ฟิล์มย่อยสลายได้ทางชีวภาพที่เติมกรด 5-อะมิโนลิวูลินิก จาก *Rhodobacter spaeroides* สำหรับการประยุกต์ทางการเกษตร

นางสาวภัทรานิษฐ์ ตรีเพ็ชร์



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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# BIODEGRADABLE FILM ADDED WITH 5-AMINOLEVULINIC ACID FROM *Rhodobacter spaeroides* FOR APPLICATION IN AGRICULTURE

Miss Phattanit Tripetch


A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
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| Thesis Title | BIODEGRADABLE FILM ADDED WITH 5-AMINOLEVULINIC ACID FROM *Rhodobacter spaeroides* FOR APPLICATION IN AGRICULTURE |
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ภัทรานิษฐ์ ตรีเพ็ชร์ : ฟิล์มย่อยสลายได้ทางชีวภาพที่เติมกรด 5-อะมิโนลิวูลินิก จาก *Rhodobacter spaeroides* สำหรับการประยุกต์ทางการเกษตร (BIODEGRADABLE FILM ADDED WITH 5-AMINOLEVULINIC ACID FROM *Rhodobacter spaeroides* FOR APPLICATION IN AGRICULTURE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.ชาลีดา บรมพิชัยชาติกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.เกียรติศักดิ์ ดวงมาลย์, ดร.จอร์จ เซริดนิกสกี้, 110 หน้า.

งานวิจัยนี้ ศึกษาการผลิตฟิล์มย่อยสลายได้ที่เติม 5-aminolevulinic acid (5-ALA) ที่ผลิตจากจุลินทรีย์ *Rhodobacter spaeroides* โดยแบ่งการทดลองออกเป็น 4 ส่วน ได้แก่ การทดลองที่ 1 การตรวจวัดปริมาณสาร 5-ALA ใน สารละลาย crude 5-ALA ด้วยวิธี colorimetric และ HPLC-fluorescence ซึ่งวิธี HPLC-fluorescence ให้ผลการตรวจวัดที่แม่นยำเนื่องจากสาร fluoresamine ที่ใช้เปลี่ยนรูป 5-ALA เป็นสารเรืองแสงมีความเฉพาะเจาะจงสูงต่อ primary amino acid การทดลองที่ 2 การแยกสาร 5-ALA จากสารละลาย crude 5-ALA ด้วย IEC สภาวะที่เหมาะสมในการแยก โดยใช้ cation resin ($H^+$ form) ใช้ sodium acetate buffer pH 4.5 เป็นสารชะ โดยปริมาณสารตัวอย่างที่โหลดเข้าสู่คอลัมน์ เท่ากับ 10% ของ Bed volume ใช้วิธีเปลี่ยนความแรงประจุ (ionic strength) ด้วยการเพิ่มความแรงประจุแบบต่อเนื่องในการชะสาร 5-ALA ออกจากคอลัมน์ จากขั้นตอนนี้สารมารถชะสารที่ต้องการได้ yield ประมาณ 43 % ในการทดลองที่ 3 การผลิตฟิล์มย่อยสลายได้ที่เติม 5-ALA ใช้สารมาตรฐาน 5-ALA เพื่อลดปัจจัยอื่นๆ ที่อาจมีผลต่อการขึ้นรูปฟิล์ม โดยโพลีเมอร์ที่ใช้เป็นวัตถุดิบในการขึ้นรูปฟิล์ม ได้แก่ บุกกลูโคแมนแนน และ ไคโตซาน และบุกกลูโคแมนแนนเติมด่างโดยสัดส่วนใช้ ในการกักเก็บสาร 5-ALA ได้แก่บุกกลูโคแมนแนน(KGM), บุกกลูโคแมนแนนที่เติมด่าง (KGOH), ไคโตซาน (CHI) และสัดส่วนระหว่าง KGOH/ CHI ดังนี้ 80/20, 75/25, 50/50, 25/75 และ 80/20 พบว่าประสิทธิภาพการเก็บกักสาร 5-ALA ในฟิล์มผสมสัดส่วน KGOH/ CHI (25/75) สูงสุด (~65.9%) รองลงมาได้แก่ ฟิล์ม ไคโตซาน (~60.3%), ฟิล์มผสมสัดส่วน20/80 (~59.5%) และ KGOH (~58.3%)ตามลำดับ โดยกลไกการอยู่ร่วมกันระหว่างฟิล์มไบโอโพลีเมอร์ และสาร 5-ALA ถูกตรวจสอบด้วย Fourier transforms infrared (FT-IR) spectroscopy พบว่า ในฟิล์ม KGM สาร 5-ALA ถูกกักเก็บด้วยโครงสร้างที่ซับซ้อนของตัวมันเอง ฟิล์มKGOH พบการปฏิสัมพันธ์กันระหว่างหมู่อะมิโน (amino group) ของ สาร 5-ALA กับหมู่อะซิทิล (acetyl group) ของบุกในส่วนฟิล์มCHI เกิดปฏิสัมพันธ์กันระหว่างหมู่อะมิโนของไคโตซานและ หมู่คาร์บอกซิล (carboxyl group) ของสาร 5-ALA สำหรับในฟิล์มผสมคาดว่า การเกิดปฏิสัมพันธ์ มี 2 กลไกคือ การเกิดปฏิสัมพันธ์กันระหว่างหมู่อะมิโนของไคโตซานและ หมู่คาร์บอกซิล (carboxyl group) ของสาร 5-ALA และอีกหนึ่งกลไกคือ สารบางส่วนอาจถูกเก็บกักอยู่ในแมทริกซ์ของฟิล์มผสม และกลไกการปลดปล่อยของสาร 5-ALA ในฟิล์ม KGM และฟิล์ม KGOH คือการละลายน้ำทำให้โครงสร้างของบุกบางส่วนถูกละลาย ทำให้สาร 5-ALA ซึ่งละลายน้ำได้ดี ถูกปลดปล่อยออกมา ในส่วนของฟิล์มไคโตซาน กลไกการปลดปล่อยที่คาดว่าเกิดขึ้นคือการบวมน้ำ ทำให้สาร 5-ALA ในโครงสร้างถูกปลดปล่อย การทดลองที่ 4 การเก็บรักษาฟิล์มที่เติม 5-ALA ที่อุณหภูมิ -20 °C, 4 °C และ 28 °C นาน 40 วันโดยเลือกฟิล์ม KGOH, ฟิล์ม CHI และ ฟิล์มผสมในสัดส่วน 25/75 ทำการศึกษาพบว่าที่อุณหภูมิ -20 เมื่อครบ 40วัน สาร 5-ALA ในฟิล์มทั้ง 3 ชนิดยังคงเหลืออยู่มากกว่า 90% เมื่อเก็บที่อุณหภูมิ 4 °C พบว่ามีการสูญเสียสาร 5-ALA อย่างรวดเร็วในช่วง 10 วันแรกของการเก็บ และเริ่มมีอัตราคงตัวเมื่อเข้าสู่วันที่ 15 ของการเก็บเมื่อครบ 40 วัน พบว่าสาร 5-ALA ยังคงถูกเก็บในฟิล์มบุกสูงมากกว่า 50% จากปริมาณเริ่มต้น ที่อุณหภูมิ 28 °C พบว่าสาร 5-ALA มีปริมาณลดลงอย่างรวดเร็วในช่วง 20 วันแรกของการเก็บ เมื่อครบ 40 วัน ปริมาณสาร 5-ALA ที่ยังคงอยู่ในฟิล์มลดลงต่ำกว่า 50% ในทุกชนิดของฟิล์ม ซึ่งพบว่าการใช้บุกเพียงอย่างเดียวมีความเป็นไปได้ที่จะนำมาพัฒนาประสิทธิภาพการเก็บกักสาร 5-ALA ได้ดี เนื่องจากจากกลไกปฏิสัมพันธ์ระหว่างโครงสร้างบุกเติมด่างและสาร 5- ALA มีความแข็งแรงที่จะตรึงสารบางส่วนไว้ได้ ดังนั้นบุกเป็นโพลีเมอร์ที่เป็นทางเลือกที่ดีในการนำไปพัฒนาปรับปรุงโครงสร้างเพื่อเพิ่มประสิทธิภาพการกักเก็บสาร 5-ALA สำหรับการประยุกต์ใช้ในอุตสาหกรรมเกษตร เภสัชกรรม หรือ ในอุตสาหกรรมเครื่องสำอางค์ต่อไป

