

Growth Effects of Caffeic Acid and Thioglycolic Acid Modified Chitosans in U937 Cells

Aytekin A.O., Morimura S.

Abstract—Chitosan is a biopolymer composed of glucosamine and N-acetyl glucosamine. Solubility and viscosity pose problems in some applications. These problems can be overcome with unique modifications. In this study, firstly, chitosan was modified by caffeic acid and thioglycolic acid, separately. Then, growing effects of these modified polymers was observed in U937 cell line. Caffeic acid is a phenolic compound and its modifications act as carcinogenic inhibitors in drugs. Thiolated chitosans are commonly being used for drug-delivery systems in various routes, because of enhancing mucoadhesiveness property. U937 cell line was used as model cell for leukaemia. Modifications were achieved by 1 – 15 % binding range. Increasing binding ratios showed higher radical-scavenging activity and reducing cell growth, in compared to native chitosan. Caffeic acid modifications showed higher radical-scavenging activity than thiolated chitosans at the same concentrations. Caffeic acid and thioglycolic acid modifications inhibited growth of U937, effectively.

Keywords—Chitosan, U937 cell, caffeic acid, thioglycolic acid

I. INTRODUCTION

CHITIN is the second most abundant biopolymer after cellulose. Chitosan is derived from chitin by deacetylation.

Chitosan has high percentage of glucosamine as 70 % in above. Chitosan and its derivatives have commonly been used in many industrial areas such as biotechnology, food, pharmaceuticals, and waste treatment; however, solubility and viscosity pose problems in some applications [1], [2]. Aqueous chitosan solutions must be prepared in acidic conditions, and depending on its molecular weight, highly viscous solutions of chitosan are observed above 1 % (g/100ml) concentration. These solubility and viscosity problems can be overcome with unique modifications such as glycolisation, thiolation, carboxylation, and so on.

Thiolated chitosan is formed by amino groups of chitosan and thiol functions with coupling reagents (Fig 1a). Thiolated chitosans have great mucoadhesiveness ability that supports it to move across from mucosal layers. Therefore, thiolated chitosans have great potential to use in drug-delivery systems [3].

Caffeic acid is naturally phenolic compound and member of flavonoids presents in coffee, olive oil, white wine, cabbage etc [4]. Caffeic acid is 3,4-dihydroxycinnamic acid which is derived from shikimic acid and similar to cinnamic acid. Caffeic acid and its derivatives, ethyl ester (CAEE) and phenethyl ester (CAPE), act as carcinogenic inhibitors and they also show antioxidant (radical-scavenging) activity *in vitro* study [5]. Tyrosinase (EC 1.14.18.1) was used to bind phenolic compounds to amine side. However, this modified chitosan had higher viscosity than native chitosan [6].

The human leukaemic monoblast U937 cell has been used as a model cell line for leukaemia. Apoptosis can result in cell termination, causing the removal of damaged cells, and the apoptosis activities of several compounds have been reported in U937 cell lines. However, few studies have addressed the apoptosis activity of CH and its derivatives [7].

In this study, initially chitosan was modified with caffeic acid and thioglycolic acid, then growth effects of these modifications in U937 cell line was investigated.

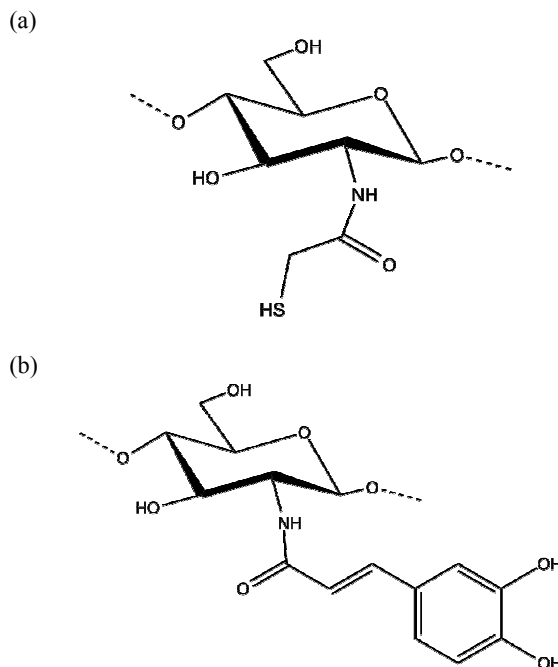


Fig. 1 Molecular schemes of thiolated chitosan (a) and chitosan-caffeic acid (b).

