CHAPTER III

RESULT AND DISCUSSION

A. Chitosan

3.1 Phthaloylation of Chitosan

When chitosan 1.2 g (degree of deacetylation = 95%) were added into a solution of 3.5 g (5-fold excess) of phthalic anhydride in 10 ml DMF. The mixture was heated under nitrogen atmosphere at 130°C with stirring. Under this condition, the reaction mixture became viscous with some remaining undissolved chitosan. After 8 hours, the reaction mixture was filtered to separate remaining undissolved chitosan.

The reason that phathaloylation could not take place in some chitosan sections may be a result of inhomogeneous deacetylation reaction of chitin into chitosan. The remaining undissolved chitosan in the reaction mixture probably was chitosan with very low % deacetylation.

When pouring the phthaloyl solution into ice-water, yellow precipitate was observed. DMF was washed out from the precipitate with excess water. Purification of phthalic anhydride and phthalic acid was done by washing with excess methanol. The precipitate was dried to give 1.5 g (51 % yield) of the product. IR Spectrum (KBr) of the product also confirmed successful phthaloylation onto chitosan structure: υ 3450 (OH), 1773 (ester C=O), 1713 (imide C=O) (see Figure 3.1). Complete disappearing of peak at 1590 cm^{-1} also indicated complete phthalimido substitution on amino groups. Since the C=O stretching of ester functionality in the IR spectrum is very small, this means that only negletable amount of ester was formed.

It was obvious that phthalimido groups on the polymer chain have made the polymer soluble in DMF and DMSO (the starting chitosan was insoluble in both solvents). This solubility had made it possible for other chemical modification on the polymer chain.

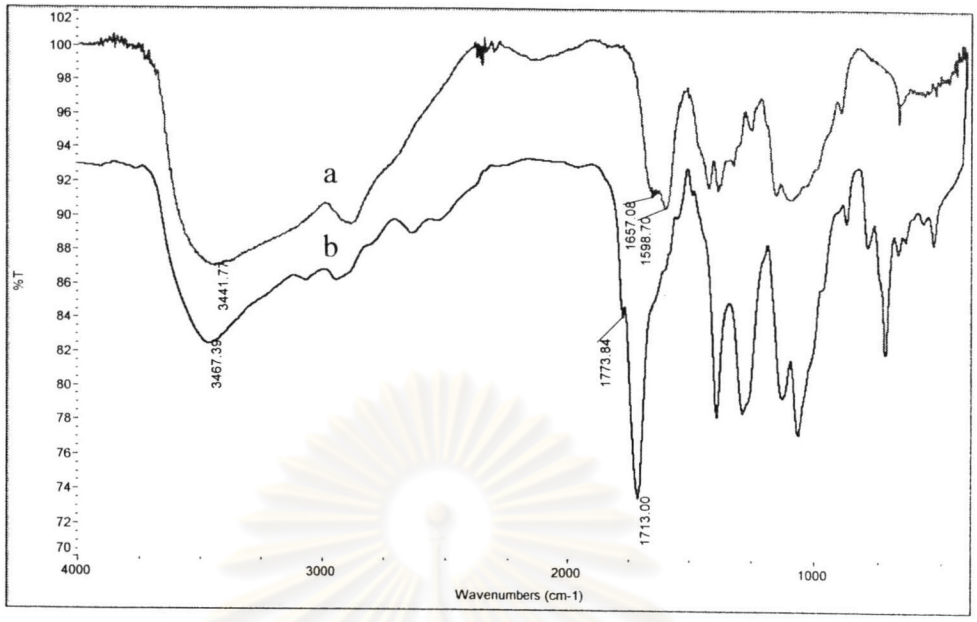


Figure 3.1 IR spectra of a) Chitosan and b) Phthaloylchitosan

3.2 Grafting of 4-methoxycinnamic acid on N-phthalimido-chitosan

When trying to graft 4-methoxycinnamic acid onto phthalimido-chitosan all four methods including 1) using *N,N'*-dicyclohexylcarbodiimide (DCC), 2) using only 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 3) using acid chloride and 4) using *p*-toluene sulfonic acid, failed to give any esterification product. Finally by using EDCI and 1-hydroxy benzotriazole (HOBt), phthalimido-chitosan grafted with 4-methoxycinnamolyl groups was obtained.

In this reaction, 4-methoxycinnamic acid (0.26 g, 0.4 mol equivalents to hydroxyl group in phthaloylchitosan) was stirred with phthaloylchitosan 1.08 g ($\sim 3.71\times10^{-3}$ equivalent of hydroxyl group left) at room temperature overnight in DMF containing HOBt 0.60 g (3 mol equivalents to 4-methoxy cinnamic acid) and EDCI 0.92 g (3 mol equivalent to 4-methoxy cinnamic acid). After 24 hours, the resulting reaction mixture appeared as yellow solution. Formation of ester linkage between 4-methoxycinnamic acid and phthaloylchitosan was confirmed by IR (Figure 3.2). *N'*-Ethyl-*N'*-[3-(*N''*-dimethyl)ethyl]urea (byproduct) was extracted from the product using 5% aqueous hydrochloric acid solution for 2-3 times. And after that the product was washed with methanol to eliminate all HOBt left. The pale yellow solid product was characterized by ^{1}H-NMR and IR. Resonance at 7.98-7.39 (Ar-**H**), 7.00-7.10 (Ar-CH=C**H**-), 8.10-8.20 (Ar-C**H**=CH-) and 3.82-3.74 (OC**H**$_3$) ppm in the ^{1}H-NMR

spectrum indicated that 4-methoxycinnamolyl group were grafted onto the chitosan polymer. Much bigger IR peak at 1776 cm^{-1} also indicated more C=O stretching from ester functionality. The reaction gave about 83 % yield. The solubility of product is shown in Table 3.1.

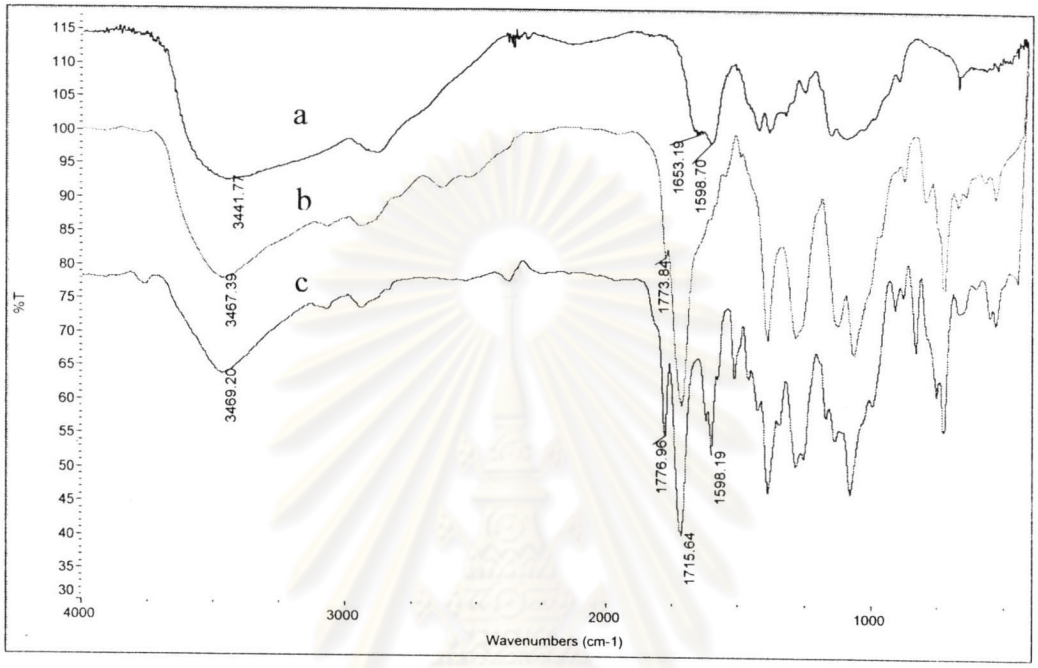


Figure 3.2 IR spectra of a) chitosan, b) phthaloylchitosan and c) 4-methoxy cinnamoyl-phthaloylchitosan

Experiments were done to obtain UV absorption properties (λ_{max}) of this product. As shown in Figure 3.4, the freshly prepared solution of the product gave one absorption band; λ_{max} of 330 nm in DMSO. Since 4-methoxycinnamic acid showed UV absorption at λ_{max} of 310 nm (see Figure 3.3), it was unexpected to see the maximum absorption of the grafted product at 330 nm (see Figure 3.4). This bathochromic shift might be a result of some interaction among chromophores on the polymer chain. Comparing with phthaloylchitosan which has absorption band (λ_{max}) at 284, this UV absorption property indicated that 4-methoxycinnamoly group was successfully grafted onto the chitosan polymer. However, after about 4.5 h, the absorption of the solution changed to λ_{max} of 310 nm (see Figure 3.5). There was no involvement of light in this shift. The blue shift from 330 to 310 nm was speculated as a result of a complete solvation around 4-methoxycinnamoyl moiety and, therefore, a disruption of interaction among chromophores.

