A STUDY OF FUNCTIONALIZATION OF CHITOSAN

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บทคัดย่อ

ไคโตแซนเป็นโพลิเมอร์ธรรมชาติชนิดหนึ่งที่น่าสนใจ เพราะว่ามีหมู่ไฮดรอกซิลและอะมิโนที่ถูกเปลี่ยนแปลงได้ง่ายโดยอาศัยปฏิกิริยาเคมี ในการทำวิจัยเรื่องนี้ ไคโตแซนเป็นสารตั้งต้นสำหรับการเตรียมโพลิเมอร์ที่สามารถจับเฉพาะอะตอมเหล็ก

ไคโตแซนเตรียมจากการนำไคตินมาต้มกับสารละลายโซเดียมไฮดรอกไซด์ที่อุณหภูมิ 110 องศาเซลเซียสและล้างทุกๆ หนึ่งชั่วโมง ปริมาณการเอาหมู่อะเซติลออกถูกวัดด้วยเครื่องมือทาง IR spectroscopy และสามารถเตรียมไคโตแซนที่เอาหมู่อะเซลติลออกประมาณ 90 %ได้

การศึกษาการเปลี่ยนแปลงหมู่ฟังก์ชันของไคโตแซนโดยการทำปฏิกิริยากับสารหลายชนิด ได้แก่ phthalic anhydride, benzoyl chloride และ salicylic acid พบว่า การสังเคราะห์ phthalimido chitosan และ benzoylated chitosan สามารถทำได้ภายใต้ปฏิกิริยา heterogeneous การเตรียม N-salicyloyl chitosan แบ่งออกเป็น 2 ระบบ คือระบบ heterogeneous และระบบ homogeneous พบว่า ผลิตภัณฑ์เตรียมได้จากระบบหลังเท่านั้นและเกิด N-acylationโดยใช้ N,N'-Dicyclohexylcarbodiimide เป็น activating agent

พฤติกรรมการจับอะตอมเหล็กของไคโตแซนที่มีหมู่ salicylate ตรวจสอบโดยการคน N-salicyloyl chitosan ในสารละลายของเฟอริกคลอไรด์ การเปลี่ยนแปลงสีของอนุพันธ์นี้จากสีขาวซีดไปเป็นสีม่วงแสดงให้เห็นถึงการมีสมบัติจับอะตอมเหล็ก

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ABSTRACT

Chitosan is a natural polymer of great interest because its hydroxyl and amino groups can be easily modified chemically. In this work, chitosan was a starting material for preparing the iron(III) specific chelating polymer.

Chitosan was prepared by treatment of chitin with 47 % sodium hydroxide solution at 110 °C with intermittent washing with water every hour during the course of the reaction. The degree of deacetylation was determined using IR spectroscopy and highly deacetylated chitosan (≈ 90 % deacetylation) was obtained.

The study of functionalization of chitosan was carried out with different reagents such as phthalic anhydride, benzoyl chloride and salicylic acid. It was found that phthalimido chitosan and benzoylated chitosan could be synthesized under heterogeneous reaction. The preparation of N-salicyloyl chitosan has been studied by two methods ; heterogeneous condition and homogeneous condition. It was found

that the product was only obtained from the latter and N-acylation was formed using N,N'-Dicyclohexylcarbodiimide as an activating agent.

Iron-chelating behaviour of chitosan containing salicylate group was carried out by stirring N-salicyloyl chitosan in an aqueous solution of $FeCl_3$. The color change of this derivative from pale to violet showed its binding of iron(III).

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CHAPTER I
INTRODUCTION

1.1 General introduction

Chelate-forming polymers are polymers that incorporate chelating ligands by covalent bonding. A chelating ligand must have at least two atoms which can be coordinated by the metal at the same time. Such atoms are usually oxygen, nitrogen and sulfur, but selenium, tellurium and possibly certain other elements may also serve the purpose.

Chelating polymers differ from ion-exchange resins in their high selectivity in sorption process and they have been used for water treatment, pollution control and recovery of metals and in analytical chemistry. A number of metal ions such as Cu (II), Cd(II), Ag (I), Zn (II), Pb (II), Fe (III), Mn (II) and Hg (II) can be removed through chelation. In Thailand, iron overload in β-thalassemia patient is increasing and iron chelation therapy is currently the most effective method of removing excess iron. Therefore our work has focused on preparation of iron chelating polymer. There are several methods of preparing chelating polymers, one of which is by chemical modification of natural polymers. Large quantities of waste lignin, chitin, cellulose and starch polymers are available and have been modified in many ways to yield chelating resins selective for metals. Our interest is to study the synthesis, properties and application of iron(III) chelating polymers which is expected to be used in the biomedical field. Chitosan which is obtained from deacetylation of chitin is our base polymer for preparing the chelating polymers through functionalization reactions.

1.2 Chitin and chitosan

Chitin(poly-β-(1→4)-N-acetyl-D-glucosamine),the chemical structure of which is shown in Figure 1.1, has been regarded as a potential marine resource because it is a useful aminopolysaccharide analogous to cellulose structurally and naturally abundant, especially in the cuticle of the marine crustacean such as crab and shrimp. Chitin exists in three polymorphic forms, α-[1],β-[2] and γ-chitins which differ in the arrangement of their molecular chains. Chitin is insoluble in ordinary solvents but if it is put into strong acids and fluoroalcohols, degradation of chitin is obtained. However, dimethylacetamide containing 5 % dissolved lithium chloride is nondegradative solvent for chitin.[3]

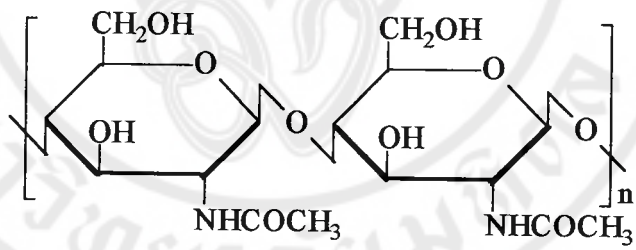


Figure 1.1 Structure of chitin

Chitin shows considerable variation in chemical and physical properties[4,5] which depend on the degree of polymerization and acetylation. The crystallographic structure and solubility from different sources and process conditions are also varied.[6-9]

Chitosan(poly(1-4)-2-amino-2-deoxy-β-D-glucan)occurs in animals along with chitin. It is a crystalline, structural polysaccharide, usually prepared by purification and N-deacetylation of chitin from natural sources with an aqueous solution of sodium hydroxide shown. The molecular structure is shown in Figure 1.2. Chitosan is slightly soluble in

mineral acids at pH below 5.8. It is soluble in some organic acids such as formic acid and acetic acid.

Chitosan is typically manufactured from the solid waste generated during shrimp and crab processing. Thus chitosan is a mixture of polymer sizes and types. The molecular weight of commercial chitosan is 10^4 - 10^6. Different conditions such as reagents, their concentrations, time and temperature employed throughout the manufacturing process may, in each case, affect the characteristics and performances of the final chitosan products.[10,11]

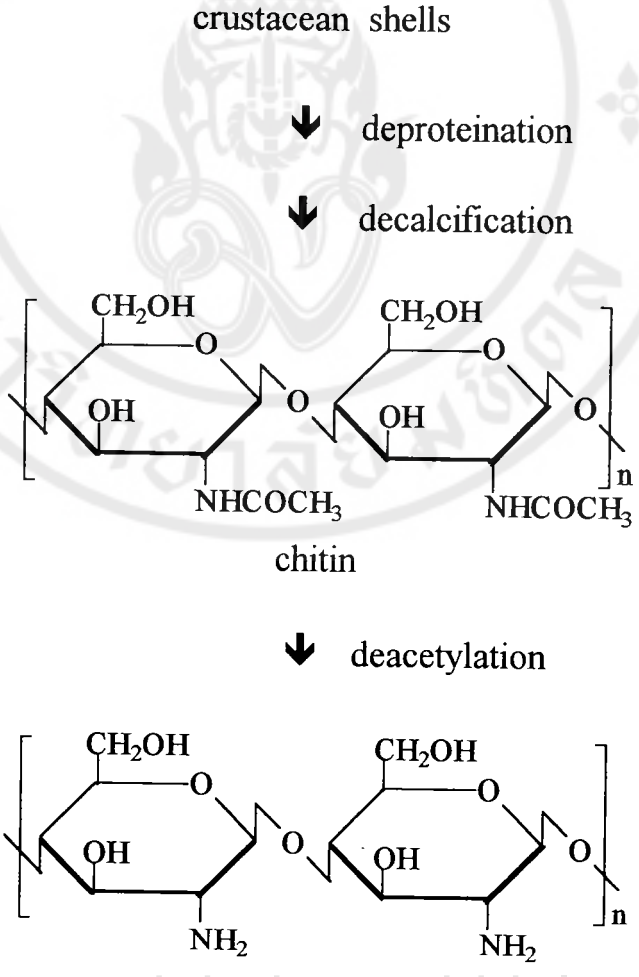


Figure 1.2 Chitosan production

Chitosan appears to be more useful than is chitin , since it has both the hydroxyl and amino groups that can be further chemically modified. Consequently, chitin, chitosan and their derivatives have recently found wide applications in the industrial and medical fields. Biodegradability, biocompatibility, low toxicity and wound healing are among many properties widely appreciated in the medical field.[12,13,14]

Apart from their applications in the medical field, chitosan has potential applications in waste water treatment, where heavy metal ions are removed by chelation. Chemical modifications of chitosan enhance chelating ability by introduction suitable groups to polymer chain to chelate specific metals ions. [15-22]

1.2.1 Preparation of highly deacetylated chitosan

Chitosan obtained from partial N-deacetylation of chitin have been extensively studied in recent years, especially for their industrial applications, as chitin is an abundant natural source of polysaccharide. Owing to the presence of reactive amino groups on the polymeric chain, the amino-polymer can be used for further modification in investigation of chelating properties. In order to do that it is necessary to prepare a highly deacetylated polymer to avoid difficulties in interpreting the mechanisms of counterion interactions. In addition, Grant et. al.[23,24] reported that the higher degree of deacetylation of chitosan results in higher degree of modification.

Several methods have been proposed for N-deacetylation of chitin and chitosan but they generally produce extensive depolymerization. One of the successful method to prepare highly

deacetylation of chitin is to react chitin in 40-50 % sodium hydroxide for a few hours at temperature higher than 100 °C. A. Domard and M. Rinaudo [25] proposed a new method for fully N-deacetylation of chitosan without much decrease in molecular weight. The main difference from previous methods is the use of thiophenol which traps oxygen thus preventing degradation and exerting a catalytic effect. This technique seems to be the most efficient for low acetyl content chitosan and results in better deacetylation with less degradation.

A method has been developed for the preparation of chitosan having a degree of deacetylation of up to 100 % by alkali treatment of chitin with intermittent washing by water instead of the continuous processing.[26] Then the product whose deacetylation was more than 90 % was transformed into threadlike pieces and further subjected to alkali treatment. The final product was easily obtained with a degree of deacetylation of up to 99 %.

Different parameters of deacetylation of chitosan have also been studied such as time and temperature of hydrolysis. It is illustrated in Figure 1.3 that the degree of deacetylation increase with increasing reaction time and temperature. [23,26,27]

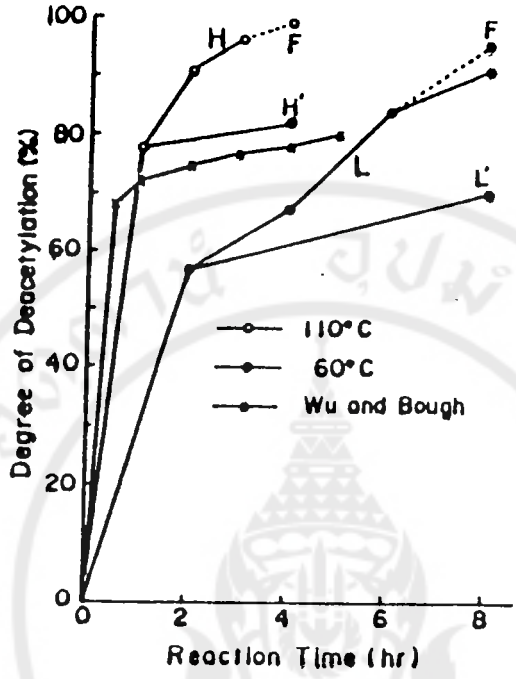


Figure 1.3 Progress of deacetylation of chitin by alkali treatment.
L and H denote reaction temperatures of 60°C and 110°C, respectively.
F indicates additional alkali treatment after the transformation of the sample form into threadlike pieces.
The prime means continuous treatment.

1.2.2 Preparation of water-soluble chitosan

The water-soluble chitosan should have a great advantage as a starting material for further modifications on the free amino groups, since the water solubility allows smooth reactions under mild conditions. Several experiments have been reported for the preparation of water-soluble chitosan starting from chitin or chitosan.

Chitin is ordinarily insoluble in water. However, during the course of the alkaline deacetylation reaction shown in Figure 1.4, it was reported that chitins with about 50 % deacetylation become soluble in water.[28,29] The solubility is strongly dependent on the deacetylation degree, and the samples with over 60 % and under 40 % deacetylation show only poor or even no water solubility. Moreover, those prepared under heterogeneous conditions are insoluble regardless of the deacetylation extent.[27] It was also found that chitosan with high degree of deacetylation and low molecular weight was insoluble in water. This suggests the importance of random distribution of a definite amount of acetyl groups for solubilization. [30,31,32]

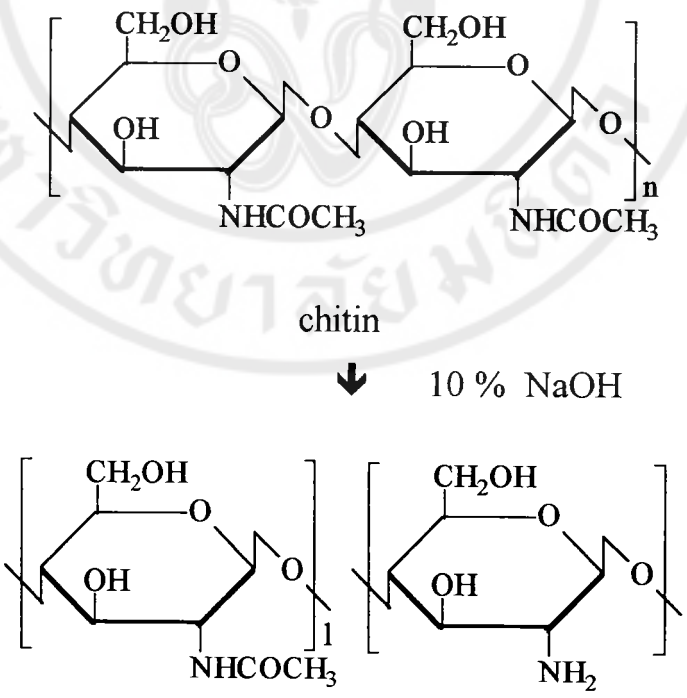


Figure 1.4 Preparation of water-soluble chitin by deacetylation reaction of chitin

The preparation of water-soluble chitin by alkaline deacetylation treatment, however, requires large quantities of solvents and long reaction time (>77 hours). This causes much trouble especially in large scale production. Furthermore it was reported that good reproducibility was obtained when the recovering of the chitin had to be carefully done in acetone at controlled temperature between 0 to 5 °C. K. Kurita et. al. found that it is possible to use water- insoluble chitosan to prepare water-soluble chitin by controlled partial N-acetylation of chitosan under homogeneous or almost homogeneous conditions to achieve random substitution as shown in Figure 1.5. [33]

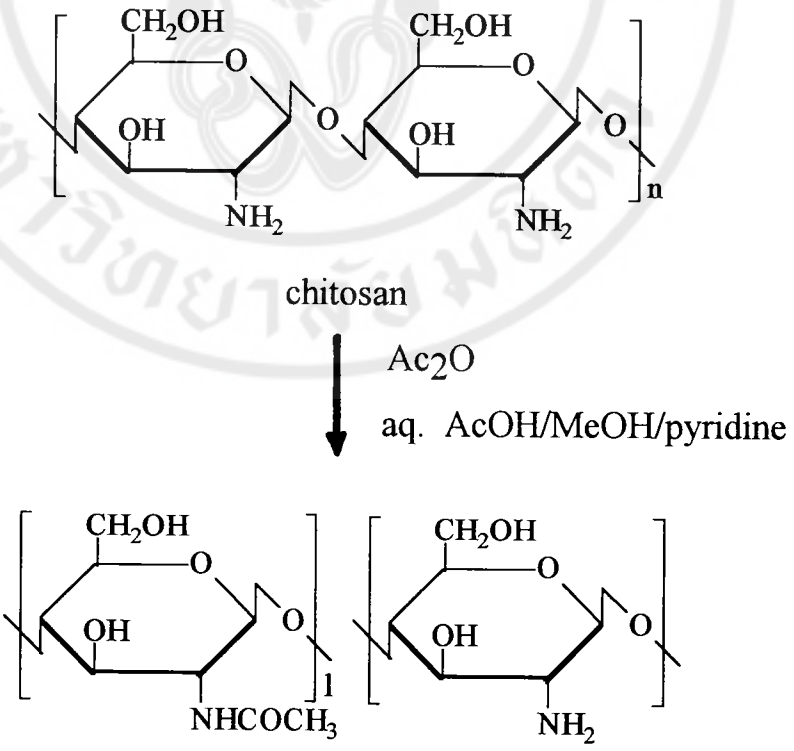


Figure 1.5 Preparation of water-soluble chitin by partial N-acylation of chitosan

In this case, a large excess of acetic anhydride is required compare to the degree of deacetylation of chitosan. The same result is obtained that water-soluble chitin can be acheived when degree of N-acylation of chitosan is between 40-60 %.

Water-soluble chitosan prepared by various acids hydrolysis were also studied.[34] Hydrolysis with hydrochloric acid gave mostly monomer or small molecule with a small degree of polymerization (DP), such as oligosaccharides with DP = 2 to 5. When nitrous acid was used, it provided chito-oligosaccharide with a DP of 9-18. Since the reaction conditions were so strong, it seemed to be difficult to obtain higher degree of polymerization than 20 M. Hasegawa et.al.[35] has reported the preparation of low molecular weight chitosan using phosphoric acid with stirring at room temperature during 1-6 weeks. It was found that two types of hydrolysis products were obtained ; water-insoluble fraction with DP of 17 and water-soluble fraction with DP of 7 with 10-20 % yield. It seems to be possible to prepare water-soluble chitosan having low molecular weight and low yield by this method.