II. MATERIALS AND METHODS

A. Materials

Chitosan was kindly provided by Koyo Chemical Co. Ltd. (Osaka). Thioglycolic acid (TGA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were purchased from Sigma-Aldrich Japan (Tokyo). U937 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). Ellman's reagent 5,5-dithiobis (2-nitrobenzoic acid) and 1,1-diphenyl picryl hydrazyl (DPPH) were obtained from Wako Chemical (Tokyo).

B. Depolymerisation of chitosan

Chitosan (1 %, 544 kDa) was dissolved in a 1 % acetic acid solution. A measured amount of 0.1 N NaNO₂ was added to yield CH/NaNO₂ molar ratios of 0.01. Chitosan was precipitated with 0.1 N NaOH at pH 8.0 after 3 h. Before lyophilisation, samples were washed three times with purified water followed by centrifugation at $9,000 \times g$ for 30 min. Molecular weight was determined using a GP4000Pw Tosoh size exclusion column and an RI detector (SEC-HPLC). A mixture of acetic acid (0.2 M, 99 %), sodium acetate (0.1 M) and sodium azide (0.008 M) was used for elution at a 0.5 ml/min flow rate. Sample concentrations were 0.1% (w/v) and PEG / PEO were used for standards. Products were characterized by ¹H-NMR [10].

C. Thiolated chitosan synthesis

Chitosan (197 kDa or 544 kDa, 0.5 g) was dissolved in acetic acid solution (1 %, 50 ml). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was added at final concentration 50 mM. Thioglycolic acid (TGA) was added with different volume between 100 to 600 μ l in a reaction vessel. pH was adjusted to 5.0 with 0.1 N NaOH and the reaction was carried out 3-h. Dialysis membrane (MWCO 12 kDa, Sigma) was used to eliminate the free acetic acid and unbound TGA from thiolated chitosan in solution. Then dialyzed was lyophilized and stored at 4°C until further use [3].

D. Chitosan – caffeic acid synthesis

Chitosan (197 kDa or 544 kDa, 10 mM) was dissolved in acetic acid solution in different concentrations. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was added at same concentration of chitosan. Caffeic acid was added with different amount between 1 to 7.5 mM in a reaction vessel. pH was adjusted to 5.0 with 0.1 N NaOH and the reaction was carried out 3-h. Dialysis membrane (MWCO 12 kDa, Sigma) was used to eliminate the free acetic acid. Caffeic acid residue was removed by centrifugation at $9,000 \times g$ for 30 min. The pellets were lyophilized to obtain chitosan-caffeic acid, which were stored at 4°C until further use.

E. Determination of thiol in thiolated chitosan

Degree of modification was determined spectrophotometrically using Ellman's reagent to measure amount of thiol groups in chitosan-thioglycolic acid compound

[9]. Firstly, Ellman's reagent 5,5'-dithiobis (2-nitrobenzoic acid) was prepared at 0.03 % concentration in phosphate buffer (0.5 M, pH 8.0). 2 ml Ellman's reagent, 1 ml phosphate buffer and each product (0.2 %) were mixed. Reaction was carried out at 2 h. Precipitated polymer was separated with centrifugation and obtained clear solution. Absorbance was measured at 420 nm. Standard curve was prepared with L-cysteine-HCl. Calculation was showed in Equation 1:

$$\text{Binding ratio (\%)} = 100 \times [\text{Thiol amount (mg)} / \text{Modified chitosan amount (mg)}]$$