สาขาวิชา เทคโนโลยีชีวภาพ
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# # 5273921623 : MAJOR BIOTECHNOLOGY
KEYWORDS: 5-AMINOLEVULINIC ACID/ ION EXCHANGE CHROMATOGRAPHY/ KONJAC GLUCOMANNAN/ CHITOSAN/ ENTRAPMENT/ BIODEGRADABLE FILM

PHATTANIT TRIPETCH: BIODEGRADABLE FILM ADDED WITH 5-AMINOLEVULINIC ACID FROM *Rhodobacter spaeroides* FOR APPLICATION IN AGRICULTURE. ADVISOR: ASST. PROF. CHALEEDA BOROMPICHAICHARTKUL, Ph.D., CO-ADVISOR: ASST. PROF. KIATTISAK DUANGMAL, Ph.D., GEORGE SRZEDNICKI, Ph.D., 110 pp.

The aims of this research are to study the process of transformation of 5-aminolevulinic acid (5-ALA) solution into a stable film. The 5-ALA was produced by a biological agent (*Rhodobacter spaeroides*). The research was divided into 4 main parts. Firstly, 5-ALA from *R. sphaeroides* suspension was determined using colorimetric method and HPLC-fluorescence method. HPLC-fluorescence was accurate and reliable to determine 5-ALA due to the direct and specific fluoresamine reaction with primary amines ($R\text{-}NH_2$). Secondly, separation 5-ALA from crude 5-ALA solution using ion exchange chromatography (IEC). The cation resin (Dowex 50×8) was used as an ion exchanger, with sodium acetate buffer (pH 4.5) as an eluent. The initial volume was loaded into column as 10% of bed volume. Changing the ionic strength using a linear elution was selected as the elution process with the yield from elution process being around 43 %. Thirdly, biodegradable film added 5-ALA, standard 5-ALA was used. Due to high purity of the 5-ALA standard, the interactions between film biopolymers and 5-ALA molecules can be thoroughly investigated and explained without interference factors. Films containing 5-ALA were made from konjac glucomannan (KGM), KGM with alkali (KGOH), chitosan (CHI) and the blend polymers (KGOH/CHI), mixed at different ratios 100/0, 80/20, 75/25, 50/50, 25/75, 20/80 and 0/100 (%w/w). The blend film (KGOH/CHI) with a ratio of 25/75 (%w/w) showed the highest percentage of entrapped 5-ALA (~65.9%), followed by CHI film (~60.3%), blend film (20/80) (~59.5%) and KGOH film (~58.3%), respectively. The mechanism for entrapment of 5-ALA within polymer was investigated by using Fourier transforms infrared (FT-IR) spectroscopy. The 5-ALA was entrapped in the matrix of the KGM film. In the KGOH film, as a result of the interaction between the amino group of 5-ALA and acetyl group of KGOH structure, some 5-ALA was entrapped in the matrix of the film. In the CHI film, 5-ALA was entrapped as a result of the interaction between amino group of CHI and carboxyl group of 5-ALA. In blend films, two mechanisms were postulated. Firstly, there was interaction between the amino group of CHI and carboxyl group of 5-ALA. Secondly, the amino group of 5-ALA was incorporated into the complexity of KGOH structure. The 5-ALA was released from the film when the film was dissolved in water. The 5-ALA was soluble when KGM and KGOH film was immersed in water, the structure was dissolved in water resulting in the release of 5-ALA. As for CHI film, the mechanism of releasing 5-ALA was related to the swelling of CHI film. Fourthly, the KGOH film, CHI and blend film (25/75 %W/W) containing 5-ALA were stored at -20 °C, 4 °C และ 28 °C for 40 days. At -20 °C, more than 90% the 5-ALA were retained in all films. At 4°C, it was found that the 5-ALA content decreased rapidly within 10 days. After storage for 40 days, more than 50% of 5-ALA within KGOH film were retained. At 28 °C, the 5-ALA content decreased rapidly within 20 days. After 40 days, the 5-ALA content retained in all films was lower than 50% with 5-ALA retained in KGOH film being ~30% higher than in other films. The reduction of 5-ALA at 4 and 28 °C followed second order kinetic reaction. It can be concluded from experiments, the konjac glucomannan has a very high potential as a biopolymer to be used for entrapment of 5-ALA. It is a good option for increasing efficiency of stabilising 5-ALA for the applications in agriculture, pharmaceutical or cosmetic industry.