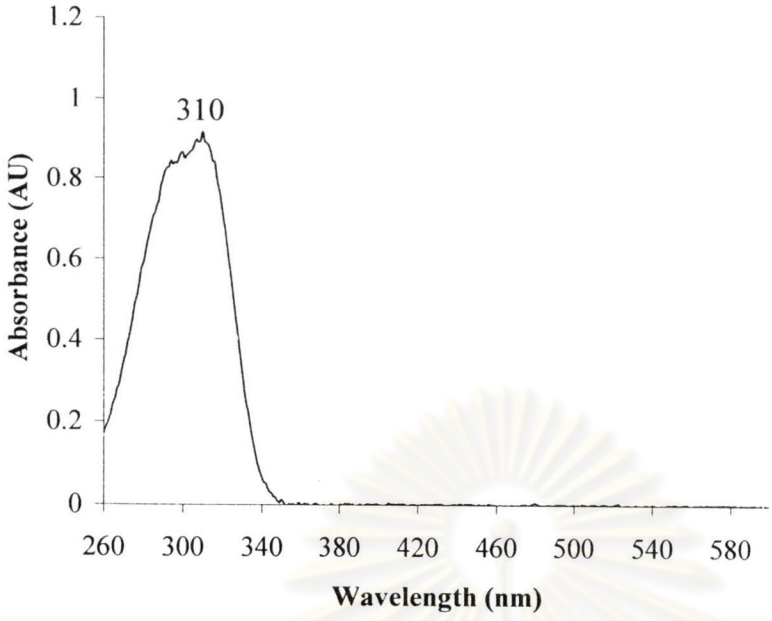


Figure 3.3 UV absorption spectrum of 4-methoxycinnamic acid (0.001 M in DMSO)

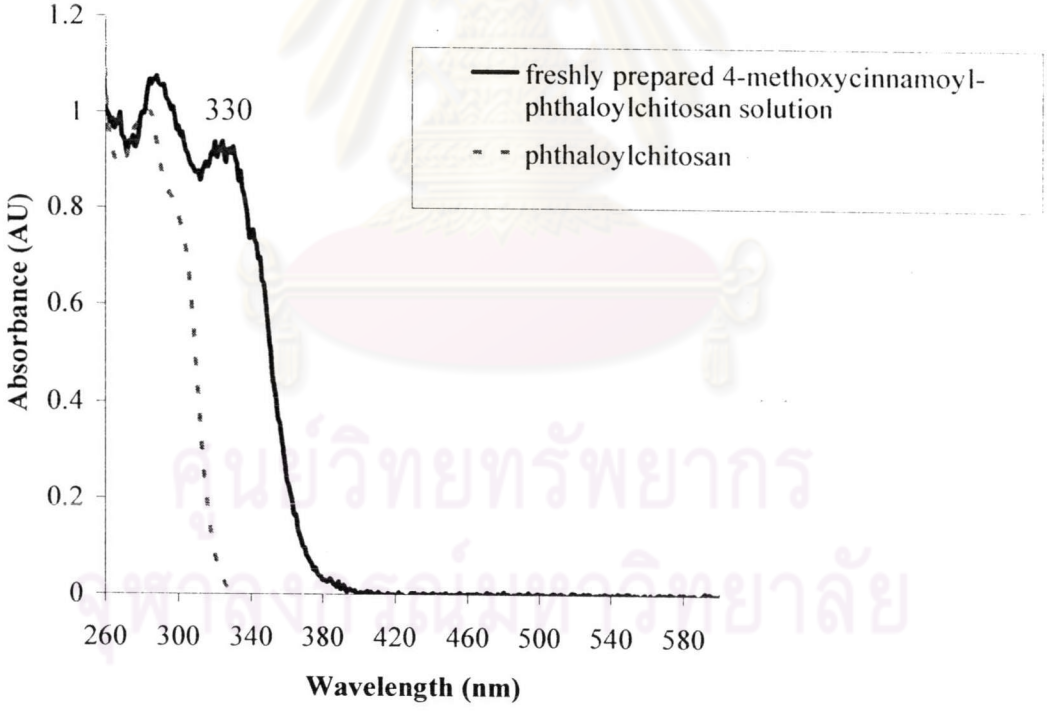


Figure 3.4 UV absorption spectra of 4-methoxycinnamoyl-phthaloylchitosan (0.1 g/L in DMSO or 1.305×10^{-4} M chromophoric units) comparing with phthaloylchitosan (0.1 g/L in DMSO)

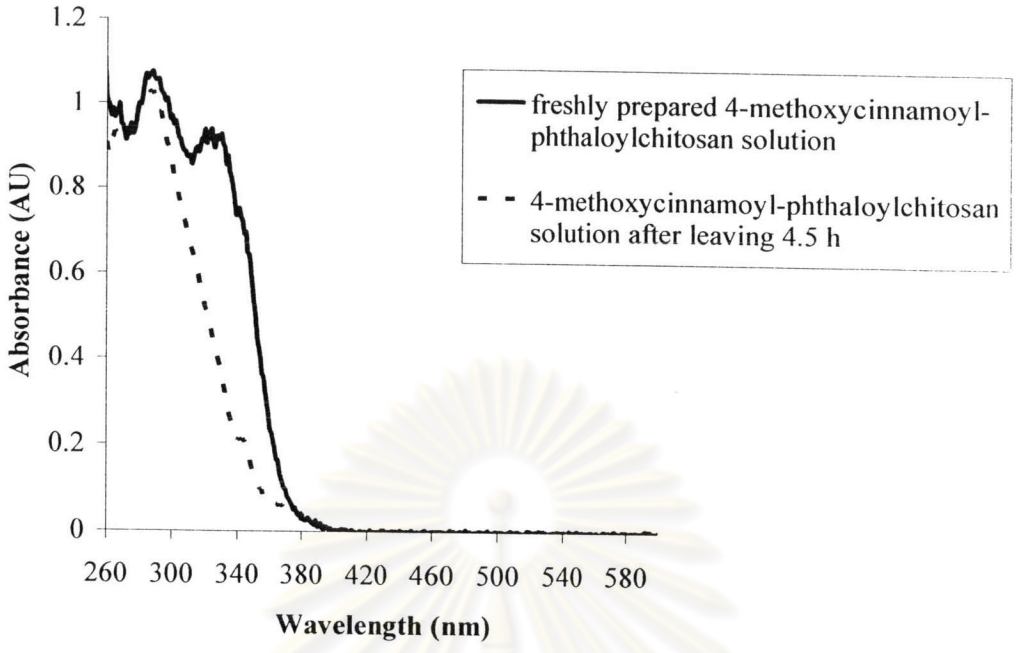
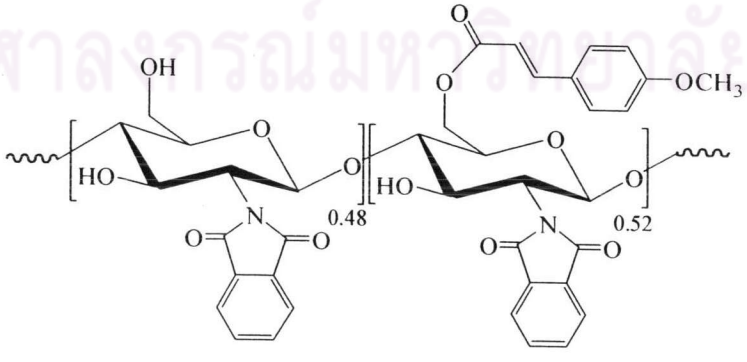