F. Determination of caffeic acid in chitosan-caffeic acid

Samples were prepared in 10 % ethanol. Samples (0.25 ml) were mixed with 0.1 % HCl in 95 % ethanol (0.25 ml) and 2 % HCl (4.50 ml) solutions. After the addition of 2 % HCl, samples were incubated at 15 min. Absorbance measured at 320 nm. Caffeic acid was used for a standard. Water was used for a blank [8]. Binding ratio was calculated by Equation 2:

$$\text{Binding ratio (\%)} = 100 \times [\text{Caffeic acid in derivative (mg)} / \text{Modified chitosan amount (mg)}]$$

G. Radical scavenging activity

Samples were prepared (200 μ l) in glass tubes and DPPH was prepared at 200 μ M in ethanol. After DPPH solution (600 μ l) was added, each sample was vortexed and incubated for 30 min at room temperature. Aliquots of 200 μ L of the samples were taken into 96-well-plate and the absorbance measured at 540 nm. Water was used as a blank [11]. The DPPH-scavenging activity was calculated by Equation 3:

$$\text{Scavenging activity (\%)} = 100 \times (1 - \text{Absorbance of sample} / \text{Absorbance of control})$$

H. U937 Passage

U937 cells were cultured in RPMI 1640 medium containing 10 % (v/v) foetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μ g/ml) and amphotericin B (0.25 μ g/ml) at 37°C under a 5 % CO₂ atmosphere. The initial cell concentration was counted and adjusted to 1×10^5 cells/ml using centrifugation at $150 \times g$ for 5 min. Subsequent passages of the cultures were prepared every 2 - 3 d.

I. Cell growth of U937

The cell concentration of U937 was adjusted to 9×10^4 cells/ml and 100 μ l aliquots were added to each well of a 96-well-plate. After a 3-h-incubation, different concentrations of thiolated chitosan and chitosan-caffeic acid samples were added and the cells incubated at 37°C with 5 % CO₂ for 2 d. After which 10 μ l of WST-8 was added with incubation for 3 h at 37°C; finally, 10 μ l of 0.1 M HCl was added to each well and the absorbance at 490 nm immediately measured in a microplate reader (Immuno Mini NJ-2300, Nalgene Nunc, NY) [12].

III. RESULTS AND DISCUSSIONS

A. Synthesis of chitosan derivatives

TABLE I
BINDING RATIOS OF MODIFIED CHITOSANS

Products	Chitosan	Modification substances	Molar ratio	Molecular-weight (kDa)	Binding ratio (% g/100g modified chitosan)
T 1	1 %	T.A.; 100 µl	-	197	0.4
T 6	1 %	T.A.; 600 µl	-	197	1.2
T 2	1 %	T.A.; 200 µl	-	544	0.4
T 4	1 %	T.A.; 400 µl	-	544	0.9
CA 19	10 mM	C.A.; 1 mM	0.10	197	1.1
CA 21	10 mM	C.A.; 5 mM	0.50	197	3.3
CA 22	10 mM	C.A.; 7.5 mM	0.75	197	9.6
CA 1	10 mM	C.A.; 1 mM	0.10	544	1.1
CA 7	25 mM	C.A.; 7.5 mM	0.30	544	3.4
CA 34	75 mM	C.A.; 37.5 mM	0.50	544	9.8

T.A: Thioglycolic acid; C.A: Caffeic acid.

Chitosan (0.04 g) was dissolved in acetic acid solution (25 ml, 1 %).

In order to investigate varies of binding ratios; medium-molecular-weight chitosan was prepared by oxidative degradation method. A molecular-weight of 197 kDa chitosan was obtained by depolymerization of 544 kDa chitosan using NaNO_2 . The medium- and high-molecular-weight chitosans were used for initial chitosans of modifications. According to $^1\text{H-NMR}$ results, acetylation degree of depolymerized chitosan was the same as high-molecular-weight chitosan (data not shown).