Field of Study: Biotechnology
Academic Year: 2015

Student's Signature .....
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# CHAPTER 1

# INTRODUCTION

## 1.1 Research background

The 5-aminolevulinic acid (5-ALA) belongs to the class of α-aminoketones (Donnelly *et al*., 2007). It is found in all living cells (Namjoshi *et al*., 2007). Nowadays, 5-ALA has attracted more attention for use in medicine since it can destroy malignant cells in photodynamic diagnosis and therapy (Fukuda *et al*., 2005). Moreover, 5-ALA is also suitable to be used in agriculture. There are many researchers who reported that 5-ALA appears to act as a hormone-like plant growth regulator. At low concentrations it can improve photosynthetic capacity, increase plant growth and yield. The 5-ALA is a natural photodynamic compound effective as a biodegradable herbicide and insecticide when used at high concentration (Hotta *et al*., 1997; Richter *et al*., 2010; Watanabe *et al*., 2000).

The productions of 5-ALA are divided into chemical synthesis and biological production. Currently, chemical synthesis has drawbacks of complication, relatively low yields and high cost. (Miyachi *et al*., 1998). Therefore, a biological production is more favour because of an inexpensive industrial production and improved production process to increase the 5-ALA yield. The photosynthetic bacterium, *Rhodobacter sphaeroides*, is often used for biological 5-ALA production. Extracellular 5-ALA was produced under anaerobic-light conditions with adding of levulinic acid (LA) (Nishikawa and Murooka, 2001; Sasaki *et al*., 1991). To separate 5-ALA from crude solution, the methods include ion-exchange chromatography (IEC) (Mauzerall and Granick, 1956) and capillary electrophoresis (CE) can be used. However, CE method

is still not popular in industrial production, because of high costs. IEC method is still widely used in industry because of it is simple to use and low cost. However, there is no study on process optimisation to separate 5-ALA from biological suspension. Therefore, optimisation of method for separation 5-ALA from *Rhodobacter sphaeroides* suspension for using in agriculture will be one of main studies in this research.

However, the stability of 5-ALA in aqueous solution is a critical problem for its use in various fields. The factors affecting the stability of 5-ALA have been found to be pH, concentration, temperature and degree of oxygenation (Donnelly *et al*., 2007). Thus, the stability of the5-ALA solution is a problem that still needs to be solved before using it in various practical. The method that is likely to prevent 5-ALA degradation is to make it in solid form, which is well known in commercial as 5-aminolevulinic acid hydrochloride, which is an expensive product and need to be always kept at -20 °C. Therefore, this research presents the new products to stabilize of 5-ALA by entrapped 5-ALA under film which made from the biopolymers. The biopolymers that are proposed in this research include konjac glucomanan and chitosan.

Konjac glucomannan (KGM) is one of the biopolymer which is extracted from the corm tissues of *Amorphophallus* konjac plant (Luo *et al*., 2013). It is a natural polymer that is environmentally friendly, therefore it has been widely used in green food and health care for its unique pharmacological functions in cosmetics and agricultural industry (Douglas *et al*., 2004). The high viscosity of the KGM solution is also required for film formation. KGM film formation is able to form strong intermolecular interactions of the hydrogen bonding and self-entanglement.

(Cheng *et al*., 2002; Luo *et al*., 2013). Recently, KGM film was defined as a novel natural polymer material applied in the fields of coating, food preservation and enzyme entrapment (Leuangsukrerk *et al*., 2014; Supapvanich *et al*., 2012; Wang *et al*., 2008). Moreover, the properties of KGM films may be easily modified by blending with other compatible polymers. At present, there are some studies showed that interactions of KGM with other biopolymers such as chitosan, sodium alginate, poly (vinylpyrrolidone), and sodium carboxymethyl cellulose have produced films with enhanced tensile strength, breaking elongation, and thermal stability (Xiao *et al*., 2000; Xiao *et al*., 2001). Therefore, it is interested in this research work that chitosan will be chosen to be blended film with KGM for entrapment of 5-ALA. Because chitosan has a good film-forming properties, and selective gas permeabilities ($CO_2$ and $O_2$). It is a non-toxic biopolymer and biodegradable. There has been a great interest in the protein drug-coating and when KGM was mixed with chitosan, the properties of films were improved (Campos *et al*., 2011; Puttipipatkhachorn *et al*., 2001). The blending of biopolymers in films (KGM/chitosan) can enhance mechanical properties of the film. However, only a few studies have been using film KGM and blend KGM and chitosan films for entrapment the compounds compatibility small molecules. There are many researches showing that the film made from KGM chitosan and blended film combining KGM and chitosan have a good property as a coating material. Therefore, the main goals of this research is to optimise separation process for 5-ALA from *Rhodobacter sphaeroides* culture by using IEC and developing shelf stable 5-ALA solution formulation in film to prevent degradation of 5-ALA.The product would have the potential to be developed for use in various industries such as agriculture, food, medicine and cosmetics. In addition,

this product is biodegradable, comes from renewable sources and is harmless to living cells and friendly to the environment.

## 1.2 Objectives

1.2.1 To optimise separation method of 5-aminolevulinic acid from *Rhodobacter sphaeroides* suspension.

1.2.2 To study the process of transformation of pure 5-ALA solution into stable and high efficiency film by microencapsulation technique using natural biopolymers.

## 1.3 Scope

The thesis research have been divided into 4 main parts

1.3.1 Determination of 5-ALA from *R. sphaeroides* suspension using colorimetric method and HPLC-fluorescence method

1.3.2 Separation of 5-ALA using ion exchange chromatography (IEC).

1.3.2.1 Selection of a suitable pH of eluent

1.3.2.2 Selection of sample loading volume

1.3.2.3 Elution process

1.3.3 Study of a suitable condition for fabrication of film containing 5-ALA

1.3.3.1 Determination of a suitable condition for the production of biodegradable film containing 5-ALA which made from konjac glucomannan and chitosan.

1.3.3.2 Characterization and determination of properties of films with and without 5-ALA using Fourier Transform Infrared Spectroscopy (FT-IR), wide-angle X-ray diffraction (XRD), Field Emission Scanning Electron Microscope (FE-SEM), water solubility and water absorption of the film.

1.3.4 Determination of stability of the 5-ALA in film

## 1.4 Hypothesis

1.4.1 The 5-ALA can be separated from *R. sphaeroides* culture using cation exchange chromatography by changing in ionic strength using a linear elution.

1.4.2 The 5-ALA solution can be transformed into a shelf stable in solid form by entrapment under biodegradable film formation.