Figure 3.5 UV absorption spectra of freshly prepared 0.1 g/L 4-methoxycinnamoyl-phthaloylchitosan solution in DMSO or 1.305×10^{-4} M chromophoric units and similar solution after leaving for 1 day

Estimation of degree of substitution of 4-methoxycinnamoyl group on phthaloylchitosan was done by assuming that 4-methoxycinnamoyl moiety on phthaloylchitosan chain of the well solvated chitosan solution possesses ε of 23,000 $M^{-1}cm^{-1}$ (equal to that of 4-methoxy cinnamic acid and octyl-4-methoxy cinnamate). The degree of substitution obtained was 0.52 (see Appendix A for calculation).

As a result, general structure of the obtained product is as follows:



M.W. 249,883

Table 3.1 Solubility of chitosan, phthaloylchitosan and 4-methoxycinnamoyl-phthaloylchitosan

	DMF	DMSO	Pyridine
Chitosan	-	-	-
Phthaloylchitosan	+	+	+-
4-methoxycinnamoyl phthaloylchitosan	+	+	+

+ Soluble*, - Insoluble, +-Partially soluble

* means about 5 mg of sample can be completely solubilized in at least 1 mL of the tested solvent

Other solvents including water, acetonitrile, methanol, acetone, ethyl acetate, dichloromethane, diethyl ether, hexane could not solubilize any of the prepared chitosan derivatives.

B. Irradiated Chitosan

3.3 Phthaloylation of irradiated chitosan

Phthaloylation of irradiated chitosan was done with the same method used for the high M.W. chitosan. The reaction gave about 48 %yield. This means that 100% imido formation had occurred on chitosan structure. Complete disappearing of peak at 1590 cm^{-1} together with an appearance of intense peak at 1713 cm^{-1} (C=O of imide) indicated complete substitution of phthalimido on amino groups. Since the C=O stretching from ester functionality is very small, only negletable amount of ester formation had occurred.

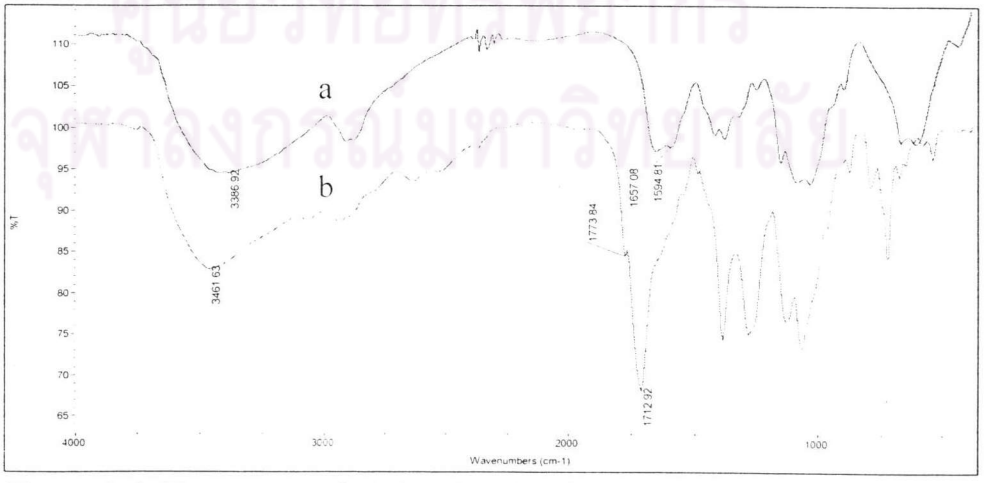


Figure 3.6 IR spectra of a) irradiated chitosan and b) phthaloyl-irradiated chitosan

3.4 Grafting of 4-methoxycinnamic acid on irradiated chitosan

Grafting of 4-methoxycinnamic acid on irradiated chitosan was successfully done using EDCI and HOBt. The pale yellow solid product was characterized by ^{1}H-NMR and IR. Intense C=O stretch at 1777 cm^{-1} indicated ester functionalities in the product (see Figure 3.7). ^{1}H NMR signals at 7.89-7.32 (Ar-H), 7.00-7.20 (Ar-CH=C**H**-), 8.00-8.20 (Ar-C**H**=CH-) and 3.61-3.92 (-OC**H**$_3$) ppm also indicated successful grafting 4-methoxycinnamoly group onto chitosan polymer.

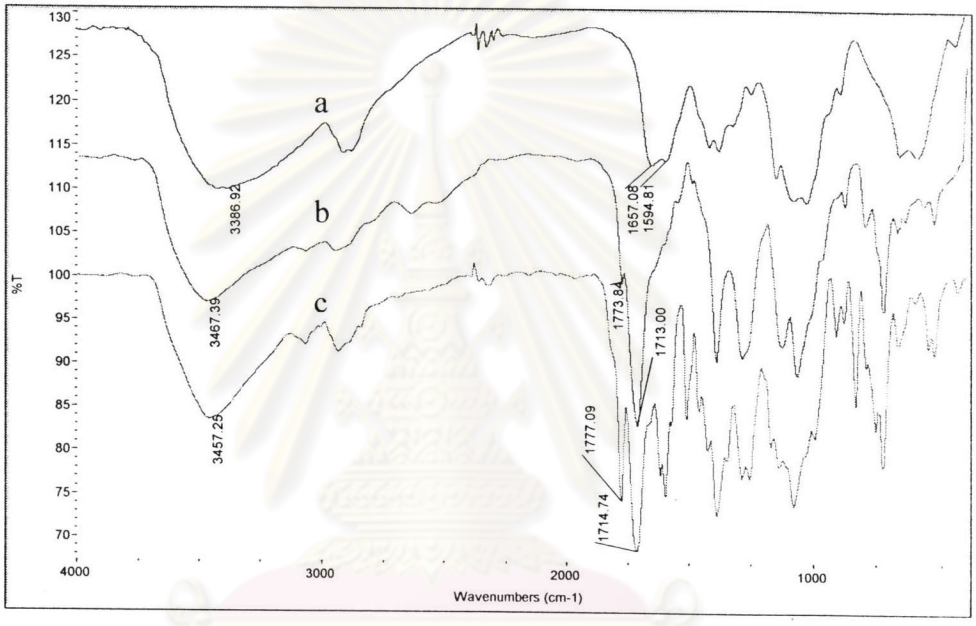


Figure 3.7 IR spectra of a) irradiated chitosan, b) phthaloyl-irradiated chitosan and c) 4-methoxycinnamoylphthaloyl-irradiated chitosan

Experiments were done to obtain UV absorption properties (λ_{max}, ε) of this product. As shown in Figure 3.8, the product gave one absorption band; λ_{max} of 330 nm in DMSO for freshly prepared solution. Similar blue shift from 330 nm to 310 nm was also found when solution was left for 4.5 hours.