Chitosans were bound with caffeic acid and thioglycolic acid. EDAC proceeds reaction to covalently link a carboxylate ($-\text{COOH}$) or a phosphate ($-\text{PO}_4$) group with an amine ($-\text{NH}_2$). In this case, an amino group of chitosan was reacted with carboxyl groups of caffeic acid and thioglycolic acid by EDAC. In this study, ten different derivatives, four of thiolated chitosan and six of chitosan-caffeic acid, were chosen to investigate the radical-scavenging activity and cell growth effect of U937 cells. Binding ratios were summarized in Table I.

Thiolation was achieved with a range of 1 %; however, binding ratios of caffeic acid were observed up to 10 % depend on initial caffeic acid concentration. Although initial thioglycolic acid concentrations were different in T1 and T2 productions, binding ratios were the same as 0.4 %. Thiolation efficiency was better in medium-molecular-weight than in high-molecular-weight chitosan. Because, di-sulphite crossing becomes stronger during the thiolation and it causes chain folding.

The binding efficiency of caffeic acid was strongly depended on molecular-weight of chitosan. The same caffeic acid content was obtained in which the increased the amounts of initial substances such as CA 7 and CA34 products. The increasing initial amount and molar ratio of caffeic acid / chitosan resulted in increased binding ratio. Binding efficiency was increased by increasing of caffeic acid concentrations in

the medium-molecular-weight chitosan. Although the CA 1 and CA 19 have different molecular-weights, the same caffeic acid contents were observed. Hence, the low initial caffeic acid

Chitosan aqueous solution is prepared in acidic water. Both modifications could be soluble in water at neutral pH and prepare in higher concentrations than native chitosan. A molecular weight of 544 kDa chitosan could not be used in radical-scavenging activity and cell growth assays, because of solubility and viscosity problems.

B. Radical scavenging activity

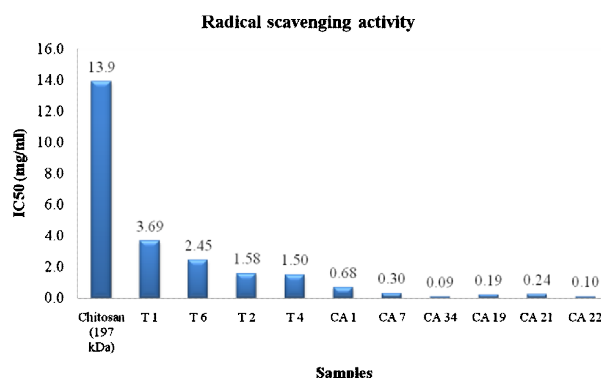


Fig. 2 IC50 of radical-scavenging activity of samples

Radical-scavenging activity was measured using DPPH. Half inhibition concentration (IC50), which is the efficient concentration required to decrease initial DPPH concentration by 50 %, of the chitosan, thiolated chitosans and chitosan-caffeic acid derivatives.

As expected, the molecular-weight of 197 kDa chitosan had the highest IC50 value. Derivatives of chitosan have higher radical-scavenging activity than native chitosan.

All chitosan-caffeic acid had higher radical-scavenging activity than thiolated chitosans. The increasing thiol content increased radical-scavenging activity in medium-molecular-weight derivatives (T1 and T6). However, the radical-scavenging activity was not different in high-molecular-weight thiolated chitosans (T2 and T4). Although, T1 and T2 have the same thioglycolic acid content, the radical-scavenging activity of T2 was higher around two-fold than T1. The high-molecular-weight chitosans showed the highest activity in thiolated chitosans. Electrostatic potential of thiolated chitosans is lower than chitosan-caffeic acid derivatives (Fig. 3). Hence, caffeic acid and amine site of glucosamine of the high-molecular-weight derivatives of thiolated chitosans (T2 and T4) showed radical-scavenging activity. The radical-scavenging activity was depended on sulfur groups in medium-molecular-weight modifications (T1 and T6). After thiolation, modified chitosans were easily dissolved in water with entire pH range and showed lower viscosity than native chitosan.