## 1.5 Expected outcomes

This research will propose the optimized condition for separation 5-aminolevulinic acid from *R. sphaeroides* suspension for use in agriculture and will present the new method to stabilise5-ALA by entrapment it under biodegradable film. The outcome of this research will guide to produce a 5-ALA in film for increasing the stability and shelf life of 5-ALA before using in agriculture or other related fields.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 5-aminolevulinic acid

### 2.1.1 Chemical structure and properties

The 5-aminolevulinic acid (5-ALA) (Figure 2.1) is a 5-carbon aliphatic amino acid with a straight chain, water soluble, molecular weight of 131.13 g/mol and dissociation constants pKa1= 4.05 and pKa2 = 8.30 (Novo *et al*., 1996). The stability of 5-ALA in aqueous solution depends on four factors, such as pH, concentration, temperature and degree of oxygenation of the solution (Bunke *et al*., 2000; De Blois *et al*., 2002; Donnelly *et al*., 2007).

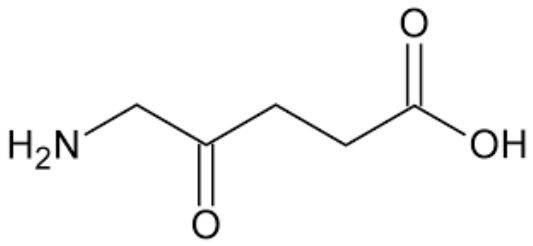



**Figure 2.1** Chemical structure of 5-ALA (Donnelly *et al*., 2006)

5-ALA is found in living cells. It is an essential precursor in tetrapyrrole biosynthesis, such as heme chlorophyll (bacteriochlorophyll) vitamin $B_{12}$ and other tetrapyrrole compounds. The biosynthesis of 5-ALAcan be formed via two distinct metabolic pathways as follows: (1) C4 pathway or shermin pathway (Figure 2.2a) is observed in mammalian cell yeast, fungi and very common among the purple non-sulfur photosynthetic group and a few chemotrophs, synthesizes 5-ALA from succinyl-CoA and glycine (Sasaki *et al*., 1990; Tangprasittipap and Prasertsan, 2002).

(2) C5 pathway (Figure 2.2b) is formed from glutamate which present in higher plant algae and several bacteria (Tangprasittipap *et al*., 2007).

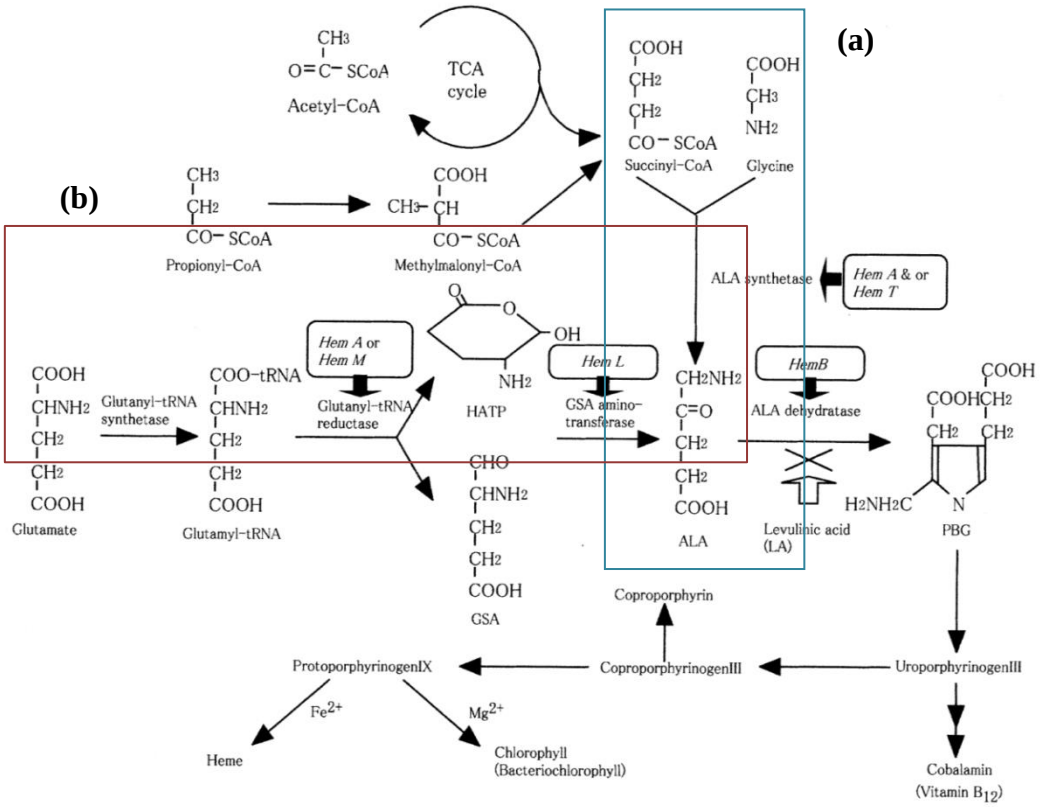





**Figure 2.2** Biosynthesis of 5-ALA and tetrapyrrole compounds in living cell
(a) C4 pathway (b) C5 pathway (Sasaki *et al*., 2002)

From the above biosynthesis pathway of 5-ALA it would be seen that, 5-ALA is animportant precursor of chlorophyll, vitamin $B_{12}$, heam, porphobilinogen (PBG) in living cells.

### 2.1.2 Application of 5-ALA

Recently, 5-ALA is used in various fields such as agriculture, medicine, biotechnology and cosmetic industry (Table 2.1). Because, it was harmless for crops, human, animals and not accumulate in the environment (Hotta et al., 1997; Sasikala et al., 1994). In medicine, 5-ALA can be used to destroy malignant cells in photodynamic diagnosis and therapy (Fukuda *et al*., 2005). Other medical applications, such as treatment of mycosis, inhibition of peptidase, rheumatoid arthritis treatment and cosmetic and dermatological applications (Sasaki *et al*., 2002). In biotechnology, 5-ALA can be used in porphyrin production, vitamin B12 production, animal cell culture, plant cell culture, and yeast, fungi and bacteria culture (Bykhovsky *et al*., 1997; Fujita *et al*., 1997; Miyachi *et al*., 1998; Sasaki *et al*., 2002). In agriculture, the concentration and the role of 5-ALA that were used in various fields was depending on the target such as in agriculture, 5-ALA was the natural photodynamic compound effective as a biodegradable herbicide and insecticide when use it at high concentration (>1000 ppm) (Rebeiz *et al*., 1984; Rebeiz *et al*., 1990). Moreover, 5-ALA appears to act as a hormone-like plant growth regulator. ALA at low concentrations can improve photosynthesis capacity, plant growth, yield, sugar content (Hotta *et al*., 1997; Richter *et al*., 2010; Watanabe *et al*., 2000). There are some research that proposed the potentiality of 5-ALA for used in agriculture as follow:

Bindu and Vivekanandan (1998) presented the effect of 5-aminolevulinic acid in callus induction and micropropagation in cowpea. They found that the role of 5-ALA in callus formation is similar to the effects of other auxins like 2,4-Dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indolebutyric acid

(IBA) or 1-Naphthaleneacetic acid (NAA). The role of auxin in the plant tissue culture is to stimulate cell enlargement and root initiation.