1.3 Determination of degree of deacetylation of chitosan

Different degree of deacetylation of chitin gives chitosan with different chemical and physical properties. It is therefore necessary to determine the degree of deacetylation of chitosan. There are several methods available such as titration technique[36,37], spectroscopic methods (UV[36,38], IR[36], ^{1}H NMR[39] and ^{13}C NMR[40]), differential scanning calorimetry[41] (DSC), thermogravimetric method[42], pyrolysis mass spectroscopy[41] and enzymatic method.[43] Among these techniques,

titration technique and spectroscopic methods which are commonly used will be discussed here.

1.3.1 Titration of chitosan hydrobromide salts [36]

Titrimetric method is a technique used to determine the concentration of free amino groups in chitosan by converting these groups to the hydrobromide salts as shown in Figure 1.6. Then the modified chitosan is titrated with standard sodium hydroxide solution using phenolphthalein as an indicator. The amount of free amino groups or degree of deacetylation of chitosan can then be determined.

Figure 1.6 Preparation of chitosan hydrobromide salts

1.3.2 First derivative ultraviolet spectrophotometry

It has been reported that the degree of acetylation of chitosan can be determined in solution of chitosan in acetic acid by first derivative ultraviolet spectrophotometry at 199 nm.[38] At this wavelength, the N-acetyl glucosamine absorbance reading is linearly dependent on concentration and are not influenced by the presence of acetic acid.

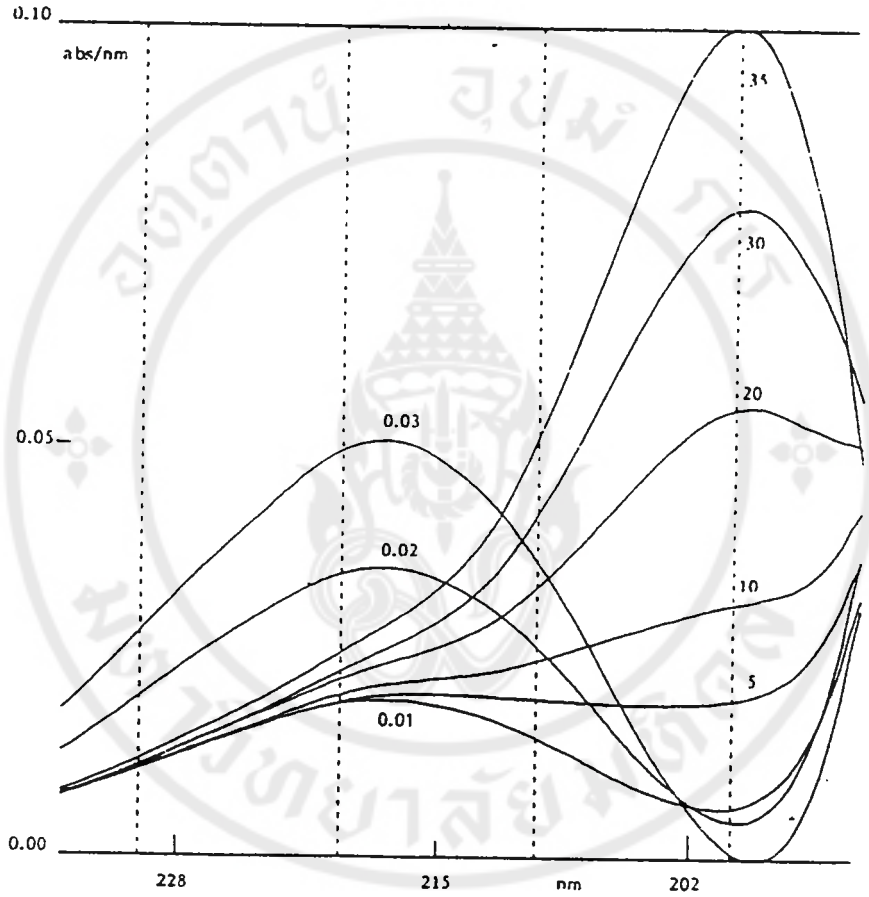


Figure 1.7 First derivative spectra of N-acetyl glucosamine at various concentrations (mg litre^{-1}) in 0.01 M acetic acid

It is also studied by G.D. Julian et. al.[36] that ultraviolet spectroscopy can be used to determine the amount of free amino groups in chitosan. They reacted these free amino groups with excess salicylaldehyde to give the yellow Schiff's base derivative N-salicylidene chitosan. Then the UV absorbance at 255 nm is measured to determine the residual concentration of salicylaldehyde.

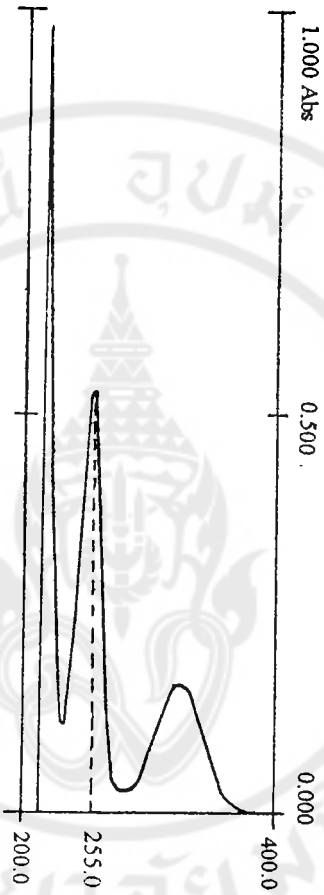


Figure 1.8 UV spectra of salicylaldehyde

1.3.3 Infrared spectroscopy

An infrared spectroscopic technique for determining the degreee of N-acetylation of chitosan was proposed by J.G. Domzy and G.A.F. Roberts.(36) This method involves the use of the amide I band at 1655 cm^{-1} as a measure of the N-acetyl group content and the hydroxyl band at 3450 cm^{-1} as an internal standard as shown in Figure 1.9. The percentage of the amino groups is given by the following equation :

$$\%\ \text{N - deacetyl} = \left[1 - \left(\frac{A_{1655}}{A_{3450}} \times \frac{1}{1.33}\right)\right] \times 100$$

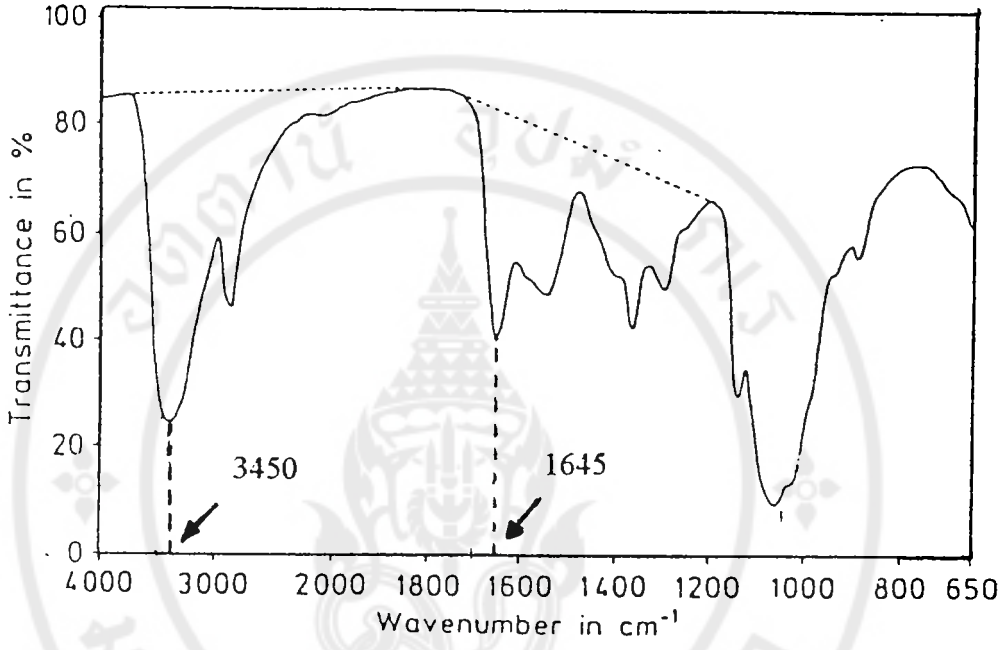


Figure 1.9 IR spectrum of partially N-acetylated chitosan showing the base lines used in determining the absorbance values A_{1655} and A_{3450} for the 1655 cm^{-1} and 3450 cm^{-1} bands

1.3.4 ^1H NMR spectroscopy

It is possible to use ^1H NMR spectroscopy to determine the degree of deacetylation of chitosan but high magnitude field NMR apparatus is necessary because the measurement can be carried out at 70 °C by using 2 wt % CD_3COOD/D_2O and 2 wt % DCl/D_2O as solvents for chitosan where signal of CD_2H residue in CD_3COOD overlaps with that of CH_3 residue in N-acetyl residue. The degree of deacetylation (D_{deac}) is evaluated by using the integral intensity, I_{CH3}, of CH_3 residue, and the sum of integral intensities,

$I_{H2\text{-}H6}$, of H2, H3, H4, H5, H6 and H6' protons as shown in the equation below .[39]

$$D_{deac}(\%) = \left[1-\left(\frac{1}{3}I_{CH_3} \div \frac{1}{6}I_{H2\text{-}H6}\right)\right]\times 100$$

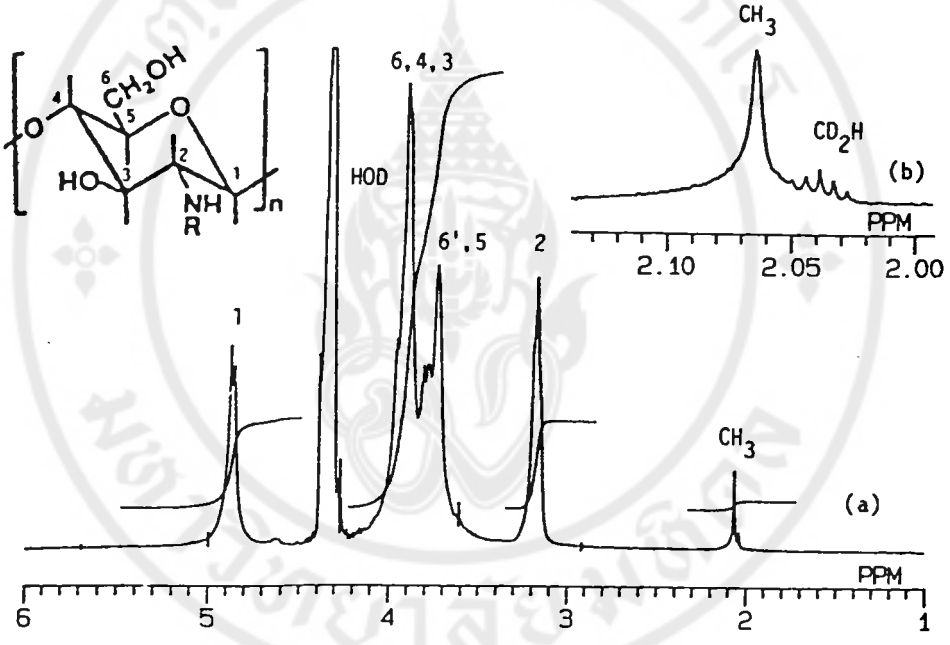


Figure 1.10 400 MHz ^{1}H NMR spectrum of chitosan (D_{deac} = 97%) in CD_3COOD/D_2O at 70 °C

1.3.5 Solid-state ^{13}C NMR

The percentage of deacetylation of chitosan was calculated from solid-state ^{13}C NMR[40] data by comparing the area of the CH_3 resonance of the acetyl group to the resonances of the glucose carbons as shown in Figure 1.11. Integration 1 corresponds to the glucose carbon atoms where integration 2 corresponds to the methyl group, which is proportional to the acetyl content. The limitation of

the cross polarisation / magic angle spinning (CP/MAS) technique for quantitative purposes becomes important at low acetyl contents. Therefore, solid-state ^{13}C NMR is recommended for determination at high acetyl content in chitosan.

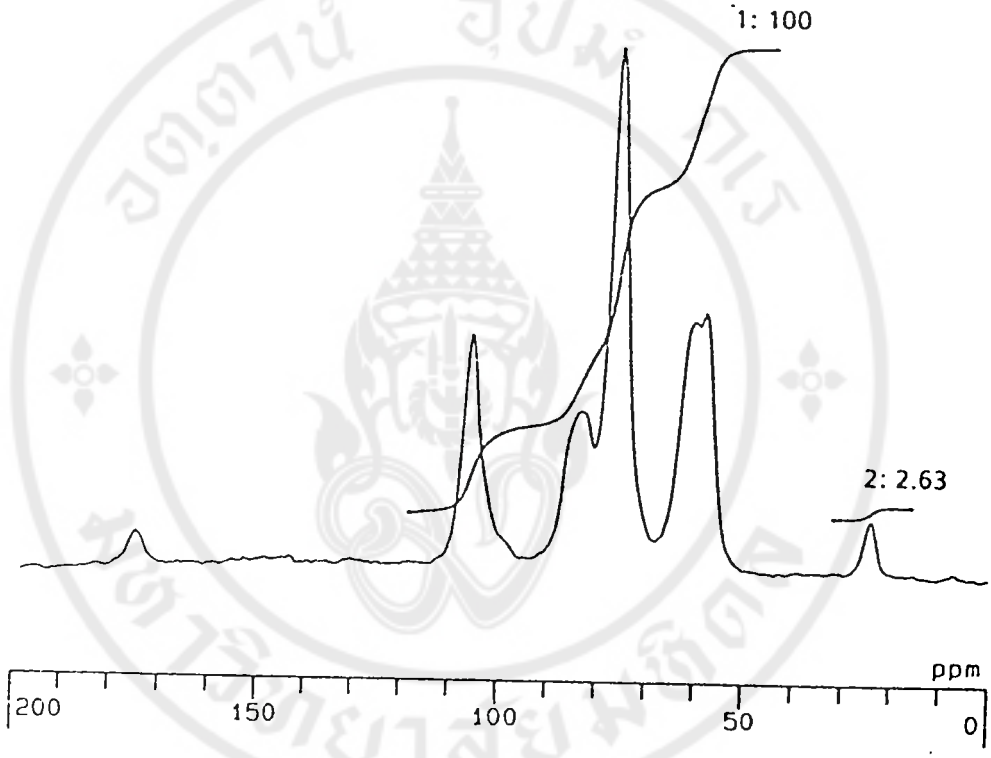


Figure 1.11 CP/MAS spectrum for chitosan

1.4 Status of iron in human body

Iron is one of the essential trace elements in man, and is present in two forms. One is essential body iron, present as hemoglobin in circulating red cells, myoglobin, tissue enzymes and plasma transferrin, the other is storage iron, present as ferritin and hemosiderin. These storage iron are found in the liver, spleen and bone narrow and are mobilized to cells when the body requires iron. A normal 70 kg man contains about 4 g of iron. Of this about 70 %

is present as hemoglobin ; 5 % as muscle myoglobin ; less than 0.5 % as tissue enzymes and plasma transferrin and the remainder about 25 % as storage iron.

Iron is obtained from the food to maintain iron balance. The iron requirement from diet in normal male is equivalent to basic iron loss of about 0.9 mg/day. Approximately 0.6 mg of iron is daily lost from the gastrointestinal tract, 0.2 mg from the skin and 0.1 mg via the urinary tract. The female needs an extra 0.4 mg/day for iron loss due to menstruation. If the body absorbs less or more iron than the requirement, it will result in iron imbalance, that is iron deficiency or iron overload. Iron deficiency is very common and 5000 million people suffer from this worldwide. Unfortunately the body lacks a mechanism for the elimination of excess iron and resulting from genetic diseases or repeated blood transfusion the buildup of iron in the tissues, leads to iron toxicity and eventual death.

The two major causes of iron overload are iron poisoning and the inherited disease β-thalassemia or Cooley's anemia.

Iron poisoning must often arises in small children through the inadvertent ingestion of iron preparations and iron-containing vitamins. The lethal oral dose of elemental iron is estimated to be 200-250 mg/kg. Because supplementary iron tablets contain up to 105 mg of elemental iron, the ingestion of several tablets can result in severe poisoning in a small child. The precise mechanism of iron toxicity is unknown but may derive from the known oxidative reactivity of the metal.

Cooley's anemia is a genetic disorder, characterized by an inability to synthesize adequate amounts of the *beta* chain of hemoglobin. As

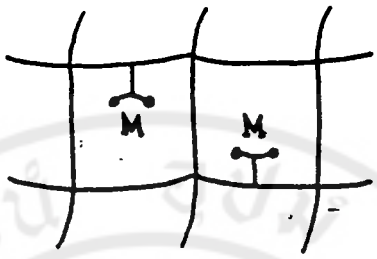
excess *allpha* chains cannot form soluble tetramers, precipitation occurs in the red blood cell precursors leading to cell death and anemia. The treatment of this diseases is hypertransfusion or supertransfusion regimen combined with iron chelation to remove the iron. Desferrioxamine-B (DFB), an effective iron chelator, reduces iron levels by forming a soluble iron complex, which is eliminated in the urine and stool. This regimen could not be used in Thailand because of insufficient blood supply and high costs of this drug. In addition to anaemia, patients with β-thalassemia also have increased iron absorption due to increase in the bone narrow activity. This leads to iron overload during the second decade of the pateint 's life, and will then require iron chelation drug theraphy. It seems to be more practical if some substances administered can bind iron in intestinal lumen and decreases the intestinal iron absorption. Such substances may be iron chelating polymers.

1.5 Characteristic of chelating polymer

Metal complexes and chelates of chelating polymer are coordination macromolecules in which an electron acceptor(e.g. hydrogen ion, metal ions) forms a coordinate covalent bond with an electron donor (a complexing or chelating ligands), in which the electron pair involved in bond formation is supplied by the electron donor.