Caffeic acid and thiol modifications have repelled hydrogen bonds between amine group at C-2 and OH groups at C-3 and C-6 positions. Caffeic acid and thiol compounds can show chemical activity in chitosan derivatives.

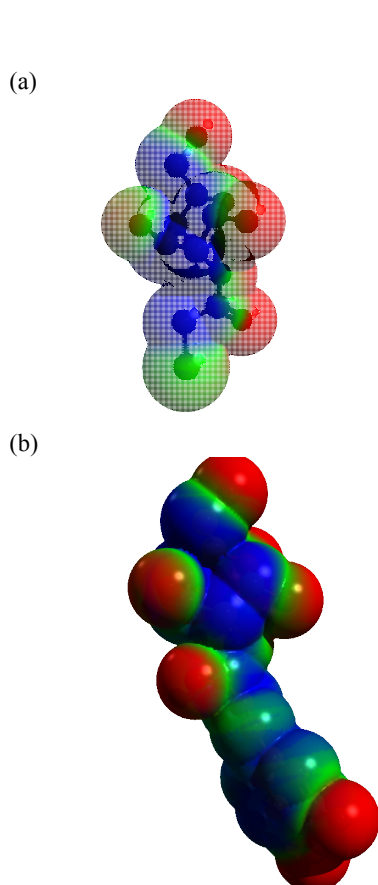


Fig. 3 Electrostatic potentials of thiolated chitosan (a) and chitosan-caffeic acid (b).

C. Cell growth of U937

The human leukaemic monoblast U937 cell was derived from the pleural fluid of a patient with diffuse histiocytic lymphoma and has been used a model cell line for leukaemia. Apoptosis can result in cell termination, causing the removal of damaged cells, and the apoptosis activities of several compounds have been reported in U937 cell lines. In this study, cell growth of U937 was observed under treatment of chitosan derivatives.

Native chitosan affected the cell death in high ratio. Depend on native chitosan; low-molecular-weight thiolated chitosans (T1 and T6) had role as protective agent in low concentrations. Increasing concentrations of thiolated chitosans showed increasing cell death.

However, increasing concentrations of derivatives The high-molecular-weight and high thiol content derivative (T4) caused the highest cell death in U937 cells in thiolated chitosans. This result was the same as radical-scavenging activity (Fig. 4a).

Low percentage of caffeic acid helped CA 1 to dissolve in biological pH. Hence, cell growth effect was almost the same in the high-molecular-weight CA 1 and native chitosan.

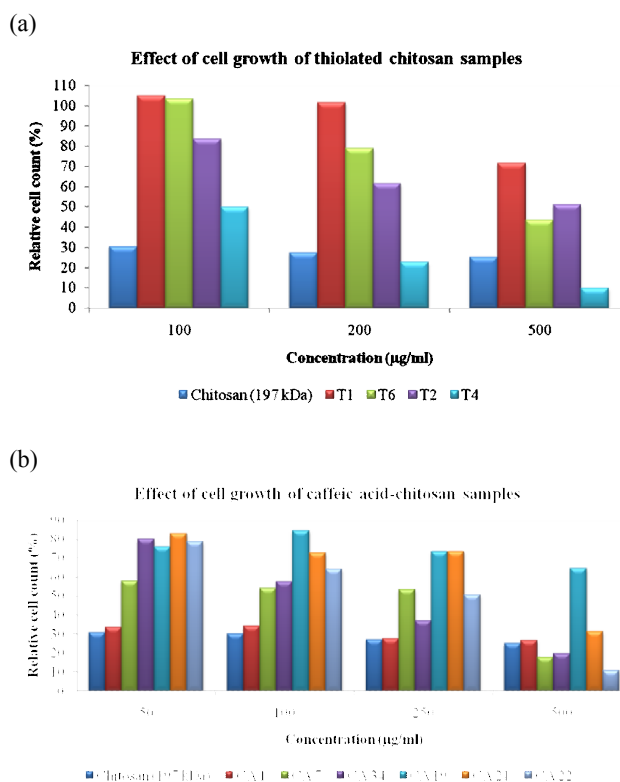


Fig. 4 Effect of cell growth of thiolated (a) and caffeic acid (b) modifications. The control was U937 cells (with a cell viability of 100 %).