Hotta *et al*. (1997) reported the effects of 5-ALA on the yield of several crops. The concentration of 5-ALA at 0.06–1.8 mM can increase the dry weight of radish root, increased the growth and yield in kidney bean, barley, potato and garlic up to 10–60%. When using 5-ALA at 6 mM, radish seedlings was injured. They suggest that 5-ALA at low concentrations can be used to enhance agricultural productivity.

In addition, 5-ALA can reduced accumulation of hydrogen peroxide ($H_2O_2$) in spinach (*S. oleracea*) and sunflower plants (Akram *et al*., 2012; Nishihara *et al*., 2003; Nishikawa and Murooka, 2001), and increased the activities of dehydroascorbatereductase (DHAR), catalase (CAT), glutathione reductase (GR), superoxide dismutase(SOD), ascorbate peroxidase (APX) under saline as well as non-saline conditions in spinach (*S. oleracea*) and Ginkgo biloba (Xu *et al*., 2009). More of applications of 5-ALA in agriculture, medicine and other fields presented in Table 2.1.

**Table2. 1** Applications of 5-aminolevulinic acid in agriculture, medicine and other fields

| Fields | Application | References |
|---|---|---|
| Agriculture | Biodegradable herbicide and insecticide | (Rebeiz *et al*., 1988) |
| | Growth promoting factor and yield enhancement, Increased growth of papaya, Promotive on the antioxidant system in ginkgo biloba leaves | Morales-Payan and Stall (2005); (Xu *et al*., 2009) |
| | Cold temperature tolerance, Promotion of pepper seed germination and seedling, emergence under low-temperature stress, Enhancing salt tolerance of potato, Enhancing photosynthetic in date palm seedlings under salinity stress | (Korkmaz and Korkmaz, 2009; Youssef and Awad, 2008; Zhang *et al*., 2006) |
| | Quality improvement of vegetables, Promotive effect of 5-aminolevulinic acid on chlorophyll and photosynthesis of pakchoi | (Memon *et al*., 2009) |
| | Growth and green-color maintenance of grass, Enhancement of growth and fruit maturity of grapevines cv. Delaware | (Hotta and Watanabe, 1999; Watanabe *et al*., 2006) |
| Medicine | Prodrug in photodynamic therapy of cancer | (Wachowska, *et al*., 2011) |
| | Cosmetic - acne treatment | (An *et al*., 2011) |
| Biotechnology | Porphyrin production | (Miyachi *et al*., 1998) |
| | Vitamin B12 production | (Bykhovsky *et al*., 1997) |
| | Induction of callusing an drhizogenesis, and shooting of *Vignaunguiculata* L. var. | (Bindu and Vivekanandan, 1998) |

The 5-ALA is one alternative compound for application in agriculture, because it has a potential on increasing agricultural productivity, harmless for human and animals and environmentally. But, the 5-ALA in commercial is very expensive due to complex production which has not been used widely in agriculture. Therefore, the production of 5-ALA should be improved to give the substance to be cheaper which it to be used in agriculture increased.

## 2.2 Production of 5-ALA

5-ALA in commerce is well known in hydrochloride form (Figure 2.3). The productions are divided into chemical synthesis and biological production.

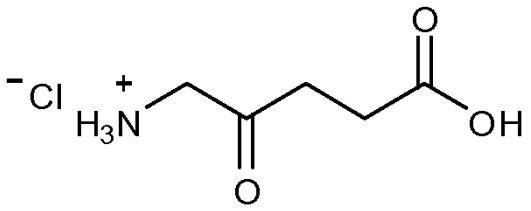



**Figure 2.3** Chemical structure of 5-aminolevulinic acid hydrochloride (5-ALA-HCl) (Donnelly *et al*., 2007)

### 2.1.1 Chemical synthesis

5-ALA can be synthesized (Figure. 2.4) from (A) levulinic acid, (B) 2-hydroxypyridine, (C) frufural, (D) furfurylamine, (E) tetrahydrofurfurylamine, and (F) succinic acid. Among them, photo-oxidation of frufurylamine (D) seems to be the most practical, albeit chemical synthesis of 5-ALA is still costly compared with biological production due to the many steps involved and relatively low yields (Miyachi *et al*., 1998).

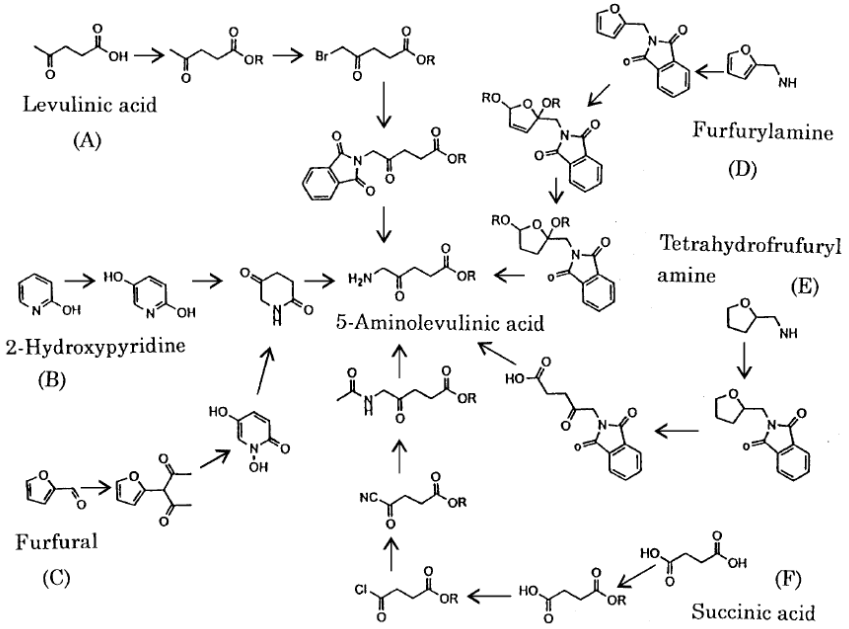



**Figure 2.4** Chemical synthesis of 5-ALA from various industrial materials (Sasaki *et al*., 2002)

In fact, the 5-ALA was produced by chemical synthesis was not popular because it has drawbacks of complication, relatively low yields and high cost. Moreover, the chemical synthesis occurred the toxic cyano compound, such as silver cyanide or copper cyanide (Miyachi *et al.*, 1998). Therefore, 5-ALA production by chemical synthesis is relatively expensive as an agricultural chemical due to the difficulties of chemical synthesis (Sasaki *et al*., 2002).