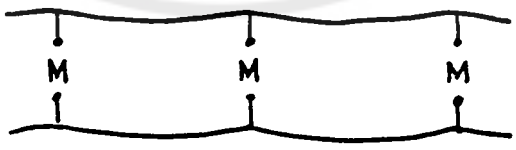
There are many types of the chelating polymers cited in literature which can be schematically presented here when M is a metal ion and ⌒• is a chelating ligand.[14]

- linear polymers containing multifunctional ligands attached at one point

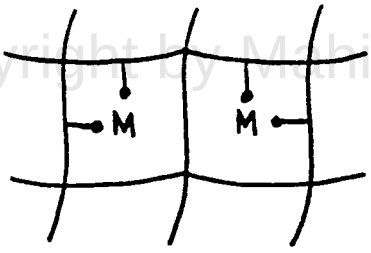


- linear polymers with pendent ligands forming intramolecular chelating groups

- linear polymers with pendent ligands forming intermolecular chelating groups

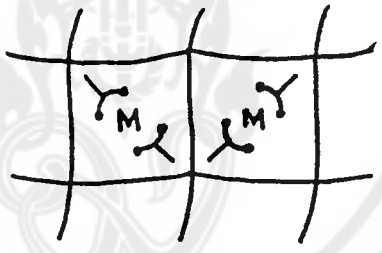


- cross-linked polymers with ligands attached at two or more positions in the network



- metallopolymers (a polymeric chelate complex is formed by metal-ion bridging of monomeric or oligomeric ligands)

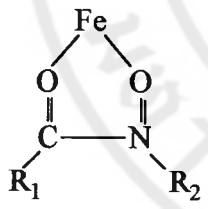
- metallopolymers (a polymeric chelate complex is formed by chemically inert polymers with adsorbed low molecular weight ligands)

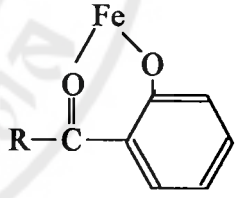


The selectivity of a chelating polymer is determined to a large extent by the nature of the chelating groups (ligands) such as the affinity of the ligands to the metal ions and the stability constant of the complexes. The chelating capacity is dependent on the ligand density and the chelating efficiency, which is strongly influenced by the nature of the polymeric matrix. It has been reported that the properties of the polymer backbone or support not only have a significant effect on the chemical and mechanical stability of the chelating resin, but also affect chelating properties such as capacity, kinetic behavior and metal selectivity.

In the case that iron chelating polymer is needed, polymers with specific ligands are required for iron. Ferric ion is a hard acid which coordinates strongly to ligands having functional groups

made up of small, nonpolarizable, highly electronegative oxygen donor atoms (hard bases). There are many substances known to be iron inhibitors such as clay, tea, coffee, egg yolk, laundry starch, bran and marl.[44] In general there are two main approaches by which chelating resins can be synthesized. One approach involves the polymerization of appropriate monomers to form a solid polymer support containing the desired chelating functionality.[45-47] The second approach is to chemically modify existing polymer supports to contain the chelating function a group of interest.[48-50] The hydroxamate, catecholate and salicylate anions are such chelators which are known to form very stable six-coordinate tris complexs with iron (III).





(a) hydroxamate (b) catecholate (c) salicylate

A natural polymer in a commercially available from which can remove metals from sea[51,52] and industrial water is chitosan. It is a powerful chelating agent and exhibited the best collection ability of all polymers because of its high amino group which acts as an electron donor. The free amino group in chitosan was considered to be much more effective for binding metal ions than the acetyl groups in the chitin.[53] Many metals such as Pb, Cr, Cu, Co, Zn, Fe, Ag and Cd can be removed through chelation.[54-60] Furthermore,

chitosan is safe for human use and low cost polymer. Therefore, it is interesting that modified chitosan may be a good inhibitor for iron absorption. It has also reported that improvement of chelating behavior of chitosan can be obtained through its chemical modification. Our attention is modification amine groups of chitosan with salicylic acid to produce iron (III) specific chelating polymer which may be a new inhibitor for iron absorption in the future.

1.6 Functionalization of chitosan

It was realized that chitosan is an easily reactive substance, on which the reactions of the primary amino group and primary and secondary hydroxyl groups can be carried out. Moreover, a number of novel solvents have been proposed and reactions in homogeneous or nearly homogeneous media have been carried out. Today, a large number of chitosan derivatives have been prepared and studied for numerous applications.

The formation of derivatives suitable for industrial applications with good solubility in various organic solvents can be effected through the introduction of hydrophobic substituents by acylation with long-chain fatty acyl halides or anhydrides. S. Fujii et. al.[61] have shown that chitosan boiled under reflux with a large excess of hexanoyl, decanoyl or dodecanoyl chlorides in dry pyridine-chloroform affords fully acylated derivatives with no apparent degradation. In fact, chitosan samples with a lower degree of deacetylation were reluctant to undergo acylation by this method.[24] The butyrylation of chitosan with butyric anhydride yields derivative soluble in water and methanol.[23] The attachment of reducing carbohydrates as side chains to the 2-amino

functions of chitosan transforms it into branched-chain water-soluble derivatives.[62] N. Shin et.al.[63,64] reported that 3-O-acetylation of the secondary hydroxyl groups of 6-O-substituted materials, with bulky substituents such as triphenylmethyl(trityl) and (*p*-tolylsulfonyl) oxy (tosyloxy) groups, gives rise to regioselectively modified chitosan derivatives showing much better solubility. Partially N-succinylated derivatives of chitosans containing both amino and carboxyl groups show variable solubility in water, dilute acid and dilute alkali depending on the degree of substitution.[65]

A number of derivatives show enhanced chelating ability, eg., the chitosan-glucan complex from *Aspergillus niger* which efficiently removes cupric and mercuric ions from aqueous solutions.[15] The controlled cross-linking with an appropriate amount of glutaraldehyde under homogeneous conditions improves the adsorption capacity of chitosan.[16,17]

Dithiocarbamate chitosan[18], obtained by treating chitosan with carbon disulfide in a mixture of ammonia and methanol, is used to extract metal ions in high yield from acidic, neutral and alkaline solutions. Condensation of chitosan with salicylaldehyde to afford the Schiff-base derivatives introduces a novel function that is effective in chelating cupric ions.[19]

N-(O-carboxybenzyl) chitosan, N-carboxymethyl chitosan, two water-soluble polyampholytes obtained from phthalaldehydic acid and glyoxylic acid and dithiocarbamate chitosan are much more powerful chelating agents in dilute solutions than chitosan.[20] Chitosan has been reductively N-alkylated with a 6-O-substituted galactose derivative bearing an iminodiacetate residue, to give a chitosan derivative having a hydrophilic branch and a metal chelating group.[21]

The products of the reaction of chitosan with mercaptosuccinic acid, thiirane, pyridoxal hydrochloride and succinamide were synthesized to be used as toxic metal-binding agents in aqueous environments.[22] E. Chiessi et.al.[66] have recently prepared novel polymeric ligands by attaching a deoxylactyl, pentanedioic acid or propanoic acid moiety to the amine of chitosan.

Moreover, many reports have been published on the use of modified chitosan in biodegradable packaging materials[37], controlled-release system[67],membrane products[68,69,70] and controlled agrochemical release.[71] And the amino groups of chitosan have been functionalized with several groups such as cyanuryl dichloride, pyrocarbonate and 2,4-dinitrofluorobenzene for preparing randomly substituted derivatives of which properties differ from native chitosan. [30,72,73]

1.7 Scope of the present thesis

This thesis is composed of 5 parts. First of all, the study on preparing highly deacetylated chitosan was carried out. Then the determination of the degree of deacetylation of chitosan was studied. The third part represented an attempt to prepare water-soluble chitosan. The functionalization of chitosan on its amine groups with different reagents i.e. phthalic anhydride, benzoyl chloride and salicylic acid was studied in the fourth part. Salicylic acid was chosen for preparation of potential iron chelating polymer. The last part of the thesis was the study of iron chelation behaviour of the prepared N-salicyloyl chitosan.

CHAPTER II
EXPERIMENTAL

2.1 Materials and Instruments

- Materials which were used as purchase in this experiment were obtained from different companies as following :

Chitin and chitosan were Sigma products.

Benzoyl chloride, phthalic anhydride, acetic acid, formic acid and N,N-Dimethylformamide (DMF) were Merck products.

Acetic anhydride, salicylic acid, sodium hydrogen carbonate, ferric chloride anhydrous, N,N'-Dicyclohexylcarbodiimide (DCC) pyridine and triethylamine were Fluka products.

Salicylaldehyde, hydrobromic acid and phosphoric acid were BDH chemicals Ltd. products.

Sodium hydroxide was an Eka Nobel product.

Absolute methanol was a J.T.Baker Inc. product.

Hydrochlolic acid (37%) was a Carlo ERBA product.

- Solvents which were commercial grade and were distilled before used were methanol, ethanol, chloroform, dichloromethane and acetone.

- Three instruments which were used in our experiment were

PERKIN ELMER system 200 FTIR spectrometer

PERKIN ELMER DSC-7 thermal analysis system

JASCO UVIDEC double beam UV/VIS spectrophotometer

2.2 Preparation of highly deacetylated chitosan

About 5 g of chitin was added to 100 ml of 47 % aqueous NaOH in a 250 ml round bottom flask fitted with conderser and nitrogen gas inlet tube as shown in Figure 2.1. The mixture was purged with nitrogen and then heated at 110 °C with stirring. After 1 hour, the product was filtered off and washed with warm water (≈8o°C) until the filtrate was neutral (pH = 7). Then it was washed with ether and dried in air.

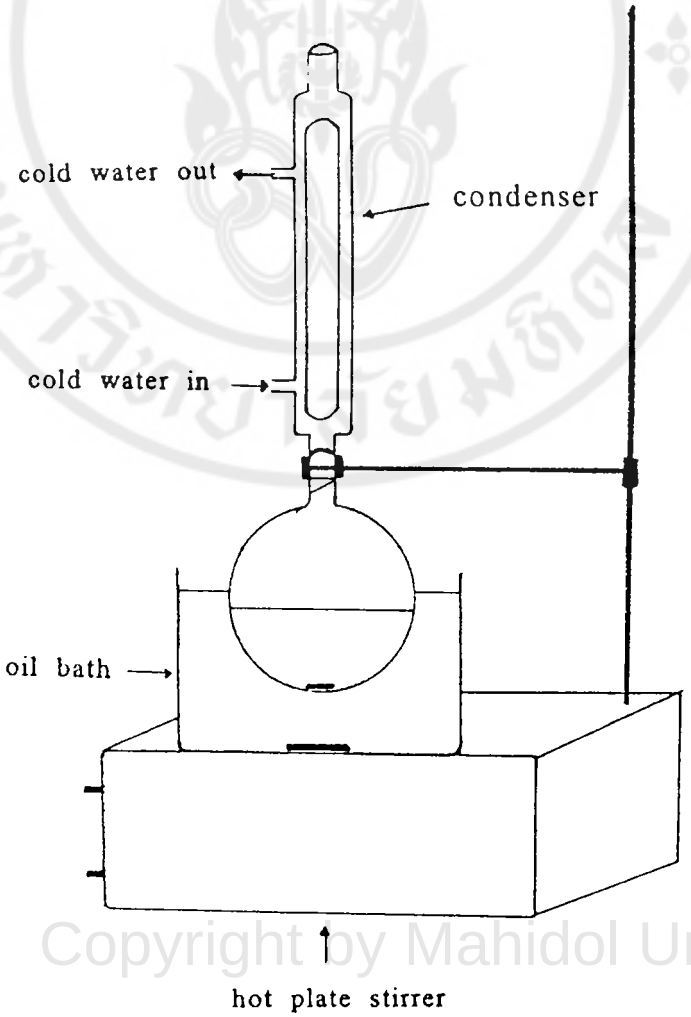


Figure 2.1 Apparatus used for the preparation of chitosan

The modified product was then put into a 250 ml round bottom flask for repeating the hydrolysis process with NaOH solution at 110 °C. The chitosan obtained was washed every hour to increase the degree of deacetylation. At the end of the reaction, the white flake product was obtained. Five batchs (chito-2,3,4,5,6) were carried for different reaction times as shown in Table 3.2. The products were characterized by FTIR spectroscopy and DSC technique.

2.3 Preparation of water-soluble chitosan

Water-soluble chitosans were carried out by several conditions as shown below :

2.3.1 Preparation of 50 % deacetylated chitin under alkali solution

A suspension of 3 g of chitin in 75 g of aqueous sodium hydroxide (30 g in 45 g water) was allowed to stand in a 250 ml round bottom flask for 3 hours at 25 °C under reduced pressure. Alkali chitin was then dissolved by stirring vigorously with 225 g of crushed ice below 0°C. The solution obtained was filtered, and the filtrate was left standing at 25 °C for 77 hours.

The solution obtained above was cooled below 5 °C and 210 g of crushed ice made from distilled water was added to avoid a rise in temperature of the solution on neutralization. To the mixture was slowly added concentrated aqueous HCl with stirring until the pH of the solution became 9. The pH of the solution was adjusted carefully to 8.7 with diluted aqueous HCl and then the solution was poured dropwise into 5 l of an acetone/distilled water mixture

with the ratio of 7 to 1 at 0 °C with efficient stirring. Cooled acetone was dropped into the precipitant simultaneously to keep the acetone/ water ratio roughly constant. The white precipitate formed was filtered off and immediately placed in a beaker containing a cold acetone/distilled water mixture and a small amount of cold distilled water was poured into the mixture with stirring to dissolve NaCl. These filtrations and washings were repeated until the washings became no longer turbid under testing with the addition of an aqueous $AgNO_3$ solution. The precipitate was finally washed with acetone and dried at 0°C in vacuum. White solid product was obtained and analyzed by FTIR spectroscopy.

2.3.2 Preparation of water-soluble chitosan under acidic condition

100 g of 85% phosphoric acid was added into 5 g sample of chitosan placed in a 300 ml Erlenmeyer flask and the flask was closed with a stopper. It usually took 1-2 days with intermittent stirring at room temperature for complete dissolution of chitosan, resulting in a brown viscous liquid. This solution was allowed to stand at room temperature for 6 weeks. After standing, chitosan was regenerated as a white precipitate by pouring the solution into excess ethanol. After stirring the mixture for 1 day, free phosphoric acid was removed with ethanol by decantation and phosphoric acid forming salt at amine groups of chitosan was removed by repeated treatments with 1% triethylamine in ethanol. The precipitate was throughly washed with ethanol.

The water-soluble fraction was separated from the precipitate by extraction with 1000 ml of water at room temperature for 2 days. This fraction was concentrated by evaporation, and was obtained as a reprecipitate by addition of ethanol to the mixture. The precipitate was treated with 1% triethylamine in ethanol, and then was washed thoroughly with ethanol by centrifugation. This water-soluble low molecular weight chitosan was collected by drying in vacuo.

2.3.3 Preparation of N-acetylated chitosan

0.3 g chitosan was dissolved in 8 ml 10% aqueous acetic acid and diluted with 8 ml methanol in a 250 ml round bottom flask. A highly swelled chitosan precipitate prepared by pouring the chitosan solution into 100 ml pyridine then was added with 0.93 ml acetic anhydride. The stirred mixture was refluxed for 5 hours. At the end of the reaction, the product was filtered, washed with ethanol and dried in vacuo. The N-acetylated chitosan was obtained as white powder and analyzed by FTIR spectroscopy. The solubility of the product was tested with water and the insolubility was found.

2.4 Determination of degree of deacetylation

Degree of deacetylation of chitosan can be determined by several methods. Among these, some are carried out here as following :

2.4.1 By hydrobromide salt titration

- Preparation of chitosan hydrobromide salt

0.5 g chitosan was first dissolved in 100 ml of 0.2 M HBr. Then 50 ml of 9 M HBr was added to the solution with vigorous

stirring to precipitate the hydrobromide salt. The light precipitate was obtained as slurry solution. It was separated by centrifugation (33 s^{-1}) and then decantation off the centrifugate. The product was washed with methanol until the filtrate was neutral then with several portions of ether before it was left to be dried in air.

The hydrobromide salt product was again washed with methanol in order to eliminate residual HBr in chitosan and air dried. It was repeated three times and at the end of the reaction, white power product was obtained.

- Titration of chitosan hydrobromide salt

An accurately weighed chitosan hydrobromide salts (≈ 0.2 g) was dissolved in 100 ml distilled water. The solution was titrated with standardised 0.1 M aqueous sodium hydroxide by using a microburette and phenolphthalein as an indicator. At the end point the solution was change from colourless to pink color. The degree of deacetylation can be calculated from the equation of N-deacetyl content

$$\text{N-deacetyl content (\%)} = \frac{0.1\ N\ X\ MW}{m}$$

N = normality of NaOH

x = volume of NaOH used at the end point

MW = molecular weight of monomer unit of chitosan

m = weight of starting chitosan

2.4.2 By UV spectrophotometry

An accurately weighed portion of chitosan approximately 0.1 g was slurried for 48 hours in a 0.02 M solution of salicylaldehyde in methanol/1% acetic acid (80/20 v/v). The volume of salicylaldehyde solution taken was dependent on the approximate extent of N-acetylation of sample. For high degrees of N-acetylation (> 50 %) 15-20 ml aliquots were used whilst for lower degrees of N-acetylation 25-40 ml aliquots were required. After stirring at room temperature for 48 hours the mixture was filtered, a portion of the filtrate diluted 400 times and the UV absorbance at 255 nm was measured to determine the residual concentration of salicylaldehyde. In this case the chitosan obtained was yellow solid. The degree of deacetylation was obtained by comparing the absorbance at 255 nm with standard curve of salicylaldehyde solution.

2.4.3 By IR spectroscopy

5 % chitosan solution in 90 % aqueous formic acid solution was prepared and cast on a smooth glass plate. The film formed was left dry at room temperature. The obtained film was then removed from the glass plate and soaked overnight in 0.05 M sodium methoxide in methanol to regenerate the free amine form. After the film was dried at room temperature, it was analyzed by FTIR spectrometer. The percentage of free amine groups was obtained by a rectilinear relationship between the N-acetyl group band at 1655 cm^{-1} and amide I band at 3450 cm^{-1} as shown in the following equation. (36)

$$\% \text{ N - deacetyl} = \left[1 - \left(\frac{A_{1655}}{A_{3450}} \times \frac{1}{1.33}\right)\right] \times 100$$

where A_{1655} = absorbance value at 1655 cm^{-1}

A_{3450} = absorbance value at 3450 cm^{-1}

Chitosan obtained from Sigma (chito-s) and prepared one (chito-p) were analyzed by three methods. The results were shown in Table 3.3.

2.5 Functionalization of chitosan

2.5.1 Reaction of phthalic anhydride with chitosan

(i) Preparation of phthalimido chitosan

A mixture of 1.5 g chitosan and 13.8 g phthalic anhydride in 100 ml DMF was heated in a 250 ml round bottom flask with a condenser and stirred at 130 °C under a nitrogen atmosphere. After 5-7 hours, the mixture became a clear and viscous solution. The precipitate was obtained by pouring the solution into ice-water and then collected by filtration and successively washed completely by Soxhlet 's extraction with ethanol. After the brown solid was obtained after drying in vacuum at room temperature. The product was analyzed by FTIR spectroscopy.

(ii) Hydrolysis of phthalimido chitosan

To a 50 ml of 1 % aqueous hydrochloride acid solution was added 0.5 g the phthalimido chitosan obtained above. The mixture was refluxed for 5 hours. The resulting chitosan was seperated by filtration,

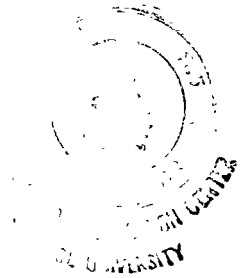
and washed with water and ethanol. After drying under vacuum, the product was analyzed by FTIR spectroscopy.