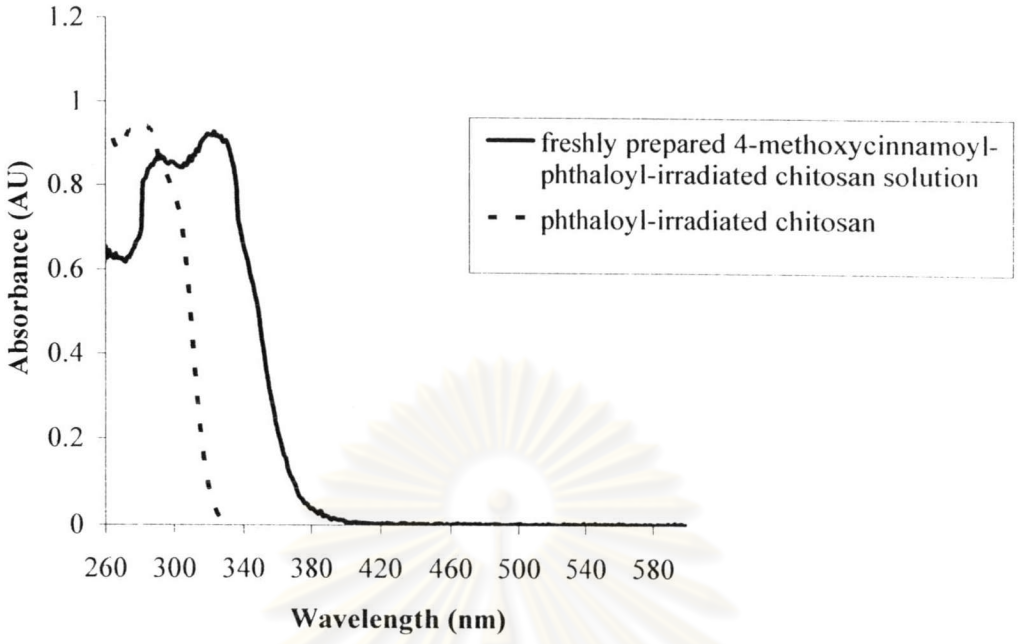


Figure 3.8 UV absorption spectra of 4-methoxycinnamoyl-phthaloyl-irradiated chitosan (0.1 g/L in DMSO or 1.518×10^{-4} M chromophoric units) comparing with phthaloyl-irradiated chitosan (0.1 g/L in DMSO)

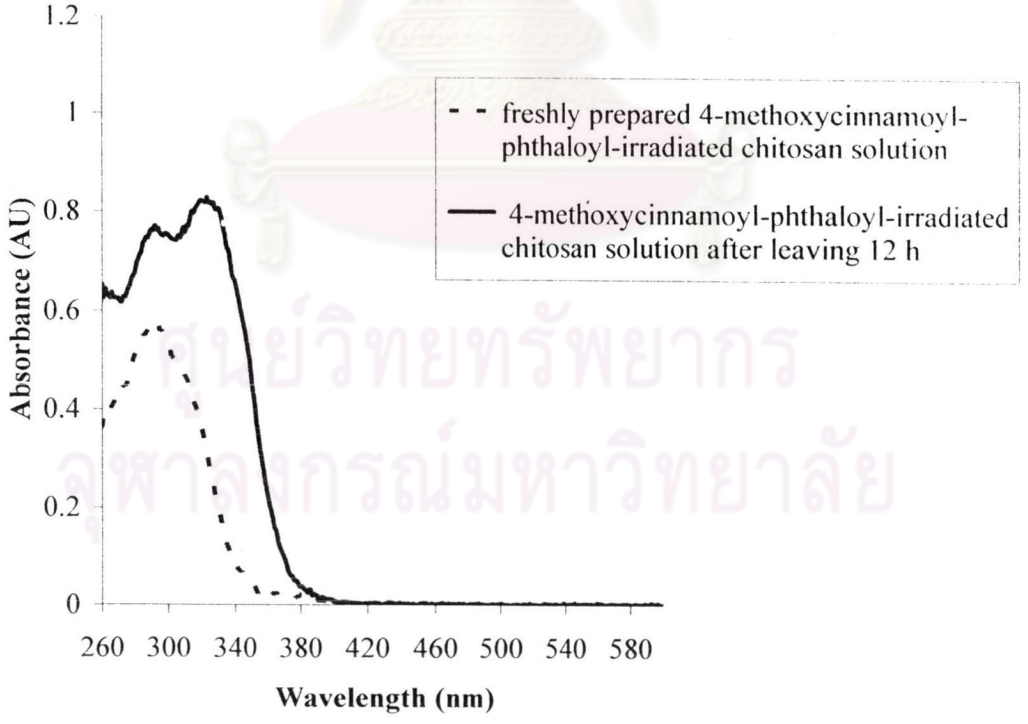
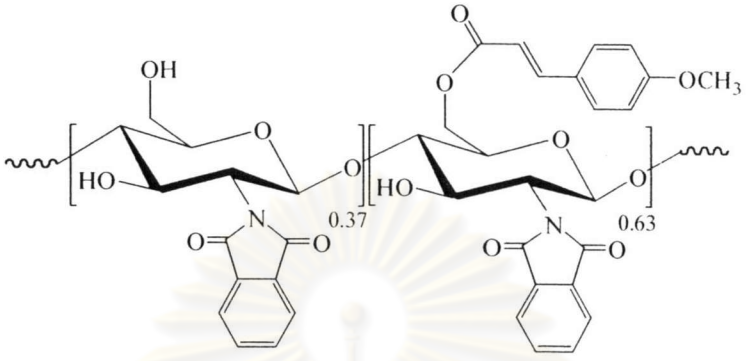


Figure 3.9 UV absorption spectra of freshly prepared 0.1 g/L 4-methoxycinnamoyl-phthaloyl-irradiated chitosan solution in DMSO or 1.518×10^{-4} M chromophoric units and similar solution after leaving for 1 day

Degree of substitution was calculated by UV absorption which gave 0.63 (399 units of 4-methoxycinnamoyl groups per 1 chain of chitosan or 632 pyranose units). General structure of the product is as follows:



M.W. 262,873

It can, therefore, be concluded that grafting of 4-methoxycinnamic acid onto chitosan could be done successfully by making chitosan soluble through phthaloylation then reacting this soluble chitosan with 4-methoxycinnamic acid using EDCI and HOBt as catalyst. Both high molecular weight chitosan (M.W. 110,000) and low molecular weight chitosan (M.W. 8,000-14,000) could be grafted successfully.

Degree of substitution for both high and low molecular weight chitosan were comparable. Both products showed UV absorption property.

3.5 Synthesis of 2,4,5-trimethoxycinnamic acid[42]

Preparation of 2,4,5-trimethoxycinnamic acid could be done successfully as described in 2.7. The %yield of reaction was 84. Both ^{1}H-NMR (see Figure B.5) and IR (see Appendix B.12) confirmed product structure.

The UV absorption spectrum of 2,4,5-trimethoxycinnamic acid is shown in Figure 3.10. It is obvious that the compound can absorb UVA irradiation.

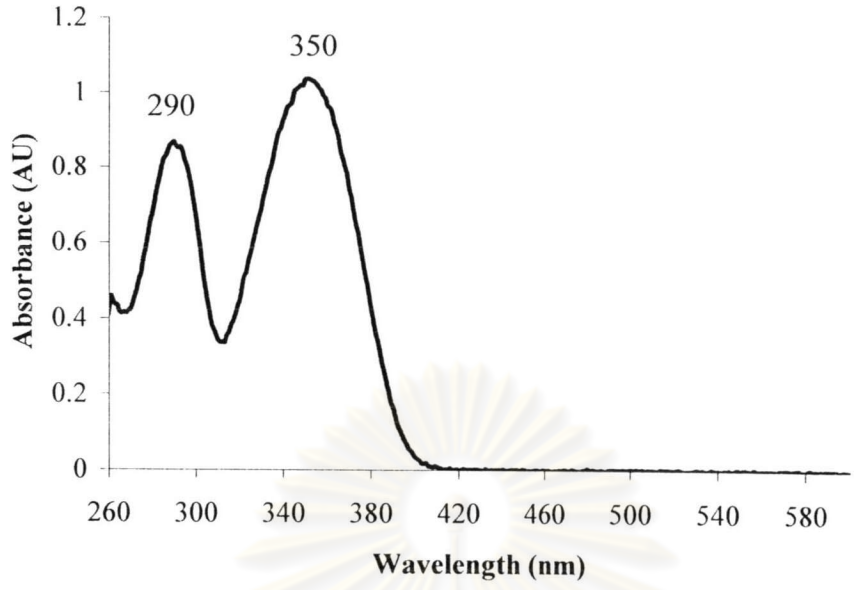


Figure 3.10 UV absorption spectrum of 0.045 M 2,4,5-trimethoxycinnamic acid solution in DMSO