### 2.2.2 Biological production

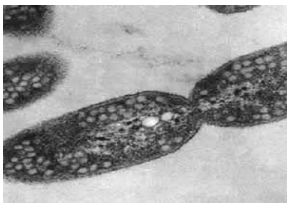
The production of 5-ALA by biological synthesis more favour because of an inexpensive industrial production and there is an attempt to improve production process to increase the 5-ALA yield. The 5-ALA can be produced by different groups of microorganisms that were showed in Table 2.2

**Table2. 2** Production of 5-ALA by different groups of microorganisms

| Group | C and N source | ALA(μM) | References |
|---|---|---|---|
| Anoxgenic photosynthetic bacteria | Glutamate | | Anderson *et al*. (1983) |
| *Chlorobiumlimicola* | Succinate and glycine | 3.95 | |
| *Rhodopseudo monaspalustris* | Succinate and glycine | 0.75<br>16000 | Sasaki *et al*. (1998) |
| *Rhodobacter Sphaeroides* | Glucose and glycine | 14300 | Watanabe *et al*.(1996) |
| *Rhodobacter Sphaeroides*CR520 | | | |
| Algae | | | |
| *Chlorella sp.* | Glucose and glycine | 2000 | Sasaki *et al*. (1995) |
| *Chlorella regularis* | Glucose and glycine | 3700 | Ano *et al*. (1999) |
| Aerobes | | | |
| *Pseudomonas riboflavina* | L-Alanine | 200 | Rhee *et al*. (1987) |
| *Escherichia coli* | Succinate and glycine | 23000 | Lee *et al*. (2005) |

The formation of 5-ALA is occurred in mostly microorganisms cells. It is tightly regulated and it does not secrete 5-ALA into growth medium. The 5-ALA could be excreted by the photosynthetic bacterium (e.g. *Rhodobactor sphaeroides*) into the medium (Nishikawa *et al*., 1999; Sasaki *et al*., 1991). Therefore, the production of 5-ALA in commercial which was established in 1998, the *R. sphaeroides* (Figure 2.5a) was interested to use to produce it by culture in an aerobic-dark culture system (Miyachi *et al*., 1998). The *R. sphaeroides*, synthesizes 5-ALA from succinyl-CoA and glycine by ALA synthetase (Figure 2.5b). A large amount of extracellular 5-ALA was produced under anaerobic-light conditions with the addition of levulinic acid (LA), a competitive inhibitor of δ-aminolevulinic acid dehydratase (Sasaki *et al*., 1998), or under aerobic conditions using a mutant of *R. sphaeroides* (Nishikawa *et al*., 1999).

(a) (b)



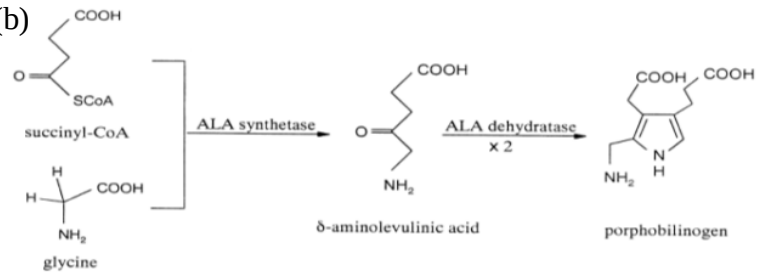



**Figure 2.5** (a*) Rhodobacter sphaeroides* (b) Biosynthetic pathway of 5-ALA and porphobilinogen (PBG) from succinyl-CoA and glycine by photosynthetic *Rhodospirillaceae* (Senge and Smith, 2004)

### 2.2.3 Separation process of 5-ALA from biological production

The production of 5-ALA from the biological process is often contaminated with saccharides, protein, amino acids, organic acids, metal ions that are coexisted in the medium (Okada *et al*., 2012). The method can be used to separate 5-ALA such as ion exchange chromatography and capillary electrophoresis. The common method that used to separate 5-ALA from crude solution is ion-exchange chromatography (IEC) (Mauzerall and Granick, 1956).



#### 2.2.3.1 Ion-exchange chromatography (IEC)

IEC is a powerful purification technique in the production of proteins in the industry. Because, IEC has high resolving power, high protein binding capacity, and versatility (there are several types of ion exchangers), type the buffer and pH can be varied, as well as easy to use (Janson, 2012). The basis for IEC is the electrostatic attraction between proteins in solution and charged groups of the ion exchanger (e.g. resin). Most elution step of ion exchange is performed in five main stages. The illustrated schematically was shown in Figure 2.6.

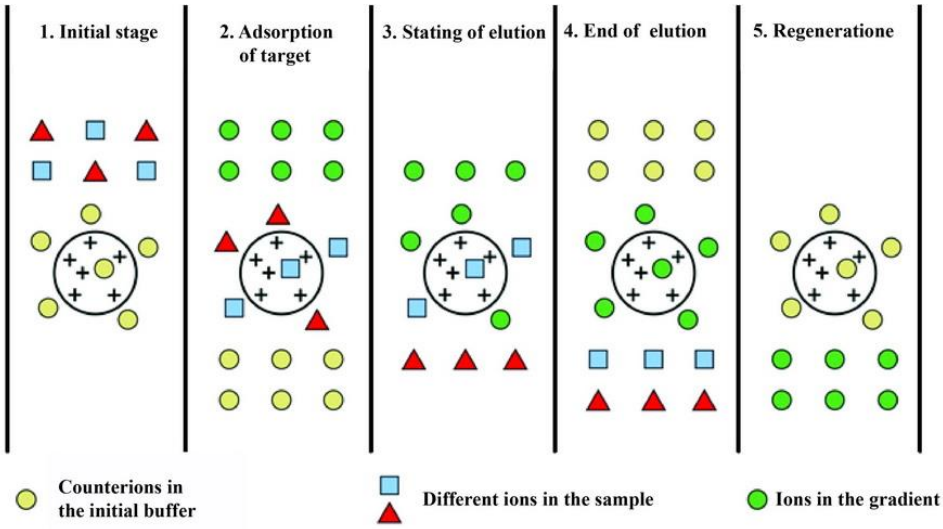



**Figure 2.6** Principle of IEC (AB, 1991)

The first stage is equilibration in the ion exchanger in which terms of pH and ionic strength, which allows the binding of the solute molecules. The exchanger groups are associated with the exchangeable counter-ions.