2.5.2 Preparation of benzoylated chitosan

1 g chitosan was soaked in 20 ml pyridine for three days to regenerate free amine after which the pyridine was evaporated under reduced pressure. The pyridine must be dried because acyl chlorides tend to hydrolyse spontaneously with water to produce HCl, and this would be a hindrance to the reaction. The chitosan was then immersed again in pyridine (30ml) and chloroform (15 ml) for a further three days. The mixture was cooled to -10 °C in an ice-salt bath, and a solution of 5 ml benzoyl chloride in 8 ml chloroform was added slowly with constant stirring. The mixture, which had changed from colourless to orange, was then refluxed for 6 hours, after which it was poured into methanol. The product was then filtered, washed with methanol and air dried. The dried chitosan was replaced in a flask with fresh pyridine, chloroform and benzoyl chloride in the amounts described above to repeat the reaction for another three times.

The acylated polymer was obtained after repeating the process for four times in order to obtain maximum amidation reaction. The product was then air dried and characterized by FTIR spectroscopy and DSC technique for examining its thermal stability.

2.5.3 Reaction of salicylic acid with chitosan



(i) Heterogeneous condition

- Condition A

1.0 g salicylic acid was dissolved in 70 ml acetone and then added with 0.2 ml conc. H_2SO_4. The mixture was divided into two fractions. Chitosan (0.5 g) was added in each fraction. The former fraction was refluxed for 24 hours while the later fraction was stirred at room temperature for 48 hours. After filtration, the product of each fraction was washed with acetone and dried in air. Then two products were characterized by FTIR spectroscopy.

- Condition B

1.0 g chitosan was soaked in 20 ml pyridine for three days after which the pyridine was evaporated under reduced pressure. The chitosan was then immersed again in pyridine (30ml) and chloroform (15 ml) for a further three days. The mixture of 1.5 g salicylic acid and 2.0 g DCC in 30 ml chloroform was stirred for 30 minutes and then was filtered. The filtrate was added to the chitosan suspended in pyridine. The mixture was refluxed for 24 hours. The product was then filtered, washed with methanol and air dried. The obtained chitosan was characterized by FTIR spectroscopy.

- Condition C

The mixture of 1.5 g of salicylic acid and 2.0 g DCC in 50 ml dichloromethane was stirred for 30 minutes and then was filtered. The filtrate was added to 0.5 g chitosan. One experiment was carried out under reflux for 24 hours and the other experiment was carried

out under room temperature for 48 hours. All product were then filtered, washed with methanol and air dried. They were characterized by FTIR spectroscopy.

(ii) Homogeneous condition

The acylation reaction of chitosan with salicylic acid can be carried out in homogeneous system.

0.434 g chitosan was dissolved in 8 ml of 10 % acetic acid and then diluted with 32 ml water. Mixture of 3.31 g salicylic acid and 4.95 g DCC in 60 ml N,N-dimethylformamide was added to the stirred chitosan solution, then the precipitation was occured. The mixture was refluxed for 20 hours. After filtration, the precipitate was put into a saturated aqueous sodium hydrogen carbonate solution for neutralization. The precipitate was then obtained by centrifugation, washed several times with water and methanol to eliminate any unreacted salicylic acid. The final pale solid product was dried in vacuum at room temperature and characterized by FTIR spectroscopy and DSC technique.

2.6 Iron chelation process

Iron chelation reaction of chitin, chitosan and N-salicyloyl chitosan were carried out. To 25 ml of an aqueous solution of $FeCl_3$ (0.1 M in 1 N H_2SO_4) was added 0.25 g of a specified sample. After stirring for 24 hours at room temperature, the mixture was centrifuged and filtered with a glass filter. The filtrate was used to measure the absorbance of the residual $FeCl_3$. The concentration of iron

was determined spectrophotometrically by using a JASCO UVIDEC double beam UV/VIS spectrophotometer. A comparison of the absorbance of $FeCl_3$ with control solution was also done by preparing stirred $FeCl_3$ solution during 24 hours. The results were shown in Table 3.6.

CHAPTER III
RESULTS AND DISCUSSION

3.1 Preparation of highly deacetylated chitosan

Chitosan which is a deacetylation form of chitin can normally be found along with chitin during the preparation of chitin from the cuticle of the marine crustacean. The presence of amino groups along the polymeric chain of chitosan provide potential for chemical modification and further application. The preparation of chitosan from chitin has been widely studied.

3.1.1 Characterization of the prepared chitosan

Preparation of high degree of deacetylation of chitin has been reported by using alkali hydrolysis method. Several factors have been found to influence the degree of deacetylation[22,26,27] obtainable including sodium hydroxide concentration, reaction time and temperature used.

In the present work, preparation of highly deacetylated chitosan was studied by using 47 % aqueous sodium hydroxide solution at temperature of 110 °C and varying the reaction time. In our case, 4 hours continuous treatment of chitin with aqueous NaOH solution was tried at 110 °C under nitrogen atmosphere. After filtration, the chitosan obtained was washed with warm water until the filtrate was neutral. Then it was washed with ether and dried in air. The chitin and the prepared chitosan were analysed by FTIR spectroscopy. The

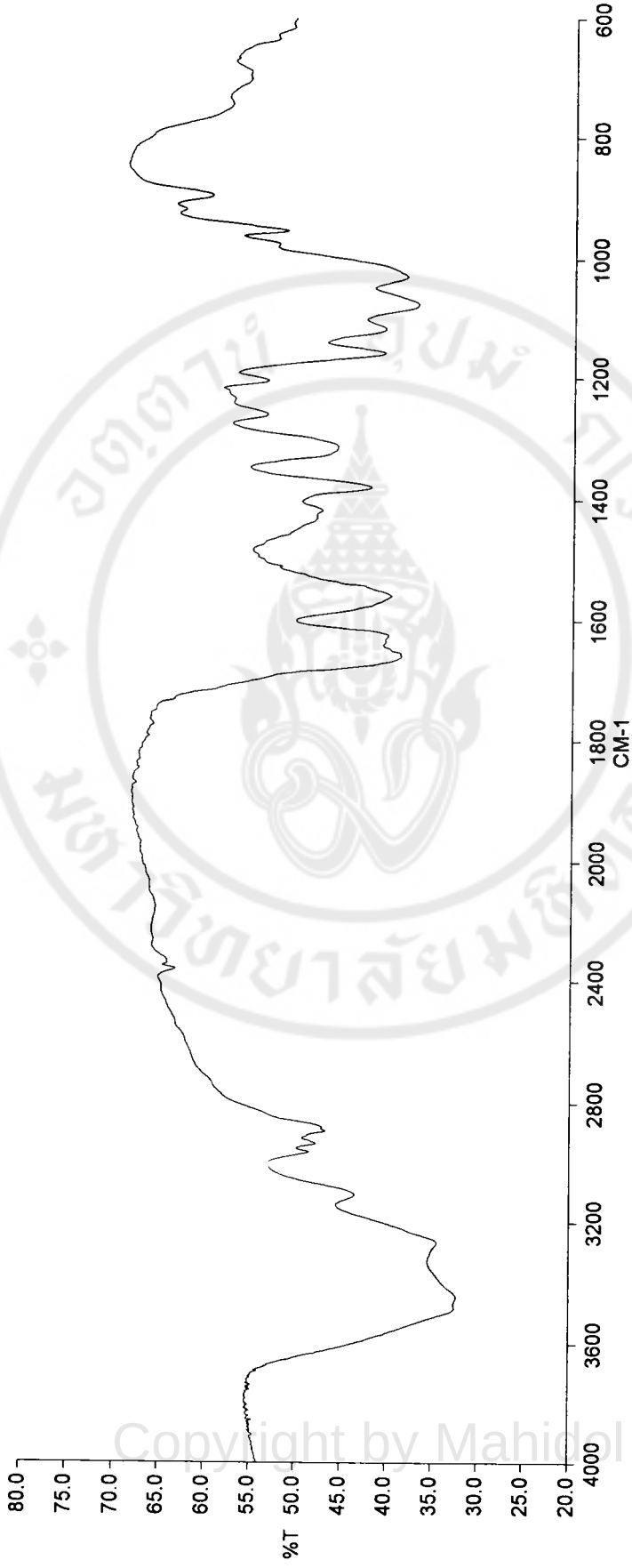


Figure 3.1 IR spectrum of chitin (KBr pellet)

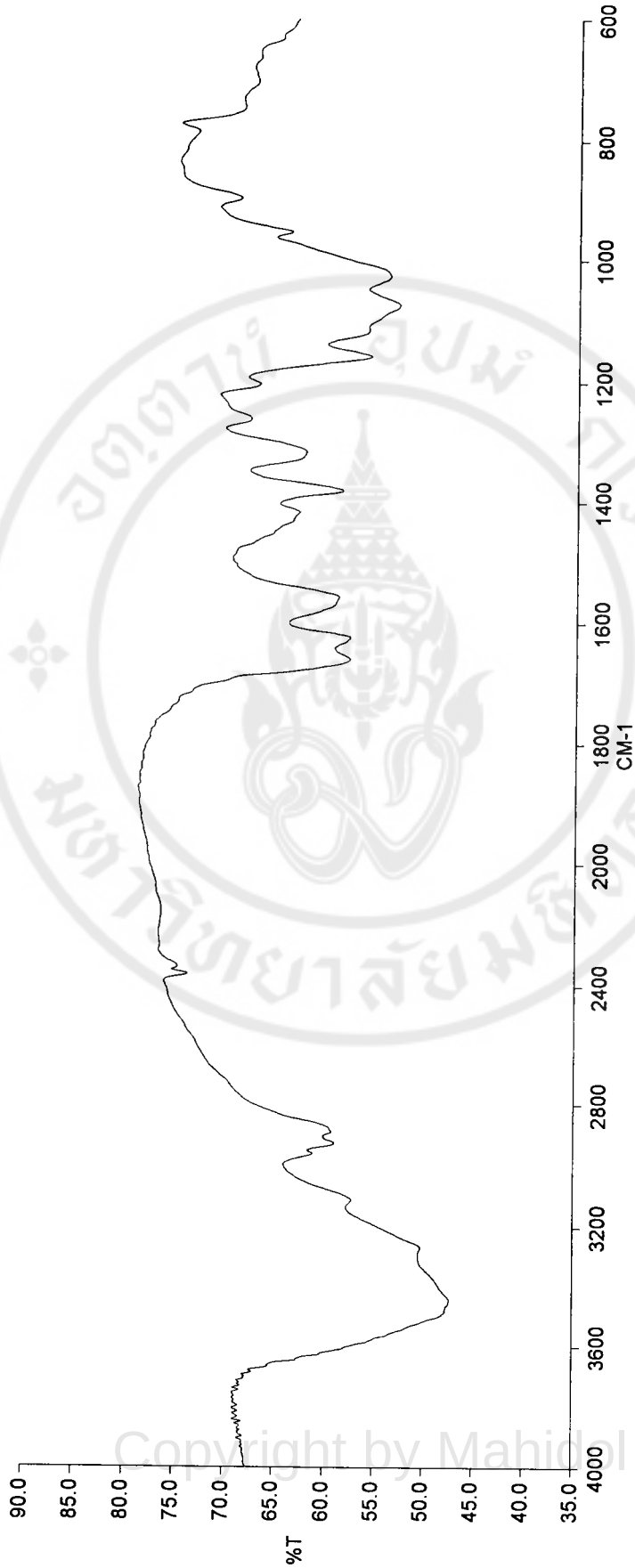


Figure 3.2 IR spectrum of chitosan (46 % deacetylation)

results obtained are FTIR spectra was shown in Figures 3.1 and 3.2 respectively. Chitin has characteristic peaks of amide I band at 1655 cm^{-1} and hydroxy group at 3450 cm^{-1}. It was found that the amide I band at 1655 cm^{-1} of prepared chitosan decreased and amino deformation peak at 1590 cm^{-1} increased as shown in Table 3.1. It was found by IR technique (discussed later in section3.3) that the degree of deacetylation was 46 % and the final product was insoluble in formic acid.

Thermal analysis of chitin and chitosan were performed by using differential scanning calorimetry (DSC). It was found that the DSC curve of chitosan (Figure 1A) (see Appendix) is different from that of chitin (Figure 2A). Chitin exhibited two DSC peaks, one of which is endothermic at 140 °C, believed to be due to the release of water. And the second peak is exothermic with a maximum at 316 °C. The latter peak is thought to be associated with degradation of the main chains.

Table 3.1 Major IR absorption bands of chitosan

Frequency (cm^{-1})	Assignment
3450	hydroxy band
2910,2870	C-H stretching
1655	amide I (C=O)
1590	NH_2 deformation

3.1.2 Study of the effect of reaction time on the degree of deacetylation

It was mentioned by several authors [26] that hydrolysis of N-acetyl group in chitin could be effected to high degree of deacetylation when chitin was intermittently washed in water, for example every hour, during the hydrolysis process. Further five experiments were then carried out by using the same condition i.e. the chitin was treated with 47 % NaOH solution at 110 °C under nitrogen atmosphere but after one hour of the reaction the modified chitin was filtered out and washed with warm water until the filtrate was neutral. Then it was washed with ether and dried in air before retreatment with aqueous NaOH solution. The total reaction time of each experiments are 2, 3, 4, 6 and 8 hours.

The obtained chitosan in each experiment was white solid and its characterization was carried out by using infrared spectroscopy. FTIR spectra (such as shown in Figure 3.3) showed significant decreases in intensity of the amide I band at 1655 cm^{-1} due to N-acetyl group in chitin and significant intensity increases of the peak at 1590 cm^{-1} characteristic of amino group in chitosan. All chitosans obtained were soluble in formic acid and acetic acid. The results of the degree of deacetylation are shown in Table 3.2.

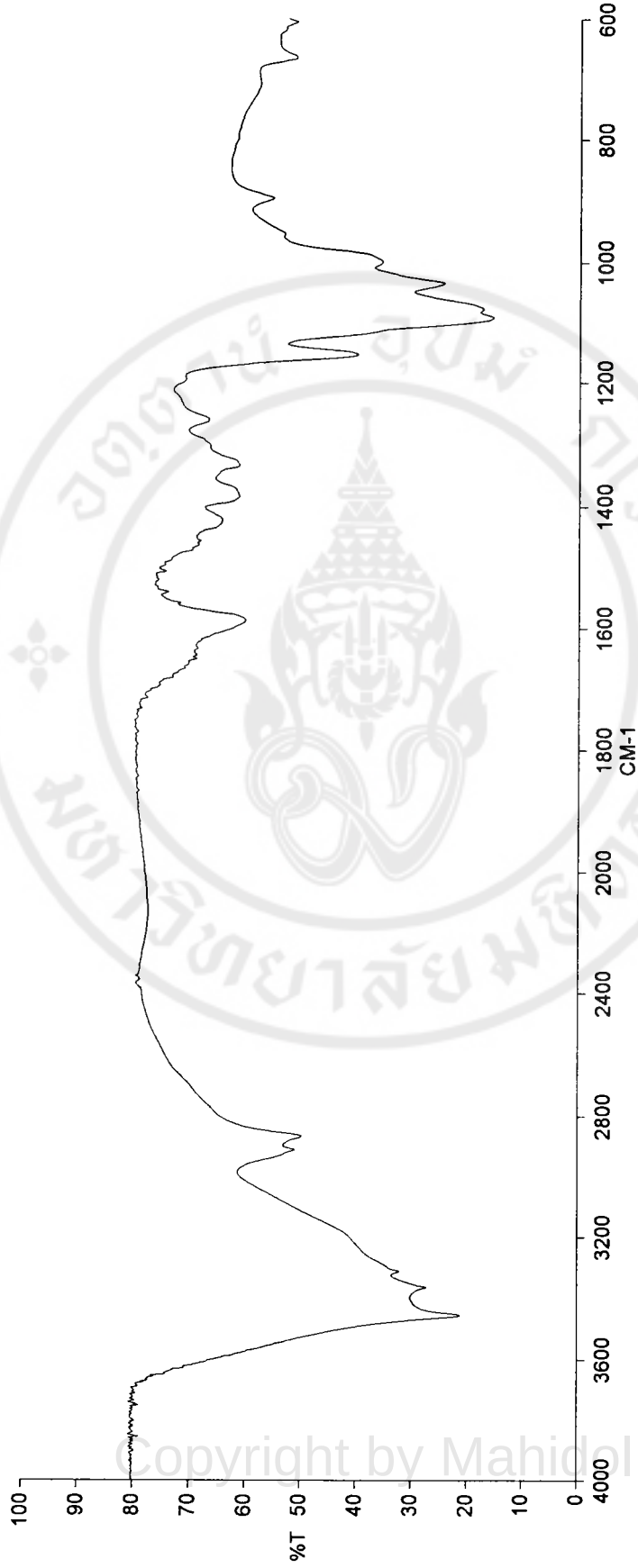


Figure 3.3 IR spectrum of chitosan (89 % deacetylation)

Table 3.2 Effect of reaction time on the degree of deacetylation of chitosan

sample	reaction time (hours)	% deacetylation [a]
chito-1	4 [b]	46
chito-2	2	89
chito-3	3	89
chito-4	4	91
chito-5	6	89
chito-6	8	92

(a) % deacetylation determined by IR spectroscopy

(b) Continuous reaction time without intermittently washing chitin with water

The chitin was treated in the form of flakes in the reaction process and deacetylation did not proceed homogeneously through the whole bulk of the sample piece. Some portion in the bulk of the sample was less deacetylated as proven by the fact that the product especially of low deacetylation (46%) contained some insoluble parts in dilute formic acid solution (section 3.1.1). Therefore intermittently washing the modified product could improve the degree of deacetylation up to 90 % or more. In addition, the effect of size and form of chitosan may also have on the degree of deacetylation in heterogeneous system reported by S.Mima et.al.[26]

It is shown in Figure 3.4 that the degree of deacetylation was 89 % after 2 hours of reaction time and it hardly increased even if the treatment time was extended to 8 hours.