3.6 Grafting of 2,4,5-trimethoxycinnamic acid on chitosan

Grafting of 2,4,5-trimethoxycinnamic acid on chitosan was done successfully using the same method used for grafting of 4-methoxycinnamic acid. The reaction gave 72% yield. The yellow solid product was characterized by ^{1}H-NMR and IR spectroscopy. Resonance at 8.00-8.21 (Ar-C**H**=CH-), 7.98-7.39 (Ar-**H**), 6.41-6.37 (=C**H**-COOR) and 3.83-3.64 (3×OC**H**$_3$) ppm indicated 2,4,5-trimethoxycinnamoyl moiety. IR peak at 1776 cm^{-1} indicated carbonyl group of ester bond (see Figure B.11). Figure 3.10 shows UV absorption spectra of 2,4,5-trimethoxycinnamoly-phthaloyl-chitosan.

Since 2,4,5-trimethoxycinnamic acid showed UV absorption at λ_{max} of 290 and 350 nm (see Figure 3.10), it was unexpected to see the maximum absorption of the grafted product at 333, 347 and 400 nm. This bathochromic shift might be a result of some interaction among chromophores on the polymer chain. When this issue was investigated, we have found out that the freshly prepared solution of 2,4,5-trimethoxycinnamoyl-grafted chitosan showed absorption at λ_{max} of 333, 347 and 400 nm. However, after about 12 h, the absorption of the solution changed to λ_{max} of 333 and 347 nm only (see Figure 3.12). These blue shift was speculate as a result of a complete solvation around 2,4,5-trimethoxycinnamoyl moiety, hence, a complete disruption of interaction amongs chromophores.

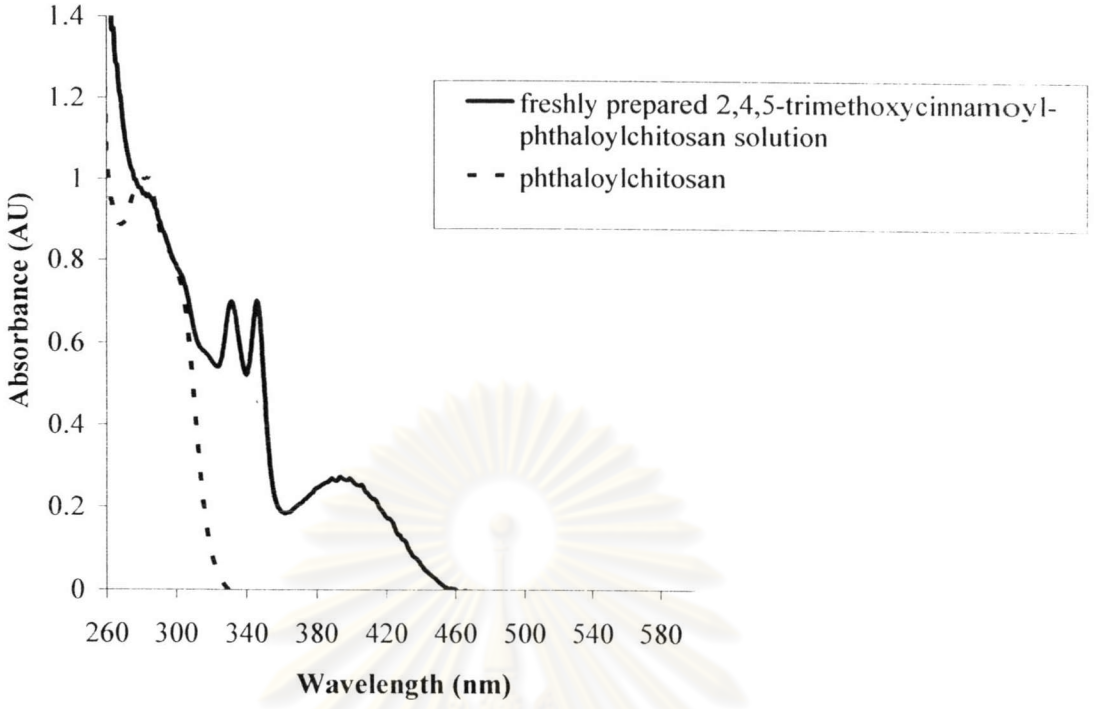


Figure 3.11 UV absorption spectra of 2,4,5-trimethoxycinnamoyl phthaloylchitosan (0.1 g/L, 1.168×10^{-4} M chromophoric units) and phthaloylchitosan (0.1 g/L) in DMSO

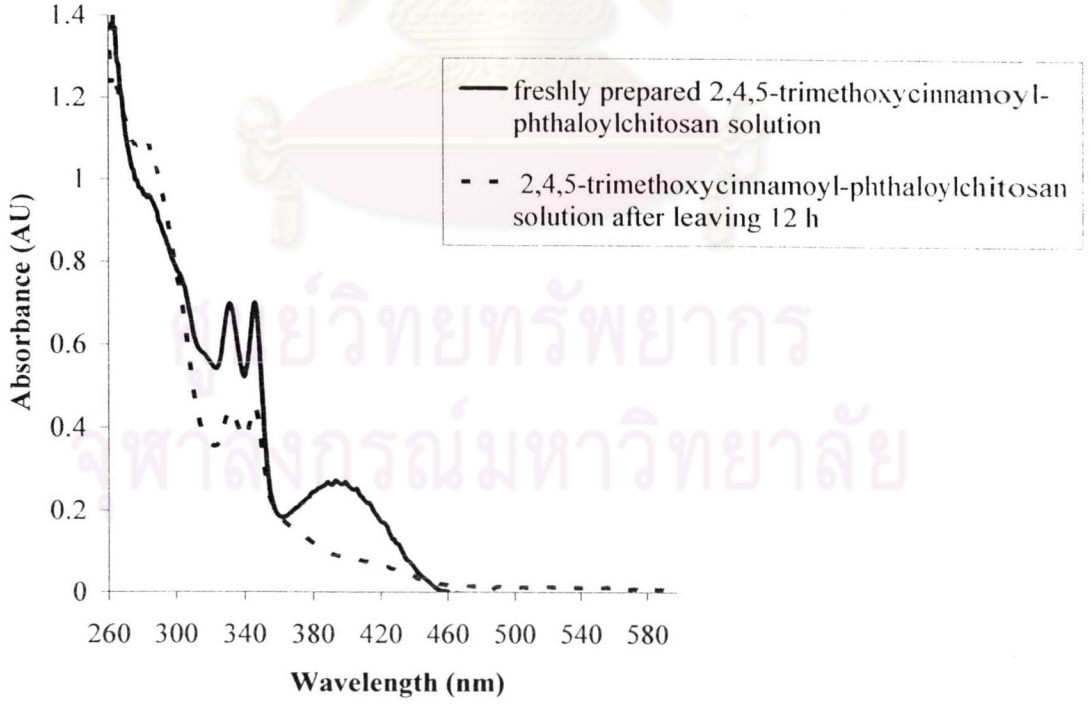
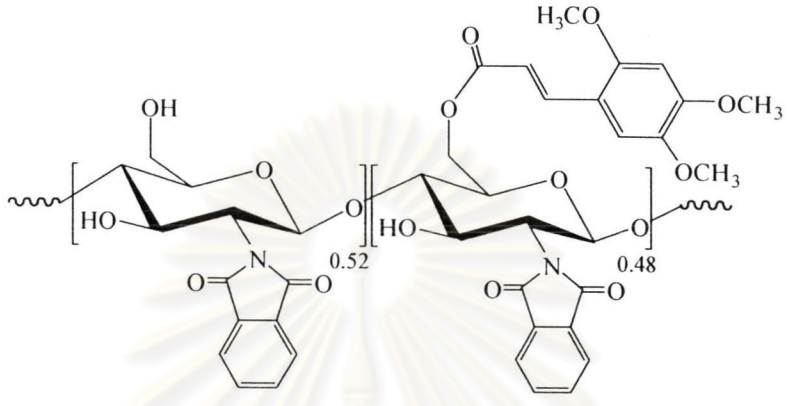