The second stage is sample application and adsorption, in which solute molecules carrying the appropriate charge displace counter-ions and bind reversibly to the exchanger. The substances have no affinity for the exchanger is removed from the column using starting buffer.

The third stage, the substances are removed from the column by changing to elution conditions which increasing the ionic strength of the changing pH or eluting buffer. The desorption is achieved by the increasing salt concentration gradient and solute molecules are released from the column in the order of their strengths of binding, the most weakly bound substances being eluted first.

The fourth and fifth stages, the substance which it is not eluted under the previous experimental conditions is removed from the column, and the column is re-

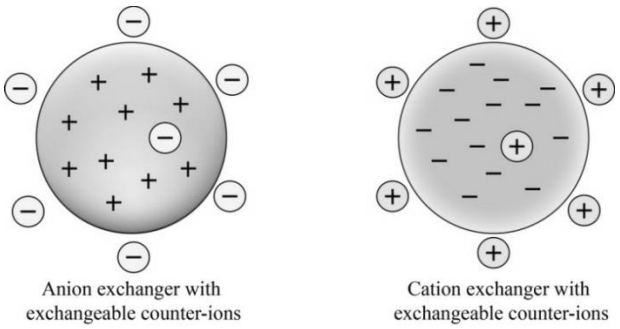
equilibrated at the starting conditions for the next purification (AB, 1991); Bollag, *et al*. 1996).

The factor that considered when using IEC for protein separation process consist of the stationary phase (type of ion-exchange group, ion-exchange capacity, particle diameter, pore structure, pore size distribution, base matrix property, etc.), elution buffer (pH, salt concentration, etc.), column parameters (length, diameter, etc.) type of elution (ionic or pH) and mode of elution (isocratic, gradient or stepwise) (Ahamed *et al.*, 2007; Ishihara and Yamamoto, 2005). Moreover, the stability of the target compound is one important factor that must be considered.

The stationary phase is an ion exchanger consists of an insoluble matrix to which charged groups are associated with mobile counter ions. These counter-ions can be reversibly exchanged with other ions of the same charge without altering the matrix (AB, 1991) (Figure 2.7).

The selected of an ion exchanger depend on a protein's net charge under chromatographic conditions. The pH at which the positive charges equal the negative charges defines that protein's isoelectric point (pI). The loading-pH should be about 0.5–1 pH unit above pI of the target protein for anion-exchange chromatography and about 0.5–1 pH below pI of the target protein for cation-exchange chromatography respectively (Figure 2.8) (Bollag *et al.*, 1996; Healthcare, 2007). At pH values of protein that is far away from the pI, proteins will bind strongly and do not desorb with low ionic strength buffers (Janson, 2012).

(a)




(b)

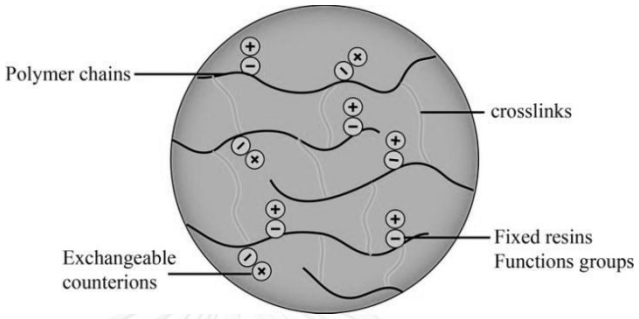



**Figure 2.7** (a) Ion exchanger types
(b) Schematic diagram of a cation exchange media particle

The selection of elution buffer can be considered in following points, when the pH of buffer approaches the pI of a target protein, the protein becomes less tightly bound to the matrix and is eluted from the column. The experiment buffer should be chosen to provide adequate buffering capacity1 pH unit away from a protein's isoelectric point (Bollag *et al.*, 1996).

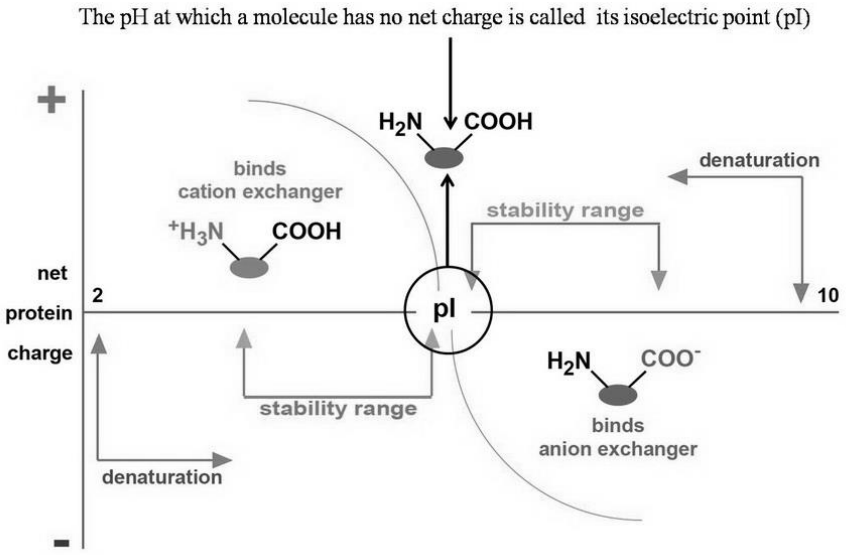



**Figure 2.8** The net charge of protein as a function of pH (AB, 1991)

Elution process, the protein that adsorb to the ion exchanger are eluted from the column either by an increase in ionic strength or by a change in pH (Janson, 2012). Firstly, increase in ionic strength, the competition for charged groups on the ion exchanger is at a minimum and substances are bound strongly at low ionic strength when increasing the ionic strength, it increases competition and reduces the interaction between the ion exchanger and the target substance, as a result, the substance is eluted (AB, 1991). Secondly, changing in the pH causes increase in the protein net charge to close the isoelectric point of a substance, then causing it to lose its net charge desorb and elute from the ion exchange (Bollag *et al*., 1996).

The protein adsorbed on an ion exchange column can be eluted by stepwise elution or gradient elution. Firstly, stepwise elution (Figure 2.9a), is frequently a simple and more rapid procedure. Stepwise elution should be used for a preliminary experiment or after the elution characteristics of the protein of interest have been established (Bollag *et al*., 1996). Secondly, gradient elution (Figure 2.9b),

removes proteins by a gradual increase in ionic strength (such as from 0.01 M to 1 M NaCl). Gradient elution should be used when starting with and unknown sample when as many components in sample as possible are bond to the column and eluted differentially (Bollag *et al*., 1996; Healthcare, 2007).