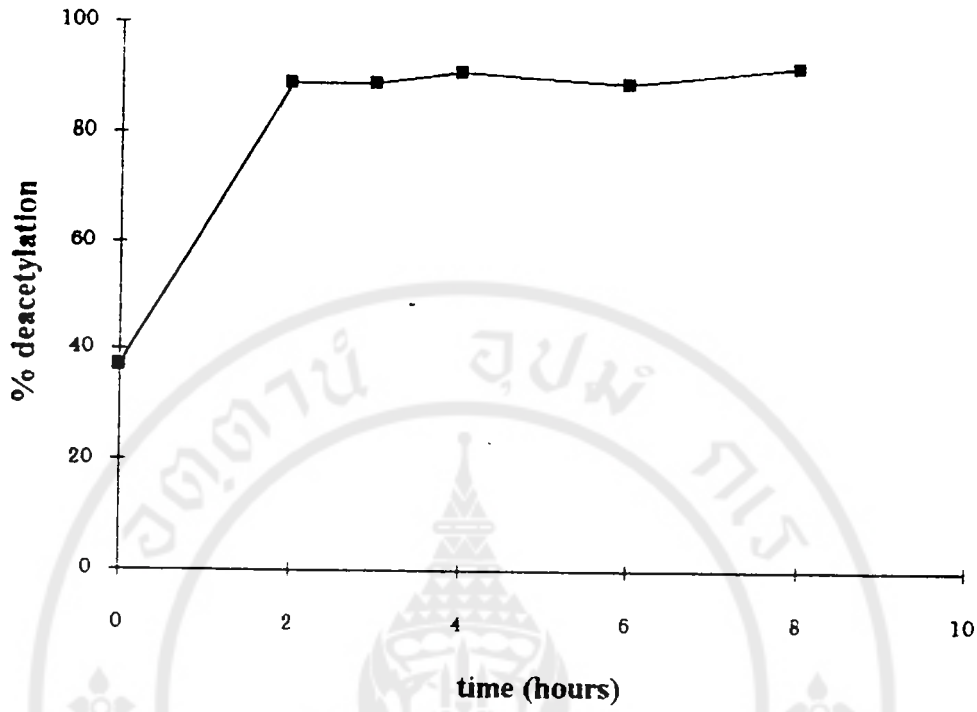


Figure 3.4 Plot of the reaction time and % deacetylation of chitosan

3.2 Preliminary study of preparation of water-soluble chitosan

The purpose of this thesis is to modify or functionalize chitosan for preparing iron-chelating polymer. Chitin and chitosan are water insoluble materials therefore the modification of these products in aqueous media are impossible. It has been reported that 50 % random N-deacetylated chitin is water-soluble. Attempt to prepare water soluble chitosan were carried out in the present experiment by three methods.

3.2.1 Hydrolysis of chitin under alkali solution

It has been mentioned in the literature that alkali treatment of chitin at 25 °C gave water soluble chitosan at various reaction times between 48 hours to 77 hours.[28] The treatment of alkali suspension of chitin with crushed ice gave a solution of alkali chitin. The clear solution

was left standing for 77 hours at 25 °C then neutralized with HCl and precipitated in acetone at temperature in the range of 0 to 5 °C to ensure the solubility in water because a small change in temperature brings about different solubility.

The pH of the solution was also strictly controlled to be 8.7 before pouring it into acetone/distilled water (7:1) at 0 °C in order to keep homogeneous solution without NaCl solid formation. The white precipitate was formed in a cold mixture of acetone/distilled water and it was washed several times with cold distilled water to remove NaCl residue. The filtrate was tested by using $AgNO_3$ solution. The precipitate was dried at 0 °C under vacuum. The FTIR spectrum of the product showed amide I absorption peak at 1655 cm^{-1}.

The solubility of the product in water was tested and it was found that the modified chitosan was partially water soluble which may be due to inappropriate degree of N-deacetylation as mention previously. The unsuccessful preparation of water-soluble chitosan by this method may arise from several factors such as the inefficient control of the temperature and pH during precipitation and recovering of the product. Furthermore this method required large quantity of solvent and long period of reaction time. Therfore, the method was thought to be unsuitable for preparation of water-soluble chitosan.

3.2.2 Hydrolysis of chitosan under acidic condition

Hydrolysis of chitosan with various acids including hydrochloric acid and nitrous acid were studied.[34] It was found that the reaction condition were so strong that low molecular weight chitosan with less than 20 degree of polymerization was obtained. Water soluble chitosan can be obtained by phosphoric acid hydrolysis

with low degree of polymerization.[35] When the chitosan symbolised as chito-s with 73 % deacetylation which was purchased from Sigma was added to 85 % phosphoric acid became completely soluble in the solution within 2 days, and the subsequent hydrolysis seemed to proceed under homogeneous condition. After 6 weeks, two fractions of modified chitosan, water-insoluble and water-soluble chitosan were obtained. The yields of the water-soluble fraction were approximately 10 % and its degree of deacetylation was found by IR analysis to increase from 73 to 83 %. Its solubility in water wastested and found that 0.5 g of chitosan was soluble in 1000 ml water which is seemed to be inpractical for further application.

3.2.3 N-acetylation of chitosan

It is known that the water-solubility of chitosan arises from the randomly distributed acetyl group at half the number of amino group in the main chain. K. Kurita et.al. prepared water-soluble chitosan by controlling the degree of deacetylation through N-acetylation reaction.[33] In our case, the acetylation reaction was therefore carried out with highly deacetylated chitosan under homogeneous or almost homogeneous conditions to achieve random substitution by dissolving the chitosan in 10 % aqueous acetic acid and acetic anhydride was used as an acylating agent. The modified product was analysed by IR spectroscopy.

It was found that the appearance of amide I band at 1655 cm^{-1} exhibited acylation reaction of chitosan. The solubility of the modified product in water was tested and it was found that the N-acylated chitosan was water-insoluble. Determination of the degree

of this product was found by IR technique to be 48 % deacetylation The amount of N-acetyl group in chitosan of the present experiment may in the same range as that of the water-soluble chitosan reported by K.Kurita et.al. but the water-insolubility was obtained.

The insolubility may be due to the distribution of N-acetyl groups. Although the method used was simpler, more efficient and less costly than the conventional method, it might fail to produce acetylated chitosan with about 50 % random substitution which exhibits water-solubility.

In conclusion for this part, it was not possible at this stage to synthesise water-soluble chitosan required as a starting material for further modification reaction as planned.

3.3 Determination of degree of deacetylation

As mentioned in the Introduction, the degree of deacetylation can be determined by several methods such as infrared spectroscopy, ultraviolet spectrophotometry, neclear magnetic resonance spectroscopy, hydrobromide salt titration and residual salicylaldehyde determination. However, each method has its own limitation depending on the N-acetyl content.

In order to find the best method suitable for our work, the chitosan prepared, symbolized as chito-p, and chitosan from Sigma, symbolized as chito-s, were analyzed by three techniques : hydrobromide salt titration, UV spectroscopy and IR spectroscopy.

3.3.1 Hydrobromide salt titration technique

This method was used to determine the amount of amino groups in chitosan by transformation of the amino groups to hydrobromide salt derivatives followed by titration of the salt with standard basic solution. Therefore, chitosan was first dissolved in 0.2 M HBr and then 9 M HBr was added to the solution with vigorous stirring to make the hydrobromide salt derivatives precipitated. The salt was filtered and washed with methanol until the precipitate was neutral and then washed with ether and air-dried.

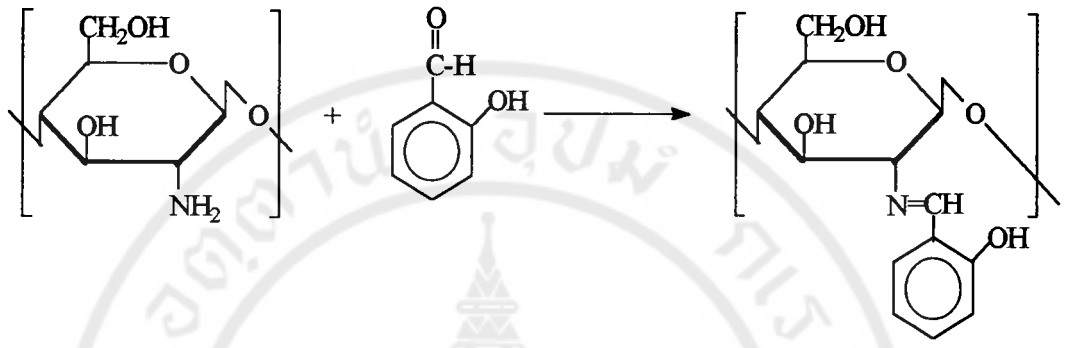
After drying the hydrobromide salt chitosan derivative, an accurately weighed portion of the salt was dissolved in distilled water then the solution was titrated with standard 0.1 M aqueous sodium hydroxide solution using phenolphthalein as the indicator. The quantity of N-acetyl groups or amino groups was then calculated.

The degree of N-deacetylation of chito-s and chito-p were found to be 66 % and 84 % respectively.

3.3.2 UV spectroscopy technique

As chitosan has active amino groups on its backbone, these amino groups can react with ketone or aldehyde to form alkylidene derivatives. It has been reported that salicylaldehyde can react with chitosan to form N-salicylidene chitosan product. Therefore the amount of residual salicylaldehyde used in the reaction can be converted to the amount of amino groups in the chitosan. Salicylaldehyde shows a significant absorption band in UV spectrophotometer at 255 nm as shown in Figure 1.8. The measurement of UV absorbance at 255 nm for

residual salicylaldehyde can ,therefore, be used to determine the degree of N-deacetylation of chitosan.



chitosan salicylaldehyde N-salicylidene chitosan

The degrees of N-deacetylation of chito-p and chito-s were determined by this method. An accurately weighed portion of chitosan was slurried for 48 hours in a 0.02 M solution of salicylaldehyde in methanol/1 % acetic acid (80/20,v/v). The volume of salicylaldehyde solution taken was dependent on the approximate extent of N-acetylation of the sample. After 48 hours the mixture was filtered and the filtrate was diluted 400 times. The UV absorbance at 255 nm of the filtrate was measured to determine the residual concentration of salicylaldehyde. The modified chitosan was yellow solid. The determination of salicylaldehyde content was obtained from the standard curve was shown in Figure 3.5. The degrees of deacetylation of chito-s and chito-p were found by this method to be 71 % and 83 %, respectively.

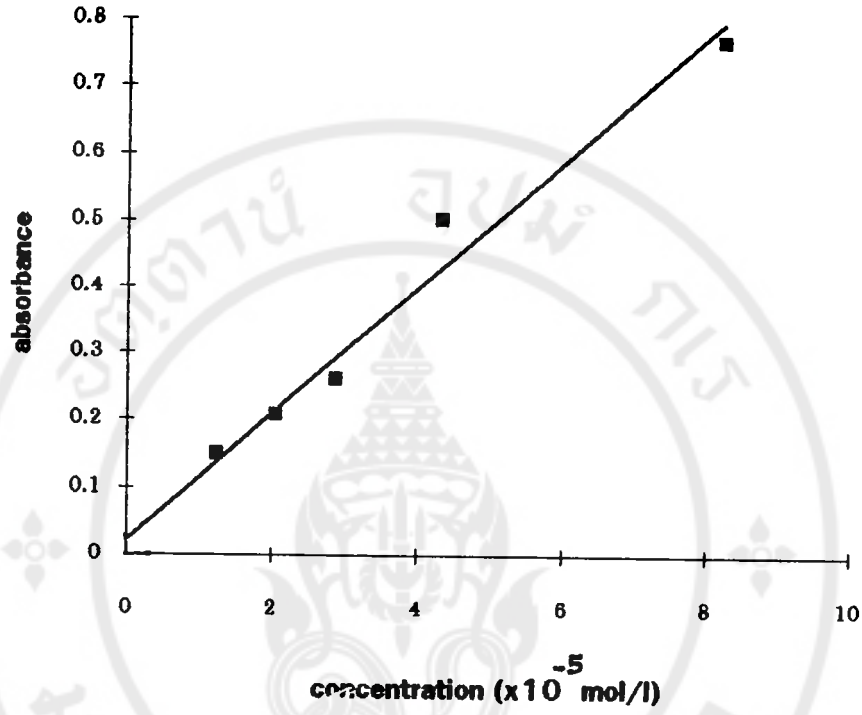


Figure 3.5 Standard curve of salicylaldehyde

3.3.3 Infrared spectroscopy technique

IR spectra of chitosans were recorded from the dried film by casting 5 % chitosan solution in 90 % aqueous formic acid or by KBr disc technique. The extent of N-acetylation of chitosan was determined from the absorbance of the amide I band at 1655 cm^{-1}. The 3450 cm^{-1} absorbance characteristic of hydroxy group of the sample was used as an internal standard to correct for the film thickness or for differences in chitosan concentration when the KBr disc technique was used. The degree of N-deacetylation of the sample was calculated from the following equation. (36)

$$\% \text{ N - deacetyl} = \left[1 - \left(\frac{A_{1655}}{A_{3450}} \times \frac{1}{1.33}\right)\right] \times 100$$

It was found that the degree of N-deacetylation of chito-s and chito-p were 73 % and 89 % respectively.

3.3.4 Comparison of the different techniques used for measurement of the degree of deactylation

The values of the degree of deacetylation obtained by IR spectroscopy, hydrobromide salt titration and UV spectrophotometry determination are shown in Table 3.3. Comparing the experimental time required for each technique, the hydrobromide salt titration and the UV spectrophotrometry methods require longer time than did the IR spectroscopy method and reagents were also required.

Table 3.3 % N-deacetyl content of chitosan samples determined by different methods

sample	% N-deacetyl content determined by		
	IR spectroscopy	HBr salt titration	UV spectroscopy
chito-s	73	66	71
chito-p	89	84	83

For these reasons, IR spectroscopy seemed to be the most suitable technique. Furthermore, all amino groups could be detected by the absorption peak at 1590 cm^{-1} whereas the determination of

N-acetylation can also be clearly evaluated by amide I band at 1655 cm^{-1}. The basis of the titration method of hydrobromide salt is due to the amount of amino groups that react with hydrobromic acid. When highly deacetylated chitosan is used, several treatments of the sample with hydrobromic acid is required, therefore, there could be error in the amount of titration afterword if the protonation is not complete. It was found that IR technique gives higher degree of N-deacetylation of chito-p and chito-s than HBr salt titration technique.

The same results were obtained when the comparison between IR technique and UV spectrophotrometry was made. The reason for the later technique giving smaller degree of deacetylation might be the same as that occurred in HBr salt titration i.e. there was a limitation to the N-salicylidene chitosan formation. In the highly crystalline chito-p sample, only the amino groups on the surface of the crystallites are accessible to react with salicylaldehyde while those within the crystalline structure are inaccessible. Infrared spectroscopy, on the other hand, can detect all amino groups. So there is significant difference between infrared technique and UV spectroscopy.

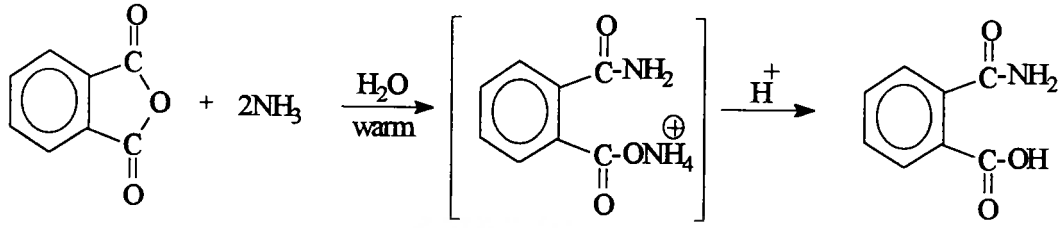
A routine technique that measures a wide range of N-deacetyl contents of chitosan is needed. Infrared spectroscopy fulfills these requirements. Furthermore, in view of the speed and ease of application of this technique, it is the most suitable method of choice in our work. Therefore, IR technique had been used in this thesis to determine degree of deacetylation of chitin or chitosan.

3.4 Functionalization of chitosan

Chemical modification of a polymer especially naturally existing polymer to make it high value added is appropriate. Chitosan which can be obtained from naturally abundant chitin has reactive amino group suitable for further modification. In order to modify it as an iron-chelating polymer, specific functional groups are to be selected. As mentioned earlier that hydroxamate, catecholate and silicylate anions are chelators for iron because of the ability to form five or six coordinate complex with iron (III). Therefore a molecule to be fixed at amino groups in chitosan molecular chain should provide such ligands. Phthalic anhydride, benzoyl chloride and salicylic acid were used to functionalise chitosan as discussed here. As water-soluble chitosan could not be prepared, highly deacetylated chitosan which is soluble in formic acid and acetic acid were used in this part.

3.4.1 Reaction of phthalic anhydride with chitosan

It has been known in organic synthesis that ammonia or amine can react with cyclic anhydride derivative to form imide compound.[74]



Our attempt has been focused on using phathalic anhydride to react with amino groups in chitosan in order to obtain a compound that is both an amide and an acid. However if phthalimido chitosan is obtained, the acid hydrolysis was expected to be carried out for ring opening reaction of the modified chitosan as shown below as six-coordinate complex was required.

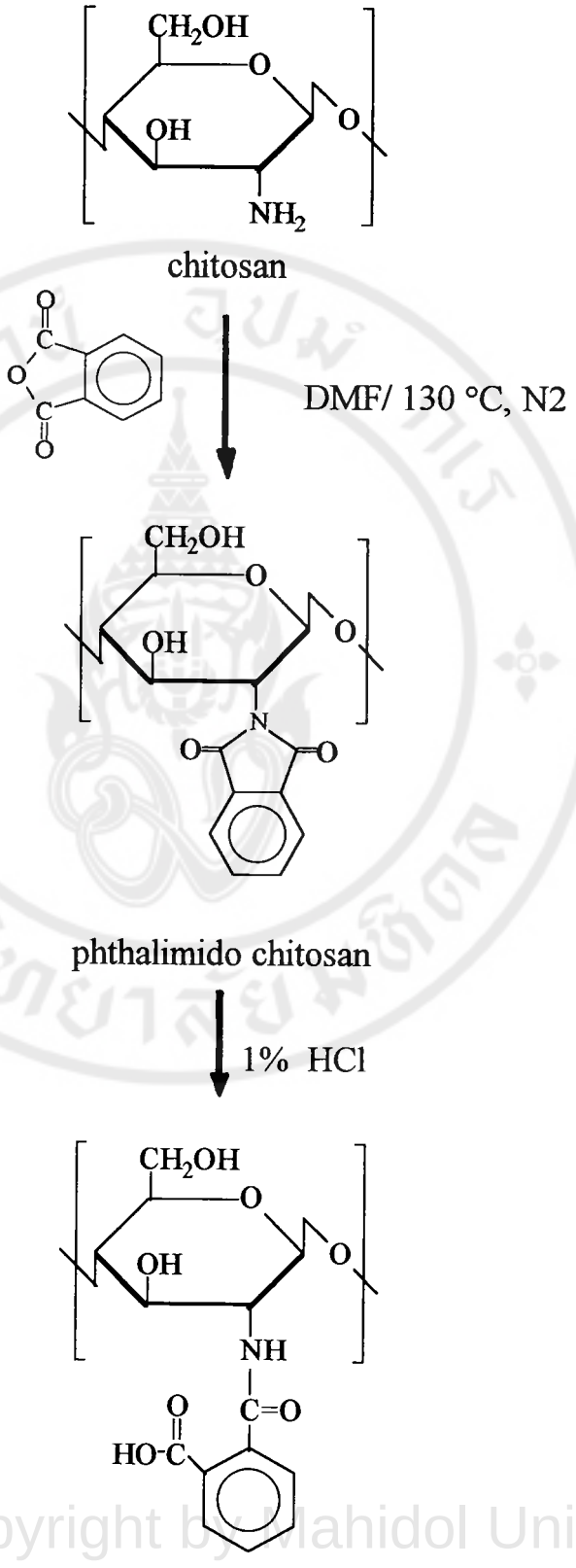


Figure 3.6 Reaction of phthalic anhydride with chitosan

The reaction between phthalic anhydride and chitosan (chito-p) was carried out in DMF at 130°C. The product obtained was brown solid and it was analysed by FTIR spectroscopy. It was found that two characteristic absorption peaks of phthalimido group appears at 1770 and 1710 cm^{-1} (Figure 3.7) as expected. As chitosan has also polyhydroxy groups along the chains but it was not found any peak in IR absorption of o-acylation from the reaction of hydroxy and phthalic anhydride.