Figure 3.12 UV absorption spectra of freshly prepared 0.02 g/L (2.34×10^{-5} M chromophoric units) 2,4,5-trimethoxycinnamoyl phthaloylchitosan and similar solution after leaving for 12 hours

Degree of substitution was calculated by UV absorption assuming that 2,4,5-trimethoxycinnamoyl moieties on chitosan chain possessed ε of 12,832 at 350 nm (equal to that of octyl-2,4,5-trimethoxycinnamate).[41] The calculation gave degree of substitution of 0.48. General structure of the product is as followed:



M.W. 259,325

3.7 Grafting of 2,4,5-trimethoxycinnamic acid and 4-methoxycinnamic acid on Chitosan

Grafting of 2,4,5-trimethoxycinnamic acid and 4-methoxycinnamic acid onto chitosan was done successfully using the same method used for grafting of only 4-methoxycinnamic acid. The reaction gave 78 %yield. The dark yellow solid product was characterized by ^{1}H NMR and IR spectroscopy. Resonance at 8.40-7.42 (Ar-**H**), 6.78-7.00 (Ar-CH=C**H**-), 7.01-7.12 (Ar-CH=C**H**COOR) and 3.94-3.80 (OC**H**$_3$) ppm indicated 2,4,5-trimethoxycinnamoyl and 4-methoxycinnamoyl moieties. IR peak at 1777 (C=O of ester) and 1613 (C=C) cm^{-1} also helped confirming successful grafting.

Since 4-methoxycinnamic acid showed UV absorption at λ_{max} of 310 nm (see Figure 3.3) and 2,4,5-trimethoxycinnamic acid showed UV absorption at λ_{max} of 290 and 350 nm (see Figure 3.10) , it was unexpected to see three bands of the maximum absorption of the grafted product at 333, 347 and 400 nm (see Figure 3.13). This bathochromic shift from 290, 310 and 350 nm might be a result of some interaction among chromophores on the polymer chain. When this issue was investigated, we have found out that the freshly prepared solution of the product (phthaloylchitosan grafted with 4-methoxycinnamoyl groups and 2,4,5-trimethoxycinnamoyl groups; double grafted chitosan) showed absorption at λ_{max} of 333, 347 and 400 nm. However,

after about 12 h, the absorption of the solution changed to λ_{max} of 310 nm and 350 nm (see Figure 3.14). There was no involvement of light in this shift. We speculated that this blue shift was a result of a complete solvation around 4-methoxycinnamoyl and 2,4,5-trimethoxycinnamoyl moieties, hence, causing complete disruption of interactions among chromophores.

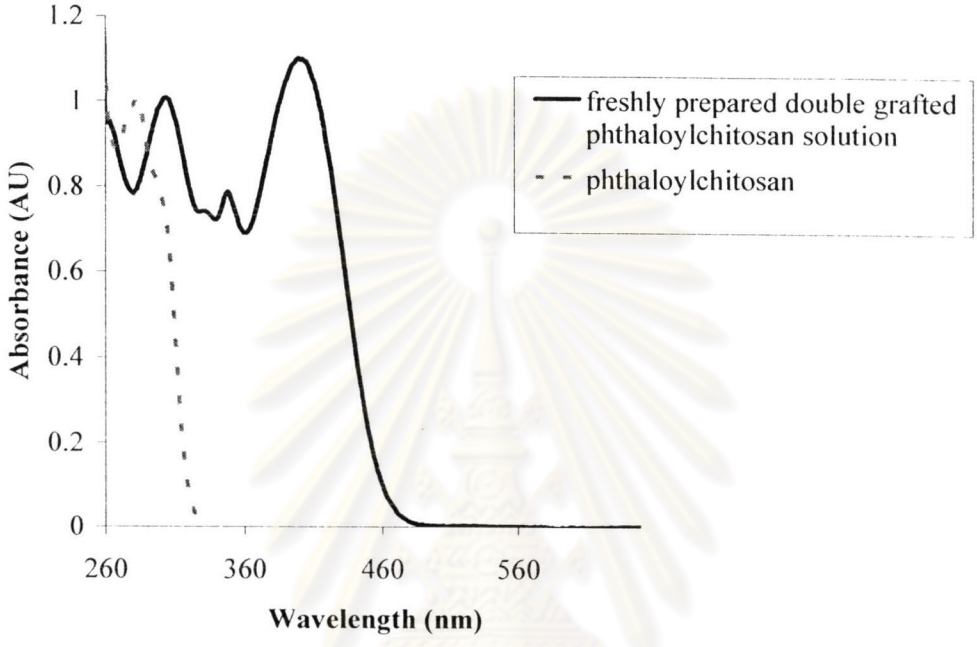


Figure 3.13 UV absorption spectra of freshly prepared 0.05 g/L (5.84×10^{-5} M chromophoric units) 2,4,5-trimethoxycinnamoyl-4-methoxycinnamoyl-phthaloyl chitosan solution comparing to phthaloylchitosan solution (0.1 g/L)

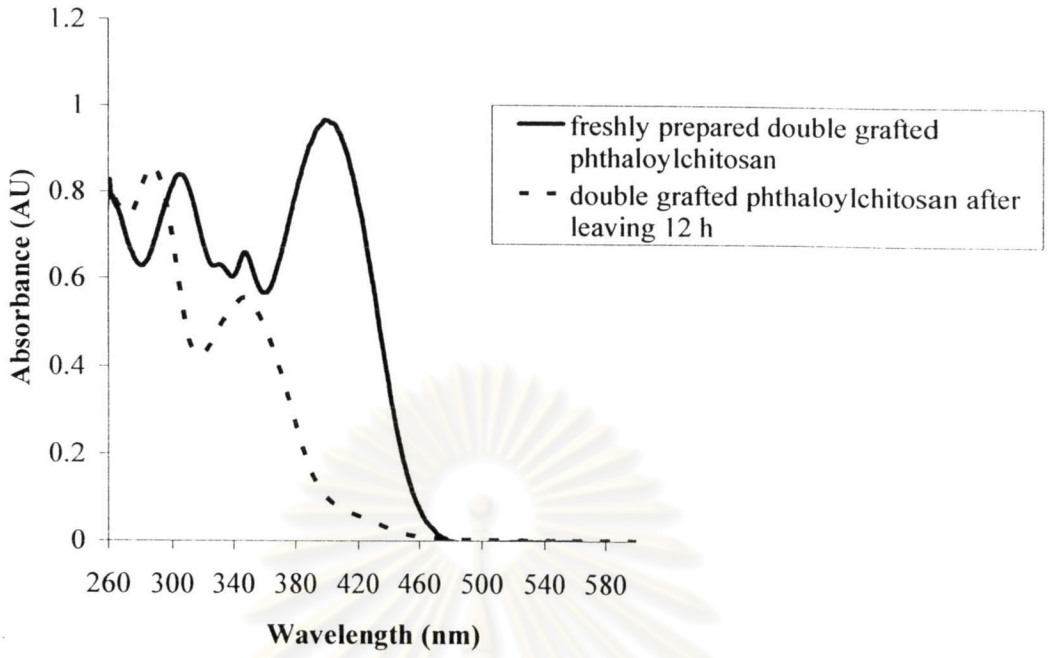
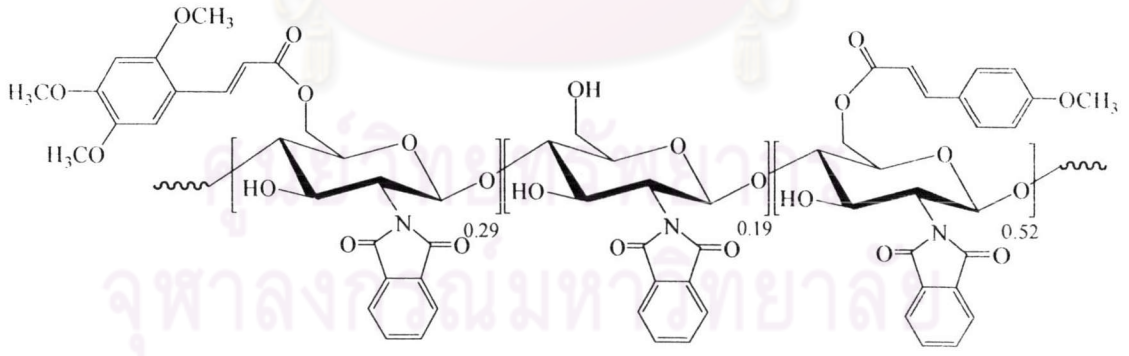