Low concentrations of chitosan-caffeic acid derivatives supported cell growth U937. showed cell death activity. Generally, high-molecular-weight chitosan-caffeic acid derivatives (CA 1, CA 7 and CA 34) had higher cell death activity than medium-molecular-weight chitosans (CA 19, CA 21 and CA 22). However, minimum cell count was obtained in CA 22 product at 500 µg/ml concentration (Fig. 4b).

IV. CONCLUSION

Chitosan is bioactive and biodegradable polymer, however; it has solubility and viscosity problems to cause problems in biological systems. These problems were overcome by thiolation and grafting of caffeic acid. Moreover, caffeic acid solutions can be prepared by water with grafting to chitosan. According to radical-scavenging activity data, antioxidant activity of caffeic acid remained after modification. Low-molecular-weight and low thiol content derivative, T1, might be used in drug delivery systems.

ACKNOWLEDGMENT

This work was supported through a research grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT).

REFERENCES

- [1] Kumar, M., Muzzarelli, R., Muzzarelli, C., Sashiwa, H., Domb, A., (2004). Chitosan chemistry and pharmaceutical perspectives. *Chem. Rev* 104, 6017-6084.
- [2] Shahidi, F., Arachchi, J., Jeon, Y., (1999). Food applications of chitin and chitosans. *Trends in food science & Technology* 10, 37-51.
- [3] Kafedjiiski, K., Krauland, A., Hoffer, M., Bernkop-Schnürch, A., (2005). Synthesis and in vitro evaluation of a novel thiolated chitosan. *Biomaterials* 26, 819-826.
- [4] Rice-Evans, C., Miller, N., Paganga, G., (1997). Antioxidant properties of phenolic compounds. *Trends in plant science* 2, 152-159.
- [5] Rajan, P., Vedernikova, I., Cos, P., Vanden Berghe, D., Augustyns, K., Haemers, A., (2001). Synthesis and evaluation of caffeic acid amides as antioxidants. *Bioorganic & Medicinal Chemistry Letters* 11, 215-217.
- [6] Kumar, G., Bristow, J., Smith, P., Payne, G., (2000). Enzymatic gelation of the natural polymer chitosan. *Polymer* 41, 2157-2168.
- [7] Harris, P., Ralph, P., (1985). Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. *Journal of leukocyte biology* 37, 407.
- [8] Rusjan, D., Korosec-Koruz, Z., (2007). A comparison of extraction methods for selected phenolic compounds from grape berry skins using liquid chromatography and spectrophotometry. *Acta chimica slovenica* 54, 114.
- [9] Lee, D., Zhang, W., Shirley, S.A., Kong, X., Hellermann, G.R., Lockey, R.F., Mohapatra, S.S., (2007). Thiolated chitosan/DNA nanocomplexes exhibit enhanced and sustained gene delivery. *Pharm Res* 24, 157-167.
- [10] Mao, S., Shuai, X., Unger, F., Simon, M., Bi, D., Kissel, T., (2004). The depolymerization of chitosan: effects on physicochemical and biological properties. *International journal of pharmaceutics* 281, 45-54.
- [11] Shimada, K., Fujikawa, K., Yahara, K., Nakamura, T., (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry* 40, 945-948.
- [12] Inagaki, S., Morimura, S., Gondo, K., Tang, Y., Akutagawa, H., Kida, K., (2007). Isolation of Tryptophol as an Apoptosis-Inducing Component of Vinegar Produced from Boiled Extract of Black Soybean in Human.