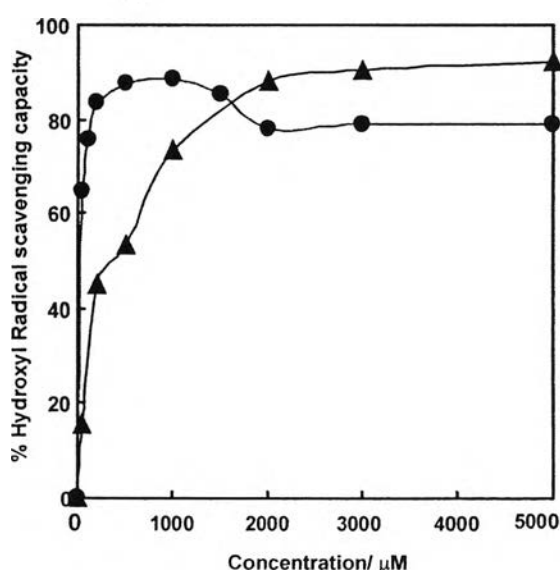


Fig. 11. Scavenging capacity of GA (●) and **3c** (▲) on hydroxyl radical.

scavenging $HO^{\bullet}$ of **3c** and GA are 350 (0.067 mg·mL^{-1}) and 50 μM, respectively. In other words, chitosan-GA, with only a certain amount of gallate groups, shows antioxidant activity on $HO^{\bullet}$ close to the pure GA. Compared to the EC_{50} in scavenging $HO^{\bullet}$ of chitosan sulfate (Xing, Liu, Yu, Guoa, Lia, & Lia, 2005) for 3.269 mg·mL^{-1}, **3c** can be considered to be a good hydroxyl radical scavenger.

It is important to note that when the concentration of GA is above 1000 μM, the $HO^{\bullet}$ scavenging capacity decreases and saturates at 80% (Fig. 11). This might be due to the reducing potential of GA to reduce Fe^{3+} to Fe^{2+}, resulting in the recycling of Fe^{2+} in the system. As Fe^{2+} initiates $HO^{\bullet}$, the higher the GA concentration, the more $HO^{\bullet}$ will be produced in the system. It is important to point out that this phenomenon could not be observed in **3c**. In the case of **3c,** the $HO^{\bullet}$ scavenging capacity increases gradually and maintains at 88% after the concentration of **3c** reaches 2000 μM. This might relate to the fact that chitosan has the ability to form a complex with Fe^{2+} and Fe^{3+}, resulting in the retardation of $HO^{\bullet}$ formation (Burke, Yilmaz, Hasirci, & Yilmaz, 2002). This result also supports that chitosan functions as a metal ion deactivator and performs as a preventive antioxidant when ferrous ions are involved in the system. We suspect that the synergistic effect in $HO^{\bullet}$ scavenging of **3c** was from the antioxidant activity of GA on the chitosan chain together with the metal ion complexation ability of chitosan.

4.5 Conclusion

The present work demonstrated a novel green antioxidant polymer by conjugating chitosan with a natural antioxidant molecule, i.e. gallic acid. The simple conjugation condition via the EDC/NSH heterogeneous system brought about a gallic acid substitution at amino and hydroxyl groups of about 15 % as evaluated by EA. chitosan-GA covered a wide range of antioxidant activity, including the carbon centered radical and hydroxyl radical. The gallic acid conjugated on chitosan not only brings antioxidant activity but also improves the water solubility of chitosan. By incorporating GA onto chitosan, chitosan performs multifunctional and has unique properties; i.e. (i) offers preventive antioxidation activity due to the metal ion

deactivator of the chitosan, (ii) is a chain breaking antioxidant due to the H-atom donating ability of the tri-hydroxyl group of GA, (iii) has water solubility due to the bulky hydrophilic group of GA, and (iv) has a synergistic effect in the $HO^{\bullet}$ scavenging activity under the Fenton reaction.

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