Figure 3.14 UV absorption spectrum of freshly prepared 0.02 g/L (3.50×10^{-5} M chromophoric units) 2,4,5-trimethoxycinnamoyl-4 methoxycinnamoyl-phthaloyl chitosan and similar solution after leaving for 1 day

Calculation of degree of substitution (see Appendix A) gave general structure of the product as followed;



M.W. 311,945

3.8 Photostability Test

It has been known that ethyl hexyl-*p*-methoxycinnamate (OMC), a widely used UVB filter, will undergo *trans* to *cis* photoisomerization under UV light exposure resulting in the decrease in UV absorption efficiency.[43] This configurational change will decrease the UV absorption efficiency of OMC because the *cis* configuration possesses ε of 12000 $M^{-1}cm^{-1}$ while the *trans* configuration possesses ε of about 23000 $M^{-1}cm^{-1}$

Experiments have also shown that the degree of *trans* to *cis* photoisomerization varied with many factors including polarity of solvents around the molecule, light intensity and concentrations. As a result, the UV-absorption chromophore, 4-methoxycinnamoyl group on chitosan chain may undergo similar photoisomerization. In this work both OMC and 4-methoxycinnamoyl-phthaloylchitosan were subjected to UV exposure. Concentrations of chromophoric unit in both solutions were similar. Results are shown in Figure 3.13 for high molecular weight 4-methoxycinnamoyl-phthaloylchitosan and in Figure 3.14 for low molecular weight 4-methoxycinnamoyl-phthaloylchitosan.

When the chromophore was grafted on both high M.W. chitosan and low M.W. chitosan, it was more photostable than OMC but when it was on low molecular weight chitosan it showed similar photostability to that of OMC (see Figure 3.15).

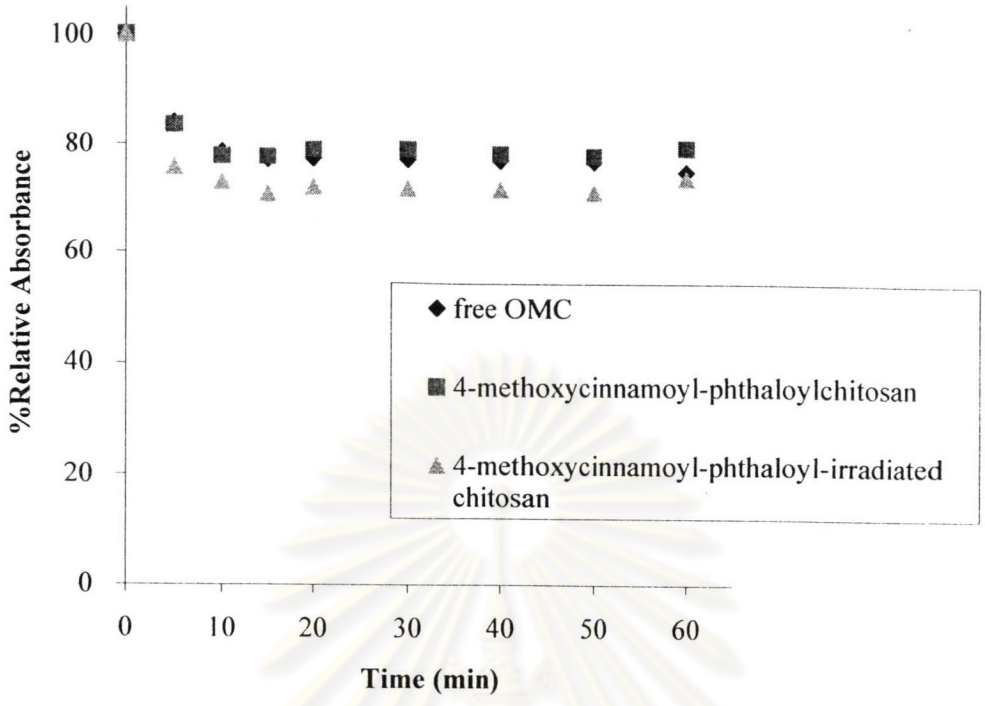


Figure 3.15 Photostability of (0.08 g/L or 1.04×10^{-4} M chromophoric units) 4-methoxycinamoyl-phthaloylchitosan and 0.09 g/L or 1.37×10^{-4} M chromophoric units) 4-methoxycinamoyl-phthaloyl-irradiated chitosan in DMSO. Concentration of OMC was 1.38×10^{-4} M. The light intensities were 5.9 mW/cm^2 for UVA and 0.478 mW/cm^2 for UVB

Similar situation to 4-methoxycinnamoyl-phthaloylchitosan was found for 2,4,5-trimethocycinnamoyl-phthaloylchitosan. In Figure 3.16, 2,4,5-trimethoxy cinnamoyl-phthaloylchitosan was equally photostable to 2,4,5-trimethoxycinnamic acid. However, when monitored at 350 nm, the double grafted chitosan was more photostable than free 2,4,5-trimethoxycinnamic acid. When monitored at 310 nm, the double grafted product was also more photostable than free 4-methoxycinnamic acid (Figure 3.17)

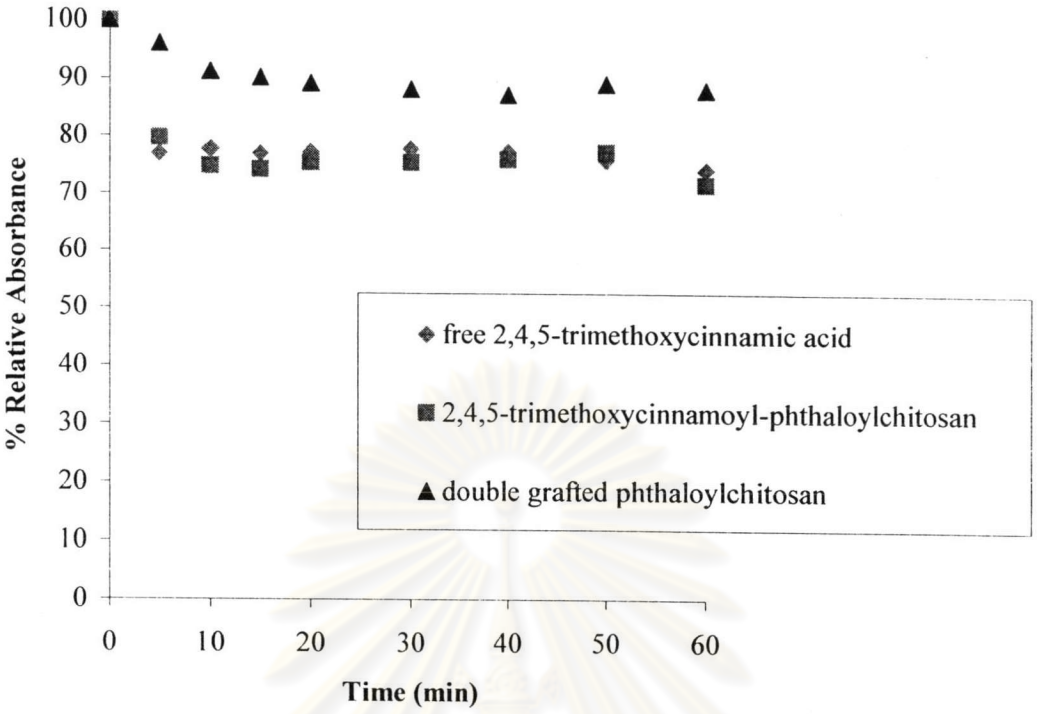


Figure 3.16 Photostability of (0.03 g/L or 1.85×10^{-4} M chromophoric units) 2,4,5-trimethoxycinnamoyl-4-methoxycinnamoyl-phthaloylchitosan (double grafted phthaloylchitosan) and 2,4,5-trimethoxycinnamoyl-phthaloylchitosan in DMSO. Concentration of 2,4,5-trimethoxycinnamic acid was 0.045M. The light intensities were 5.8 mW/cm^2 for UVA and 0.47 mW/cm^2 for UVB