Selectivity of the reaction at amino groups of chitosan have been studied and also reported.[53,54] Selectively N-substituted chitosans are formed by reaction with phthalic and related anhydrides with highly swollen precipitated chitosan in pyridine-dimethylacetamide. The product that remains in the swollen state has probably undergone preferential N-acylation with some o-acylation as shown in Figure 3.8. However, heat treatment leads to imidization from amic acids and loss of o-acyl substituents through regeneration of acid anhydrides.

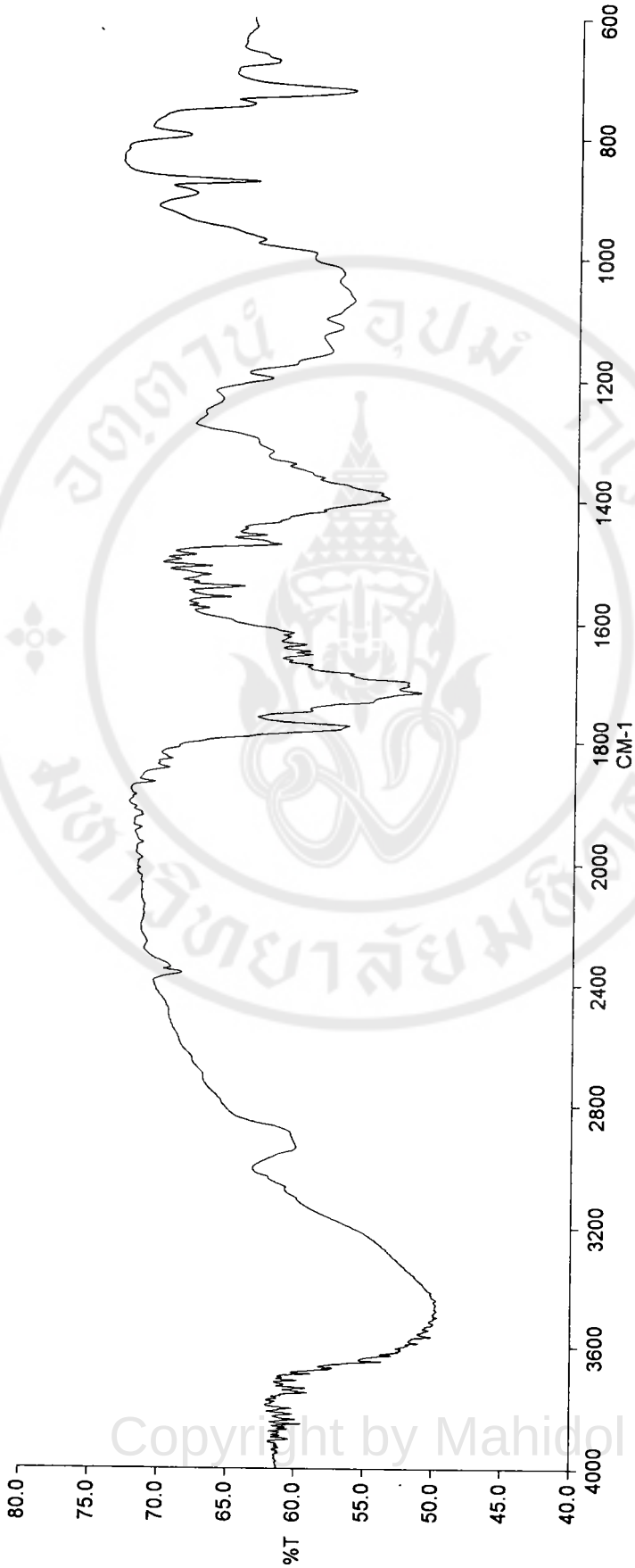


Figure 3.7 IR spectrum of phthalimido chitosan

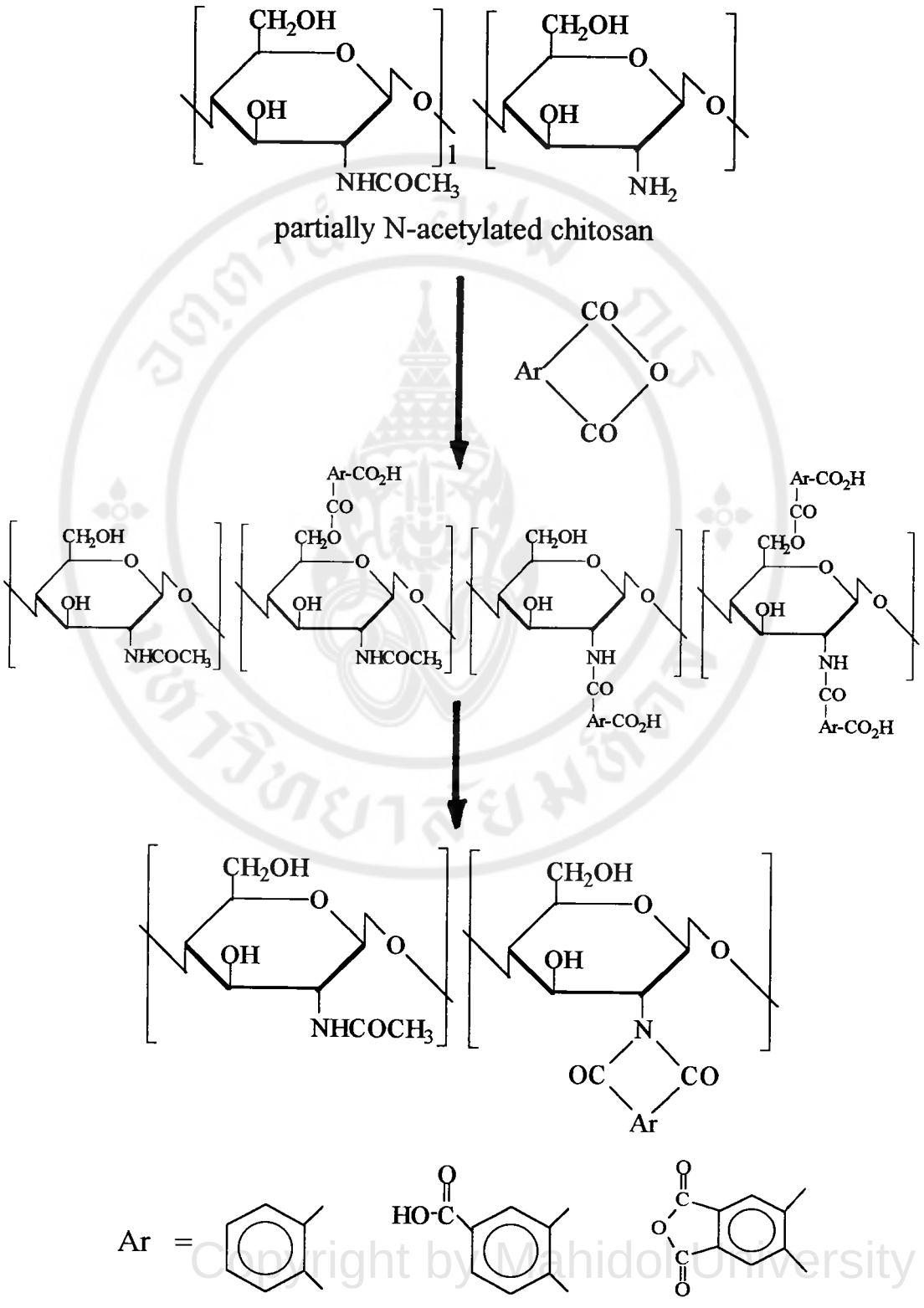
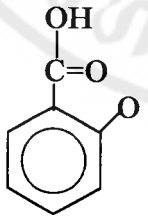


Figure 3.8 Reaction of chitosan with cyclic acid anhydrides and subsequent imidization

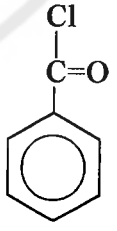
Then the modified product was treated with 1% HCl under reflux for 5 hours. The resulting chitosan was obtained and characterized by FTIR spectroscopy. No difference between the IR spectrum of phthalido chitosan and the hydrolysis one is shown in Figure 3.9. It can be concluded ring opening reaction was not occurred and our expected product could not be obtained.

3.4.2 Reaction of benzoyl chloride with chitosan

As mentioned in previous chapter that salicylic acid was chosen as the iron(III)-specific chelating agent for modifying amine groups in chitosan. The chemical structure of salicylic acid consists of two types of functional group ; carboxylic acid and hydroxy functions. Therefore it is recommended to study the reaction condition of chitosan with simple molecule such as benzoyl chloride.

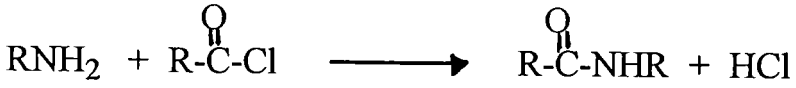


Salicylic acid



Benzoyl chloride

Reaction between amine and acid chloride derivative has been known active to produce amide compound under high temperature. This reaction can be catalyzed by base such as pyridine.



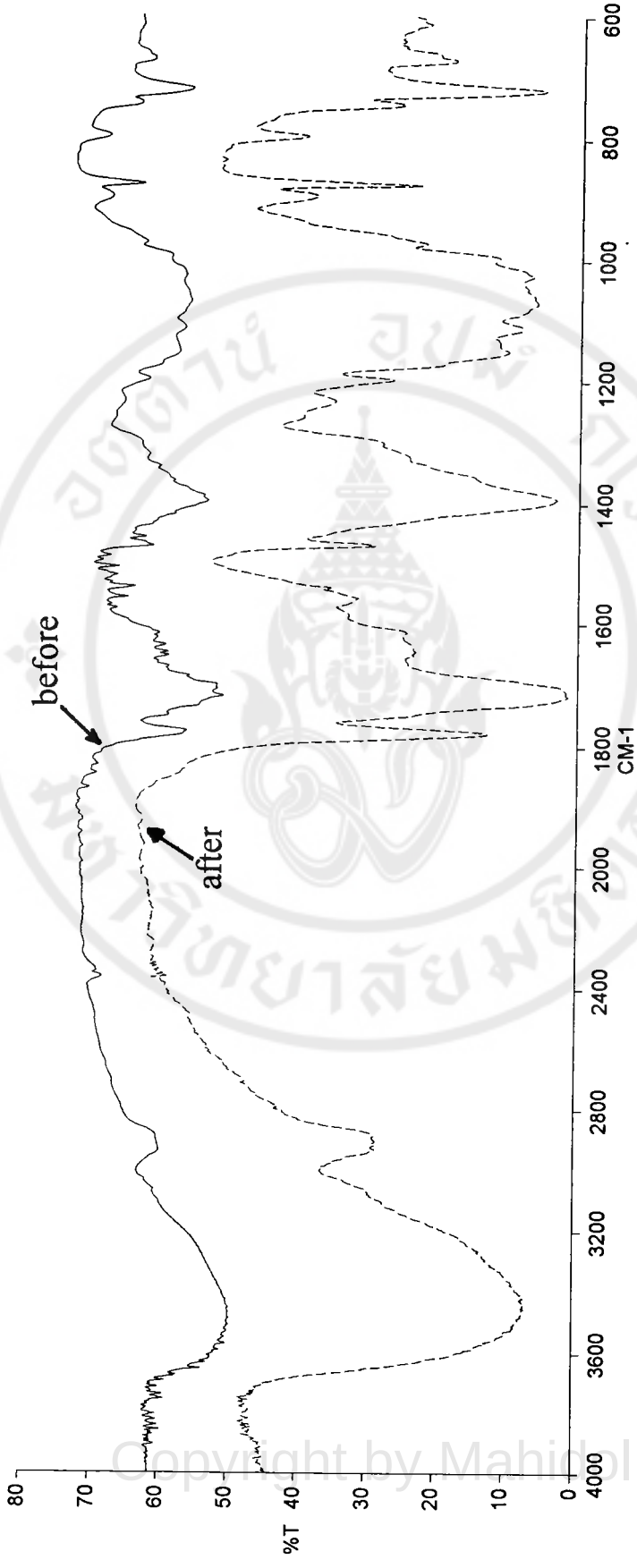


Figure 3.9 IR spectrum of phthalimido chitosan before and after acid hydrolysis

As chitosan is in solid flake form and insoluble in organic solvent. It is needed to soak chitosan in pyridine at least three days before the reaction to allow the pyridine to fully penetrate the polysaccharide structure. It was an important step in the formation of the product,since it acted as proton acceptor in the reaction, and thus facilitated acylation. The experimental procedure was interrupted every six hours and restarted with the addition of fresh acyl chloride, pyridine and chloroform. It was reported by S.Grant et.al.[24] that this type was more effective than the continuous one, with the occasional addition of fresh acyl chloride. The reaction scheme is shown below :

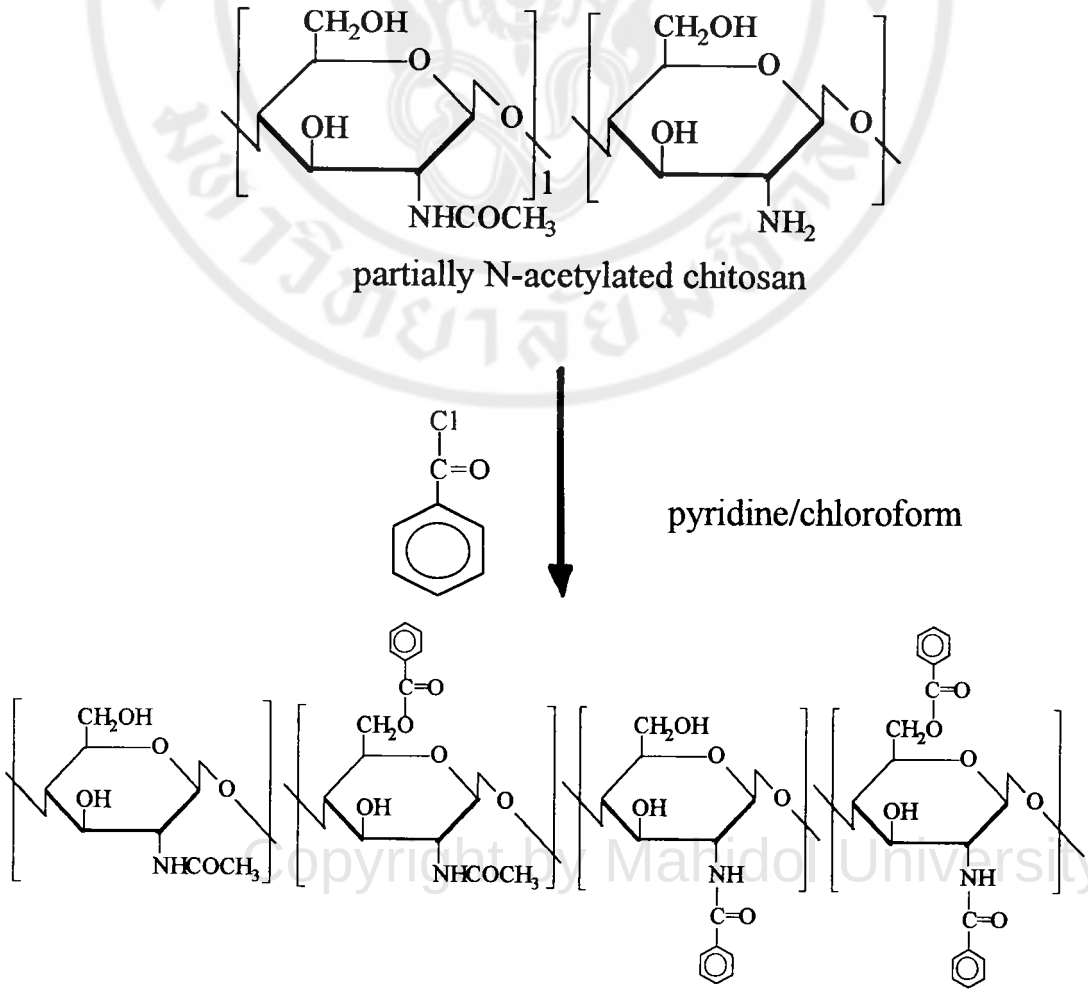


Figure 3.10 Reaction of benzoyl chloride with chitosan

The light brown solid was obtained and characterized by IR spectroscopy. The IR spectrum of the modified product is shown in Figure 3.11 and assignment of the peaks summerized in Table 3.4. From this spectrum,there was also a new peak at 1730 cm^{-1} which is indicative of an ester group, thus confirming o-acylation reaction. And the C-O stretching peaks of ester was noticed from the bands at 1270 and 1110 cm^{-1}. The peaks at 1645 and 1540 cm^{-1}can be attributed to the NH of the amide. Characteristic absorptions at 1600, 1580, 1500, 1450 cm^{-1}due to C=C stretching vibration of aromatic ring and the monosubstituted phenyl groups are observed at 800, 710 cm^{-1} in the spectrum.

The DSC thermogram for benzoylated chitosan is shown in Figure 3A (see Appendix). Its two exothermic decomposition peaks occuring at 217 and 240 °C which are lower than that of chitosan occuring about 316 °C. The data showed that this derivative is less thermally stable than chitosan possibly because of the disruption of the crystal structure of the substituted product. This product was light brown in colour and did not dissolve in organic solvents such as DMSO chloroform and DMF. The results obtained indicated that N-salicyloyl chitosan could be prepared from chitosan in heterogeneous system.