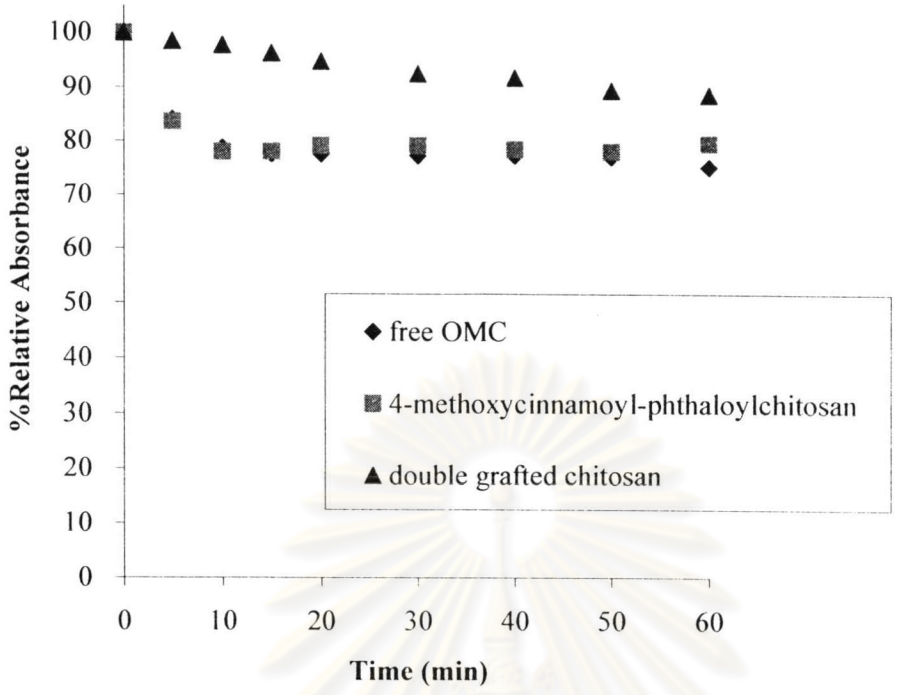


Figure 3.17 Photostability of (0.03 g/L or 1.85×10^{-4} M chromophoric units) 2,4,5-trimethoxycinnamoyl-4-methoxycinnamoyl-phthaloylchitosan (double grafted phthaloylchitosan) and 4-methoxycinnamoyl-phthaloylchitosan in DMSO. Concentration of 2,4,5-trimethoxycinnamic acid was 0.045 M. The light intensities were 5.8 mW/cm^2 for UVA and 0.47 mW/cm^2 for UVB

Since the double grafted product was more crowded with chromophores than single grafted product, therefore, chances of hydrophobic interaction amongs chromophoric units on chitosan chain would be increased. Such interaction probably helped stabilize the configurational change of each chromophoric unit. It has been known that in more hydrophobic environment, less *trans* to *cis* photoisomerization will be observed.[44,45]

Percutaneous absorption of UV filters

Penetration through the human skin was measured in Franz-type diffusion cells using baby mice skin as the membrane. Cumulative diffusion of grafted UV filters through the baby mice skin into the receptor fluid was measured and compared with OMC penetration.

Franz-type glass diffusion cells (~2.27 cm^2 and receptor volume ~13 ml, accurately measured for each cell) were used. Full thickness baby mice skin was placed on the lower halves of the cells with the stratum corneum facing the donor chamber then the upper halves of the cells were added and clamped together. The cells were heat in constant temperature water bath at 37.0°C throughout the experiment. Receptor chamber contents were continuously stirred by submersible magnetic stirrers. The receptor phase was phosphate buffered saline (pH 7.4).

Since one baby mice gave 2 pieces of skin, every sample test was done in compared to OMC. In other word, differences of skins from different mice would not be a problem because the penetration of sample was reported in comparison to OMC penetration using skin from the same mouse.

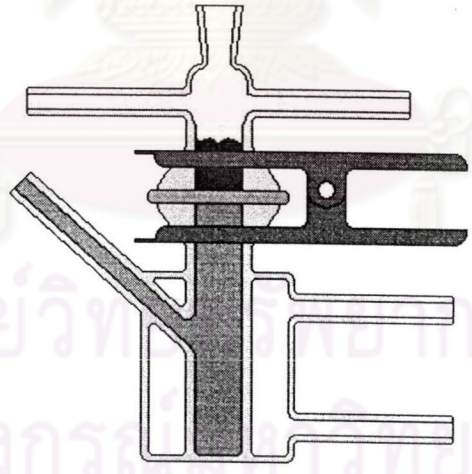


Figure 3.18 Franz-type glass diffusion cells

Figure 3.19 shows the skin penetration result of 4-methoxycinnamoyl-phthaloylchitosan and 4-methoxycinnamoyl-irradiated phthaloylchitosan comparing with OMC. It can be seen clearly that after 5 h about 80% of OMC had penetrated into the receptor fluid while grafted chitosan could not be detected in the receptor fluid.

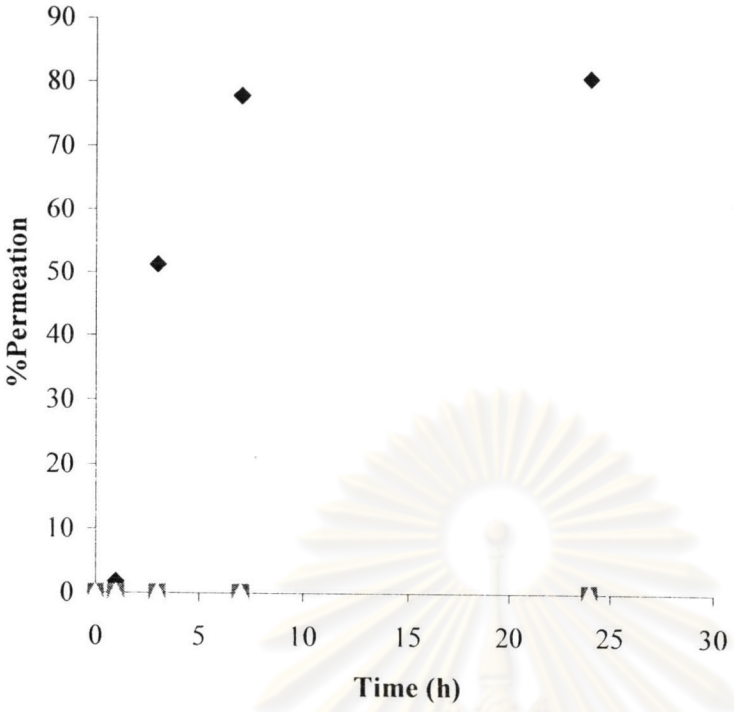


Figure 3.19 Permeation of ♦; OMC, ■; 4-methoxycinamoyl-phthaloylchitosan and ▲; 4-methoxycinamoyl-phthaloyl irradiated chitosan in DMSO by Franz-diffusion cell