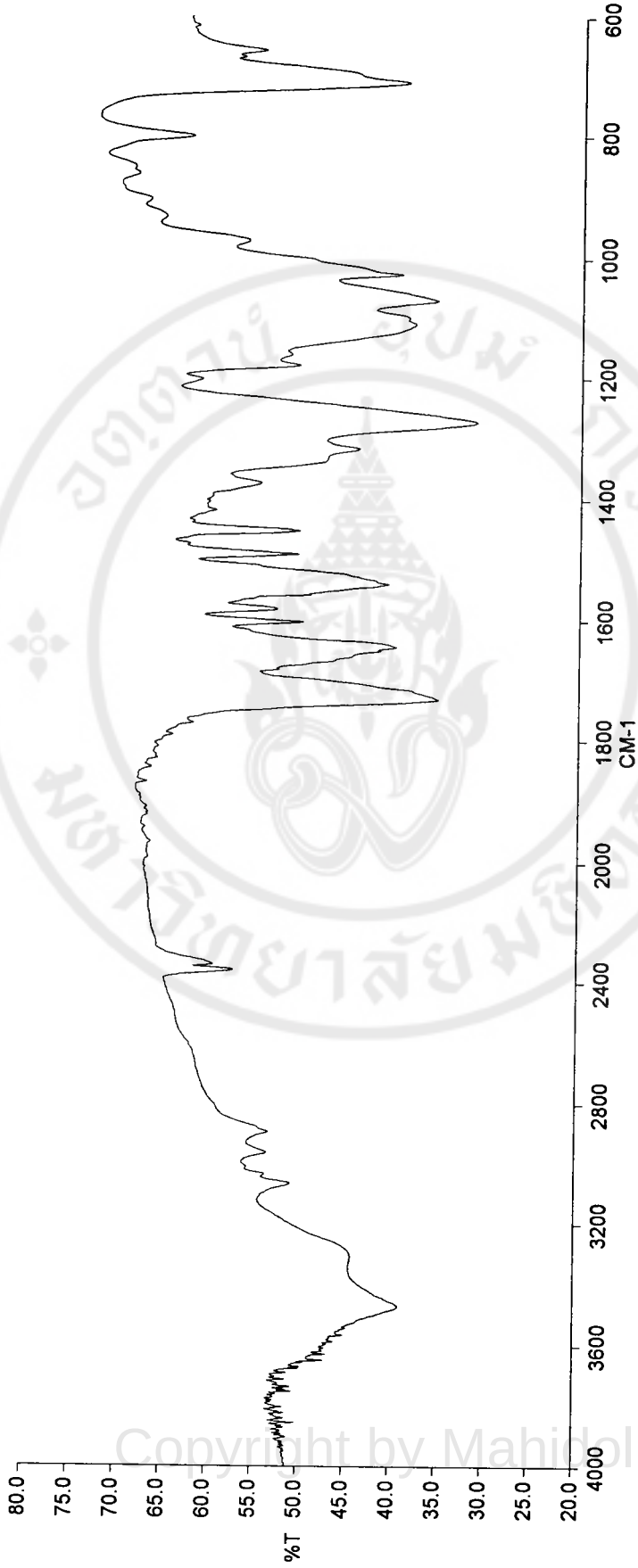


Figure 3.11 IR spectrum of benzoylated chitosan

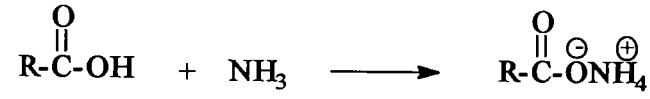
Table 3.4 Major IR absorption bands of benzoylated chitosan

Frequency (cm^{-1})	Assignment
1730	C=O stretching of ester
1270,1110	C-O stetching of ester
1645	amide I (C=O)
1540	amide II (NH deformation)
1600,1580,1500, 1450	C=C streching of aromatic ring
800,710	C-H deformation of monosubstituted phenyl group

3.4.3 Reaction of salicylic acid with chitosan

The chelating ability of chitosan for metal ions can be enchanced by reacting the amino groups of this polymer with suitable chemicals. Various chemical derivatives of chitosan have been reviewed in the introduction part. In our work, salicylic acid was chosen as the reagent to produce a novel iron chelating polymer through the reaction between salicylic acid and amino group to produce amide.

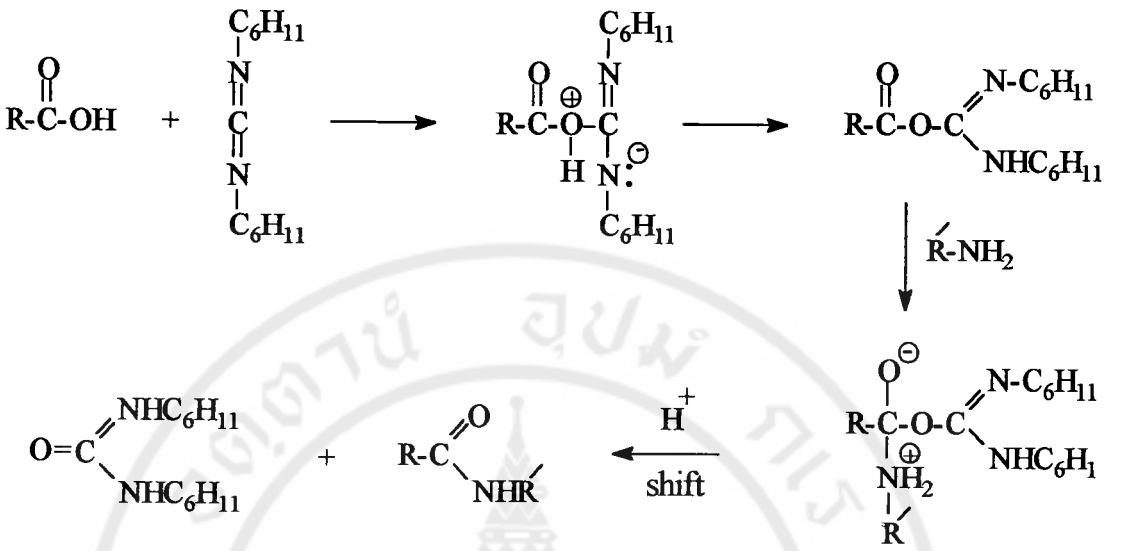
Aqueous ammonia reacts with carboxylic acid to form ammonium salts. When water is evaporated, dehydration produces an amide.



an ammonium carboxylate

Because of the low reactivity of the carboxylate ion toward nucleophilic substitution, amide formation from the reaction between carboxylic acid and amino compound does not usually take place. A better method is to convert the acid to an acyl chloride before treatment with ammonia or amine. It has been known that hydroxy group of carboxylic acid can be converted to a more reactive leaving group under acidic condition which is usually carried out for ester synthesis.

A new mild condition for amide synthesis was studied by using an activating agent such as dicyclohexylcarbodiimide, C_6H_{11}-N=C=N-C_6H_{11} (DCC). It has been found that DCC promotes amide formation by reacting with the carboxyl groups of an acid and activating it toward nucleophilic substitution. (74)



N,N-Dicyclohexylurea an amide

In order to find the condition for the preparation of N-salicyloyl chitosan, many systems were investigated. The previous study of synthesis of benzoylated chitosan illustrated that under heterogeneous condition substitution of bulky groups took place at the amine and alcohol groups. Such treatment of acid chloride with amines does not directly give amides but also ester groups. In the case of salicylic acid, heterogeneous and homogeneous conditions were carried out.

(i) Heterogeneous condition

Many heterogeneous conditions were investigated in an attempt to prepare the desired chitosan derivatives. First of all chitosan flake was put to react with salicylic acid in acetone under acid condition at room temperature and under reflux. The product obtained was analyzed by FTIR spectroscopy. No difference was found in the IR spectra of the obtained products compared to the starting chitosan.

The reaction between salicylic acid and chitosan which was suspendend in pyridine was carried out in the presence of DCC dissloved in chloroform. The experiment was carried out under reflux. The results from IR spectra of the products obtained did not show any amide peak which could indicate the amide formation. Chitosan flakes were again reacted with salicylic acid in the presence of DCC by using dichloromethane as the solvent. All of the foregoing experimental evidence from IR spectroscopy indicated that despite the activating agent used, the conditions used did not afford the required derivatives.

Two factors could be resposible for the inability of the amine groups in chitosan to react with salicylic acid under heterogeneous conditions. The first is intermolecular steric hindrance arising from too close packing of the polymer chains which results in a lack of accessibility of segments of the polymer chain to the reagent. The second is the low reactivity of salicylic acid compared with benzoyl chloride.

(ii) Homogeneous condition

Because of the problem of inhomogeniety of the reaction mixture, it was necessary to find more suitable reaction media. Acetic acid was found to be a common solvent for chitosan.

Therefore, 10 % acetic acid was used to dissolve chitosan. The solution was then diluted with water. Salicylic acid and DCC in 60 ml of DMF were added to the chitosan solution. After refluxing for 20 hours, the mixture was filtered and the filtrate was neutralized with saturated aqueous $NaHCO_3$. The modified product was a pale solid and its structure was determined by IR spectroscopy. (see Figure 3.12 and Table 3.5)

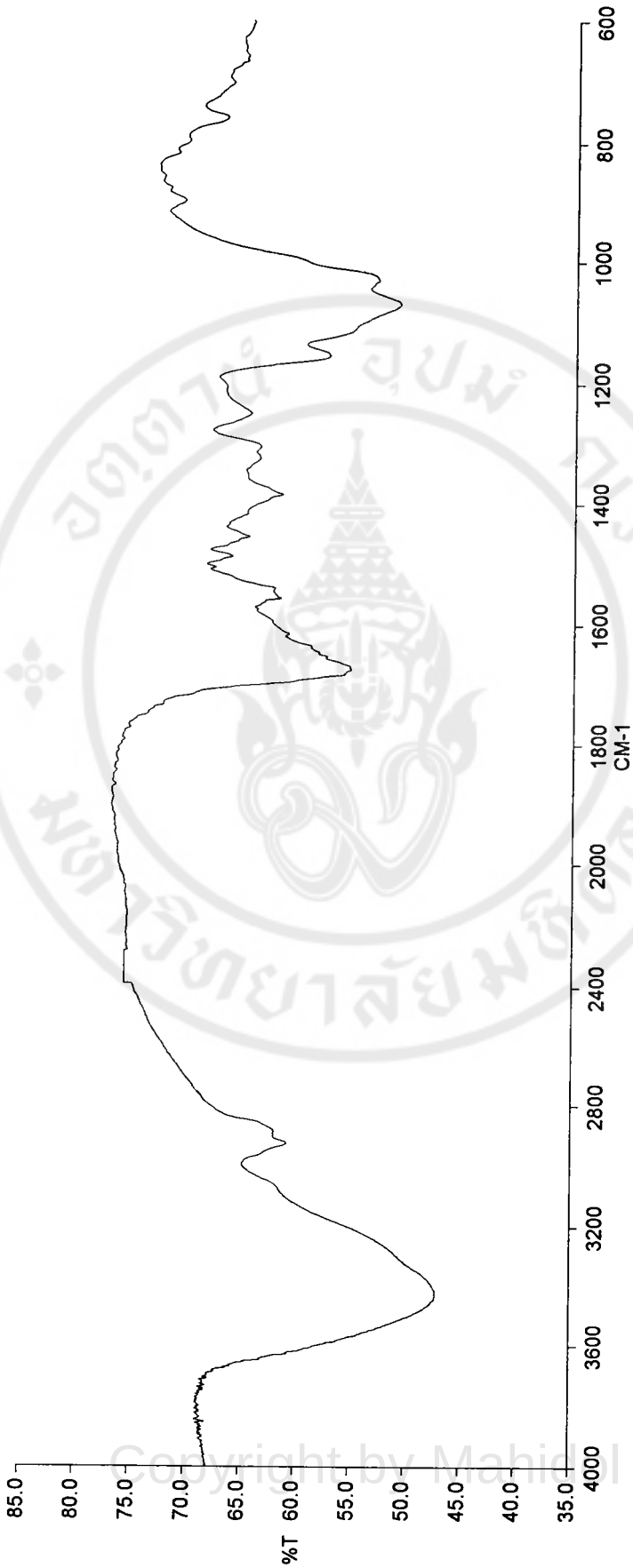


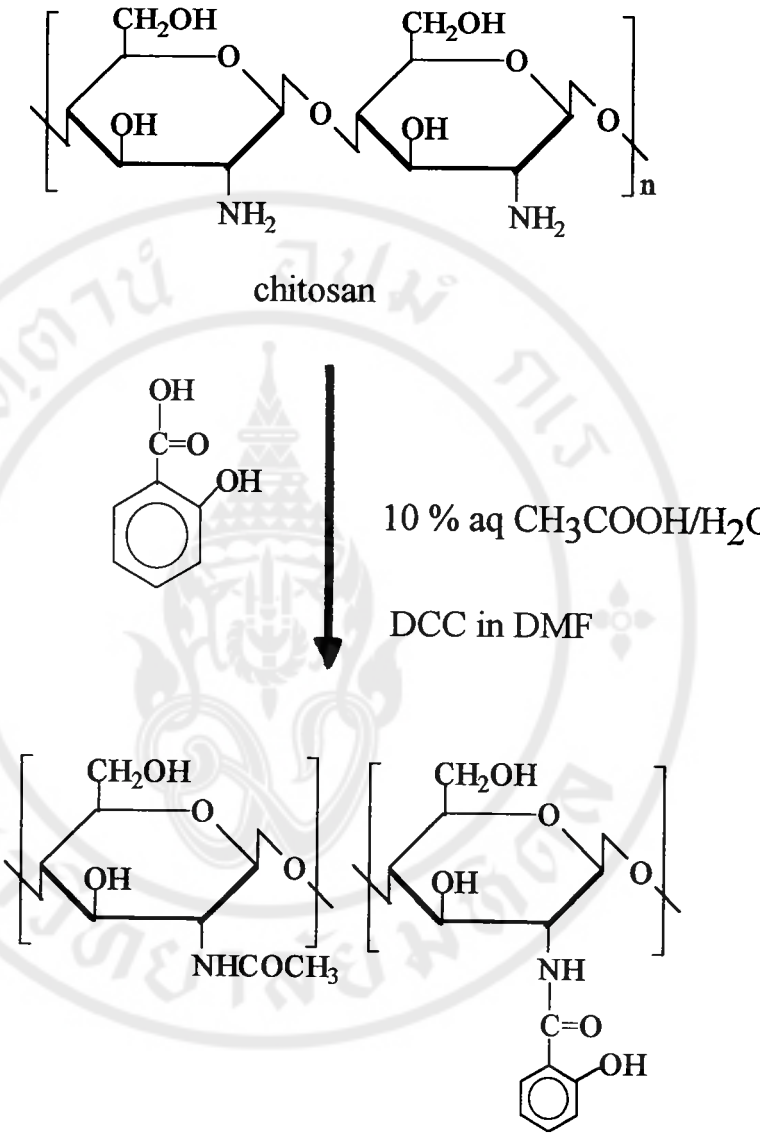
Figure 3.12 IR spectrum of N-salicyloyl chitosan

Table 3.5 Major IR absorption bands of N-salicyloyl chitosan

Frequency (cm^{-1})	Assignment
1670	amide I (C=O stretch)
1560	amide II (NH deformation)
760	C-H deformation of disubstituted phenyl group

The IR spectrum of the modified chitosan showed the appearance of extra three peaks at 1670, 1560 and 760 cm^{-1} compared with that of unmodified chitosan. The peak at 760 cm^{-1} can be assigned to the disubstituted benzene ring. The absorbance of the amide bands at 1670 and 1560 cm^{-1} increased but no peak appeared in the region of the ester band. Thus the chemical modification of chitosan with salicylic acid using DCC resulted only in N-substitution. Figure 4A (see Appenaix) shows the DSC thermogram obtained for this derivative which differed significantly from those of the starting materials, chitosan, and chitin.

The results obtained demonstrated that reaction between chitosan and salicylic acid took place under the condition used. Intermolecular steric hindrance was not a factor in preventing the reaction to take place in solution. However there are two types of acids, acetic acid and salicylic acid, present in the system. The former acid was used to dissolve chitosan and the second acid used as the required reagent. Therefore, acetic acid, the molecules of which is smaller than salicylic acid molecule, could react with amine groups of chitosan, as shown below :



N-salicyloyl chitosan

Figure 3.13 Reaction of salicylic acid with chitosan in the presence of DCC

The mechanism of the reaction of carboxylic acids with amine mediated by DCC has much in common with the nucleophilic catalysis mechanism. The acid is converted to a compound with a better leaving group and this intermediate is then attacked by another molecule

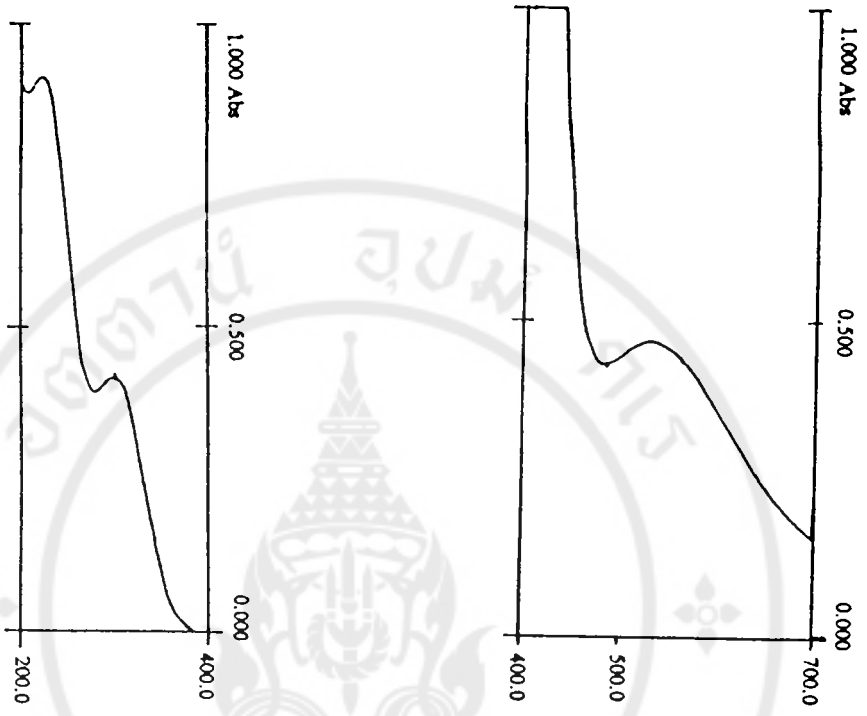
of $RCOO^-$ to give the anhydride, $(CO)_2O$, which is the actual acylating agent in the amine. In contrast to other reactions for carboxyl activation involving mixed anhydride formation, the reaction is not sensitive to moisture. Indeed, it can be carried out in aqueous solution.

3.5 The study of iron-chelation behavior

In section 3.4.3 it was shown that N-salicyloyl chitosan could be synthesized under homogeneous condition. Therefore additional periment was conducted to study the iron binding capability of this derivative.

Iron(III) binding was determined in a batch method by stirring samples of chitin, chitosan and N-salicyloyl chitosan in an aqueous solution of $FeCl_3$ for 24 hours to attain equilibrium. The pH of the solution containing $FeCl_3$ was controlled to be less than or equal to 3 to avoid precipitation of $Fe(OH)_3$. After 24 hours, the solid product was separated by filtration and the filtrate containing iron remaining in solution was determined by UV-Vis spectrophotometry.

Salicylic acid can form violet complexes with ferric ion which can be detected by UV spectrophotometry. It was found that Fe(III) has its λ_{max} at 222 and 303 nm, while Fe(III)-salicylate complex has its λ_{max} at 525 nm.



Fe (III) Fe(III)-salicylate complex

Figure 3.14 UV spectra of $FeCl_3$ and Fe(III)-salicylate complex

Measurements of residual iron in the three filtrates by UV spectrophotometry were carried out. It was found that there was no difference in the UV spectra of $FeCl_3$ solution both before and after adding samples. This may be due to the sensitivity of this technique is not great enough. It has been reported that[40] photoacoustic spectrometry (PAS) could be used to obtain UV-Vis spectra of iron(III) bound to solid samples of modified polymer supports. Based on the λ_{max} values determined by UV-Vis-PAS on samples, an interpretation of the mode of iron(III) binding as a mono,bis or tris chelate complex is made. However, this equipment was not available in the laboratory. Thus the physical characteristic can only be obtained visually. The chelating ability

of chitin, chitosan and N-salicyloyl chitosan with ferric ion is quite evident from their physical characteristics, as presented in Table 3.6.

Table 3.6 Physical characteristic of products obtained from reaction between $FeCl_3$ and chitin, chitosan and N-salicyloyl chitosan

Sample	Products obtained	
	before reaction	after reaction
$FeCl_3$-chitin	light brown flake	light brown flake
$FeCl_3$-chitosan	white flake	white flake
$FeCl_3$-N-salicylyol chitosan	pale brown powder	violet powder

The N-salicylyol chitosan gave a violet-coloured powder when the iron was added to this derivative whilst chitin and chitosan under the same condition showed no apparent change in colour. Although salicylic acid itself forms violet complex with ferric iron, excess acid was completely removed by washing several times with methanol and the filtrate was analysed by UV spectrophotometry. Since the violet colour is a well known characteristic of complex formation between salicylic anion and iron(III), it was believed that N-salicyloyl chitosan could be prepared and its binding of iron(III) demonstrated.

CHAPTER IV
CONCLUSION

1. Highly deacetylated chitosan (chito-p), 89 % deacetylation, could be prepared by treatment of chitin with 47 % sodium hydroxide solution at 110°C with intermittent washing during the reaction every hour.
2. Preliminary study of preparation of water-soluble chitosan was studied and found that water-soluble chitosan could not be synthesised.
3. Studies of the determination of the degree of deacetylation was studied by three different methods, hydrobromide salttitration,UV spectrophotrometry and infrared spectroscopy and showed that infrared spectroscopy was the most practical method.
4. Phthalimido chitosan and benzoylated chitosan could be synthesized under heterogeneous reaction but in latter case, N,O-acylation was also formed. This modified chitosan was less thermally stable than chitosan.
5. N-salicyloyl chitosan could be modified from chitosan by reacting chitosan with salicylic acid under homogeneous condition and only N-substituted chitosan was formed using DCC as an activating agent. But as the media used was acetic acid, N-acetylation with acetic acid also occurred. It was found that the product would act as iron chelating polymer.

SUGGESTIONS FOR FURTHER WORK

1. It should attempt to prepare water-soluble chitosan by controlling several factors which influence water solubility of product.
2. Study of the suitable condition for preparing N-salicyloyl chitosan by varying reaction time, temperature and amount of reagent to obtain high yield and high degree of modification should be made.
3. Iron binding of derivatives should be detected by other methods which give high sensitivity such as atomic absorption spectroscopy.

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APPENDIX

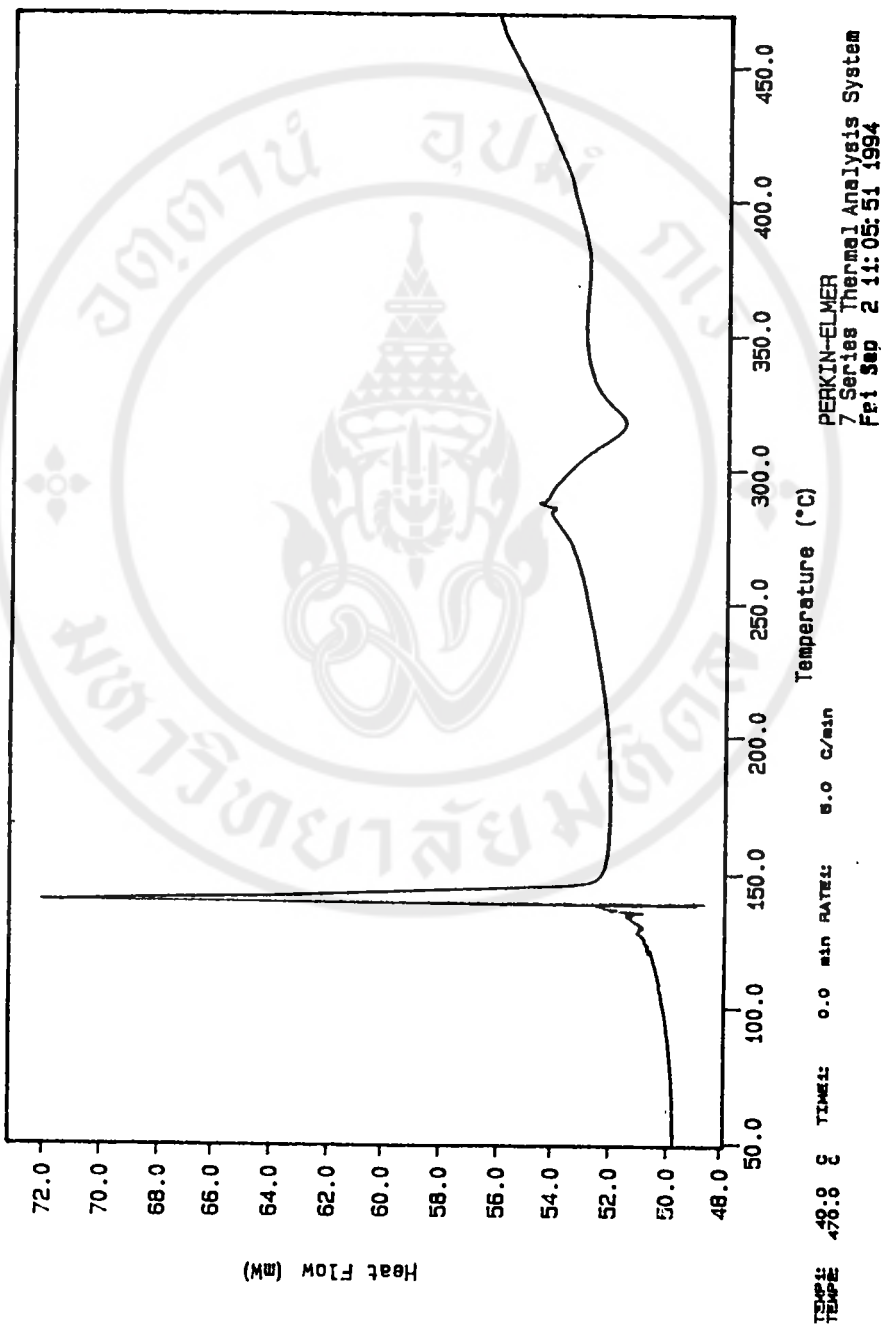

Figure 1A DSC thermogram of chitosan

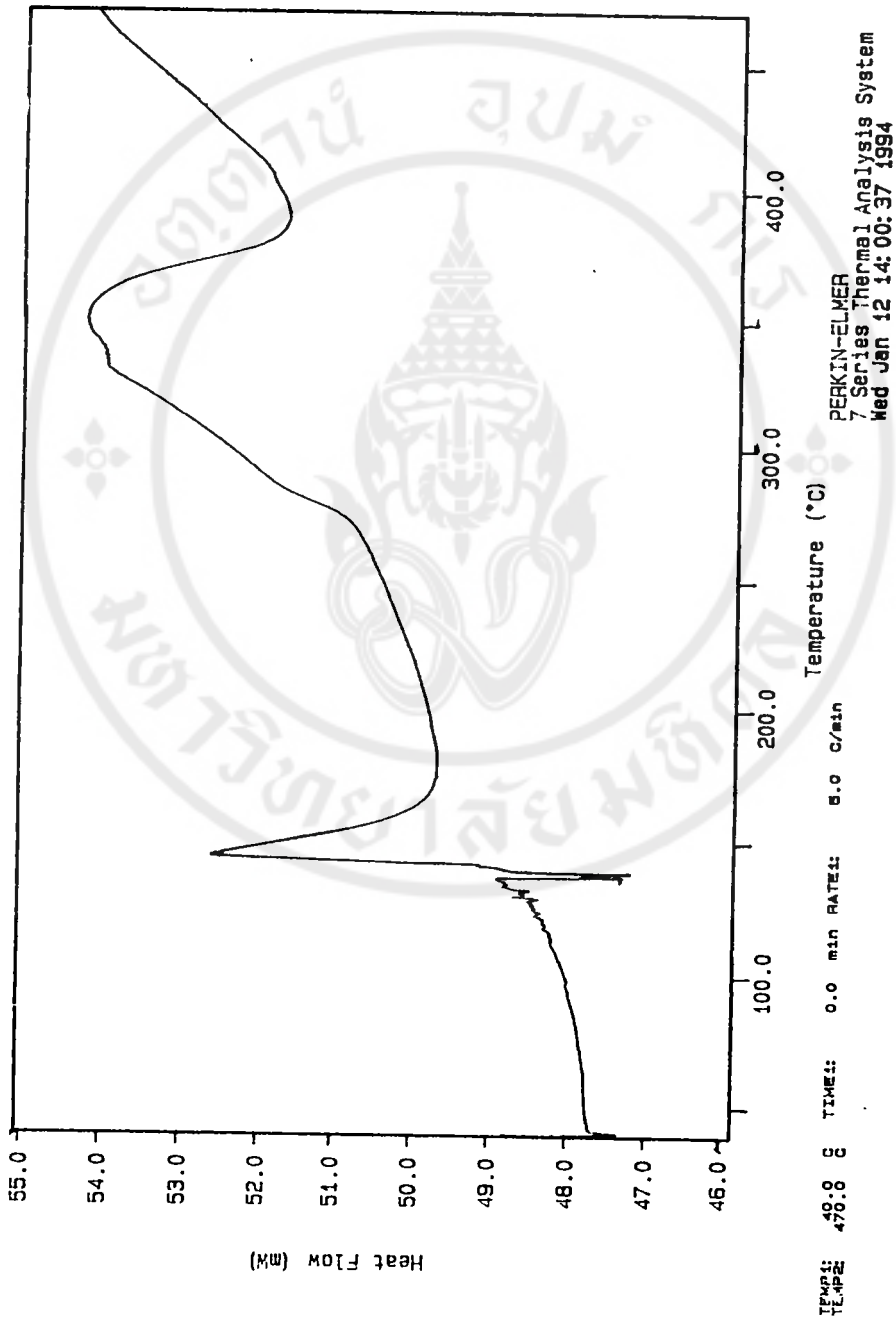

Figure 2A DSC thermogram of chitin

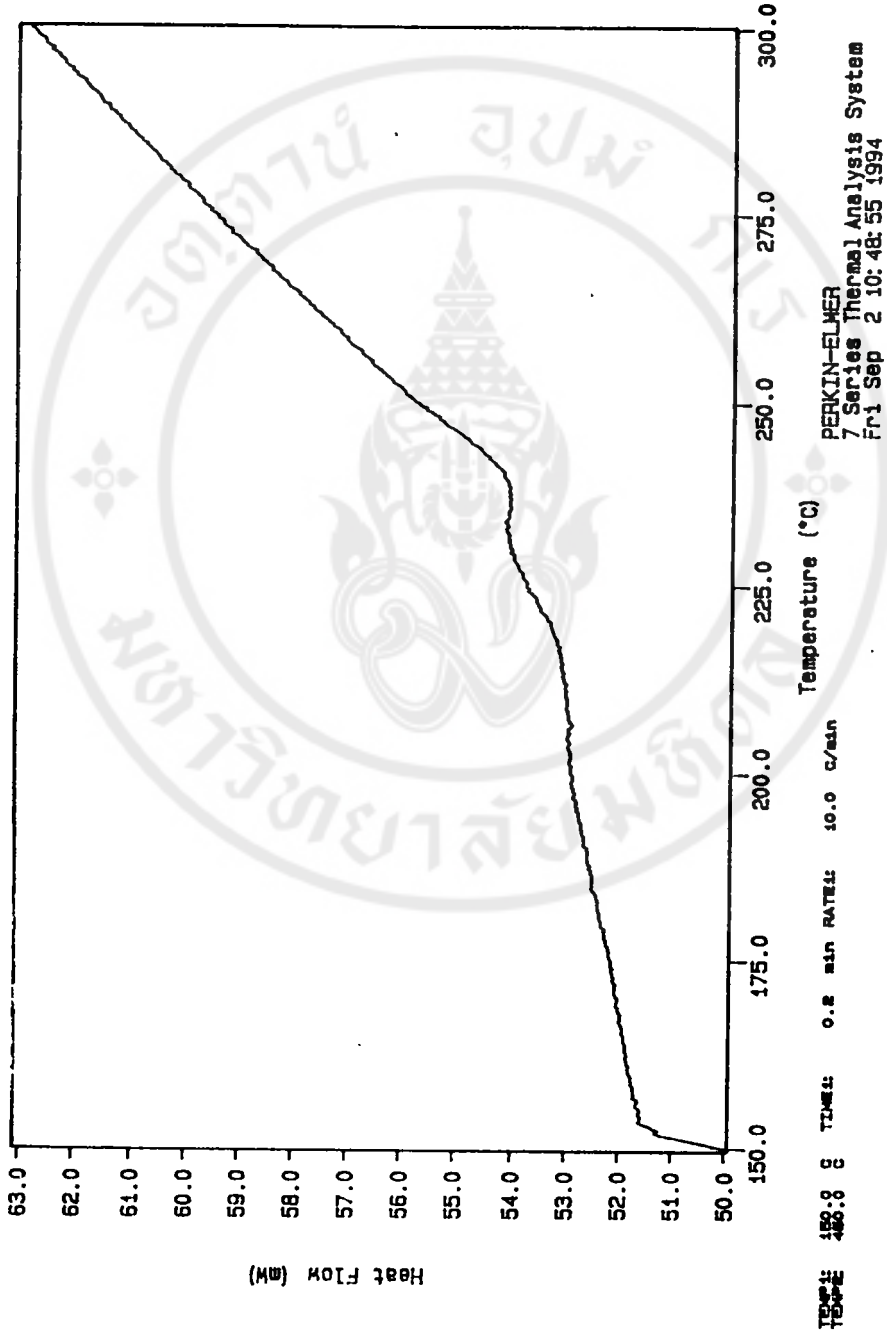

Figure 3A DSC thermogram of benzoylated chitosan

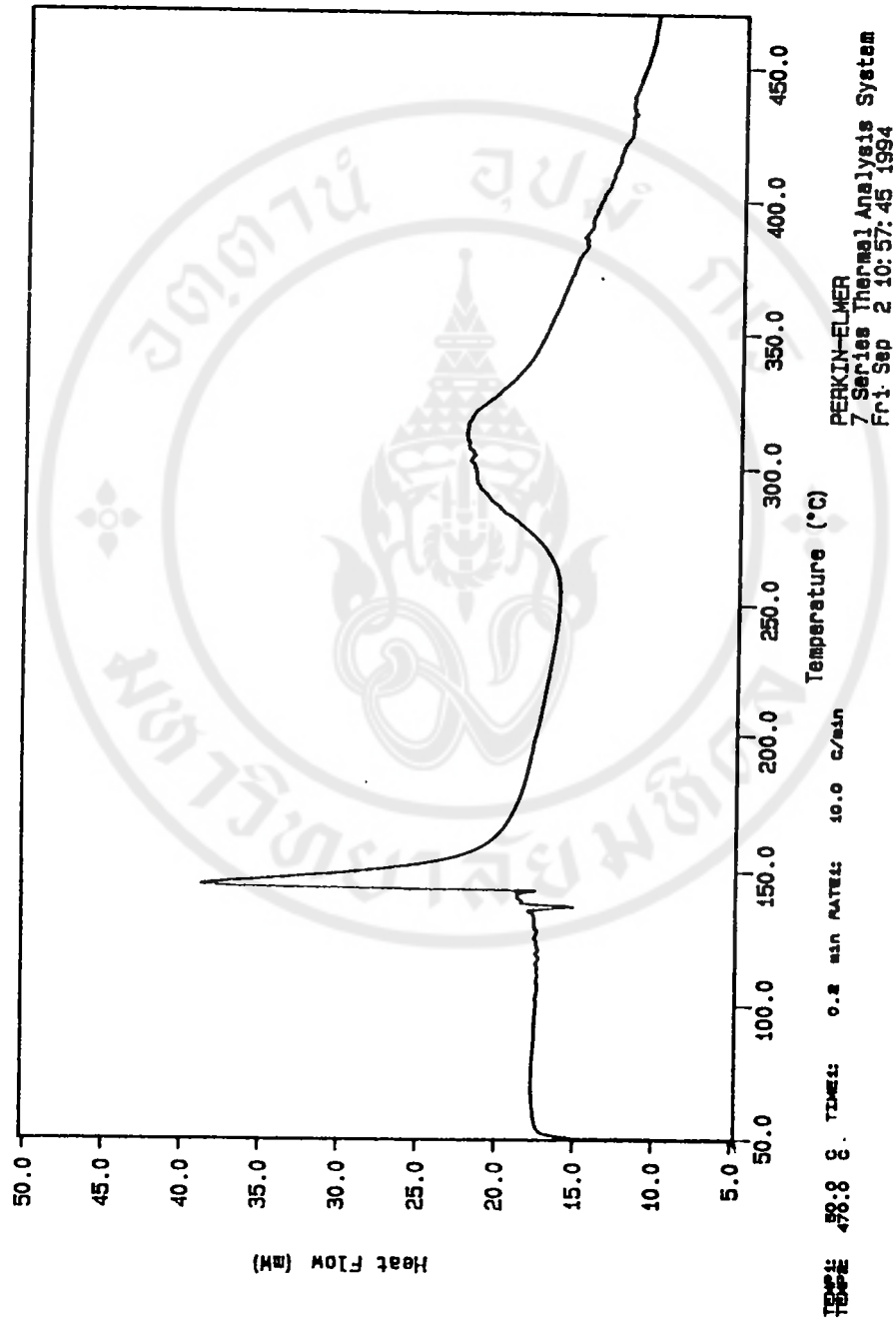

Figure 4A DSC thermogram of N-salicyloyl chitosan