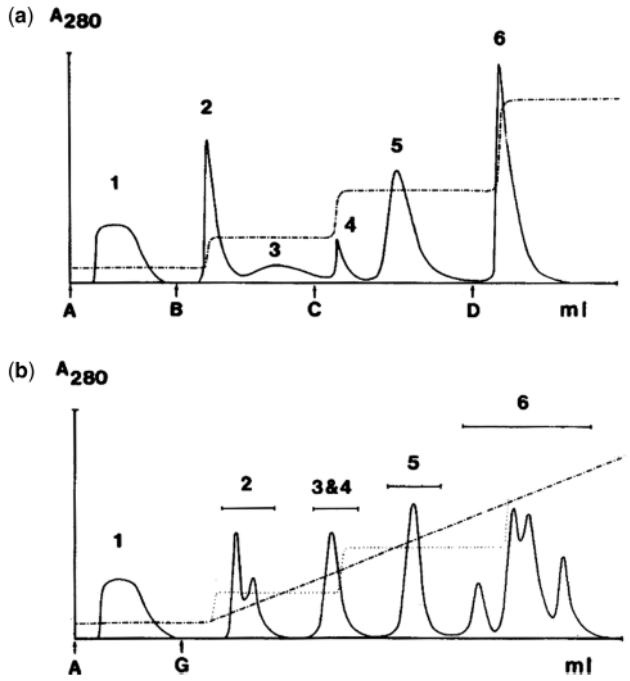



**Figure 2.9** Elution of an ion exchange column (Janson, 2012).
(a) column profile after stepwise elution
(b) column profile after gradient elution. The gradient starts at G.

Di Venosa *et al*. (2004) employed a Dowex 50X8 hydrogen form resin to separate 5-ALA from ALA derivatives. They found that 90+4% of the total 5-ALA was eluted using 1 M sodium acetate, which as3-9% of the 5-ALA derivatives was eluted.

Okada *et al*. (2012) proposed the method for separation 5-ALA from *R. sphaeroides* culture broth. They used an IEC and desorption with and aqueous

solution containing ammonium ion as an eluent. Then, the impurity in 5-ALA solution was eliminated by changing in pH. Moreover, they presented the method for production 5-ALA in solid form as known as 5-ALA hydrochloride (5-ALA-HCl).The production of 5-ALA-HCl is obtained by adding chloride ion to the 5-ALA solution. The crystallization step was obtained by mixing the 5-ALA solution containing chloride ion with the poor solvent (ethanol, acetone). The precipitated 5-ALA-HCl was recovered and undergo air-drying. The process was illustrated in Figure 2.10.

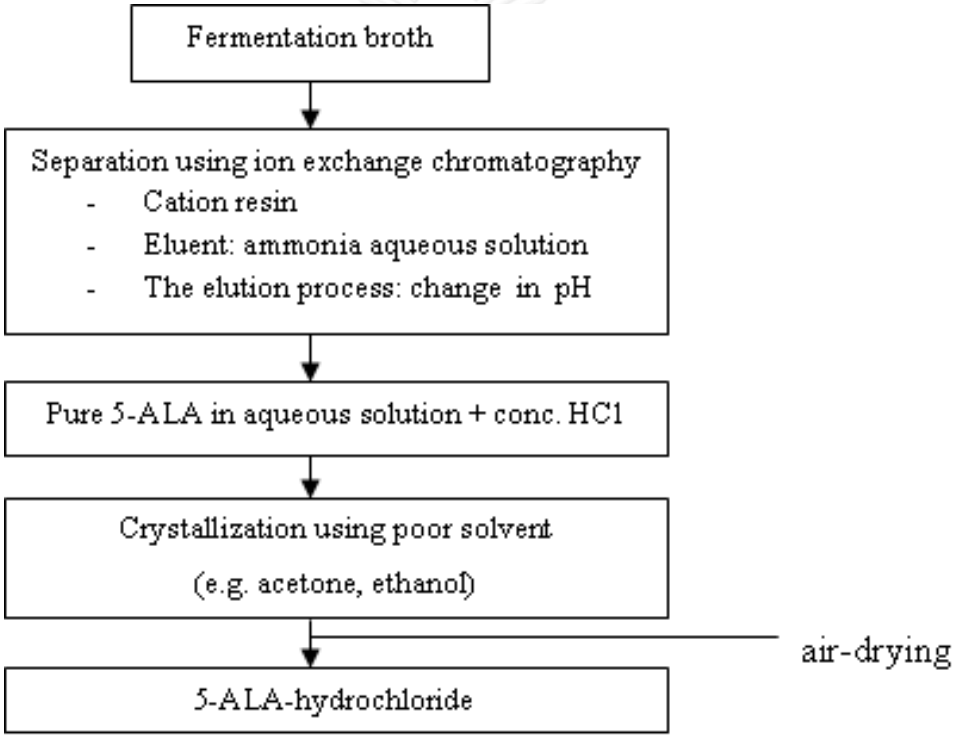



**Figure 2.10** Process flow chart of 5-ALA-HCl production in industry (Okada *et al*., 2012)

#### 2.2.3.2 Other methods for separation the 5-ALA

The capillary electrophoresis (CE) is the method for separation the compounds which the principle is depending on the differential migration of particles in an applied electric field.

Kim *et al*. (2001) established separation conditions of 5-ALA, porphobilinogen (PBG), levulinic acid (LA) and glycine from culture both of *R. sphaeroides* by CE. They found that each component was separated well using 30 mM sodium tetraborate buffer with a constant voltage of 20 kV. However, CE method is still not popular in industrial production, because of high costs and the limitation of the equipment. The advantage and disadvantages of IEC and CE method is shown in Table 2.3.

**Table 2. 3** The advantage and disadvantages of IEC and CE method.

| | **Ion exchange chromatography** | **Capillary electrophoresis** |
|---|---|---|
| Efficiency | Complex and<br>time consuming | rapid<br>powerful separating<br>reproducible separation |
| Purity | Glycine and similar structure are difficult to remove from 5-ALA | high-resolution with small sample |
| Remarks | Popular for used in industrial | cannot identify neutral species |

Source: Kim *et al*. (2001); Zhang *et al*. (2010); Di Venosa *et al*. (2004); Okada *et al*. (2012)

There are some researches that proposed the other methods to separate the 5-ALA from complex solution system as follows:

Zhang *et al*. (2010) proposed a novel separation procedure combining membrane permeation, reactive extraction and crystallization. Large molecular weight impurities in fermentation broth were removed by ultra-filtration, and then the filtrate was concentrated by reverse osmosis and the 5-ALA recovery. Moreover, they presented the process for production 5-ALA-HCl was shown in Figure 2.11.

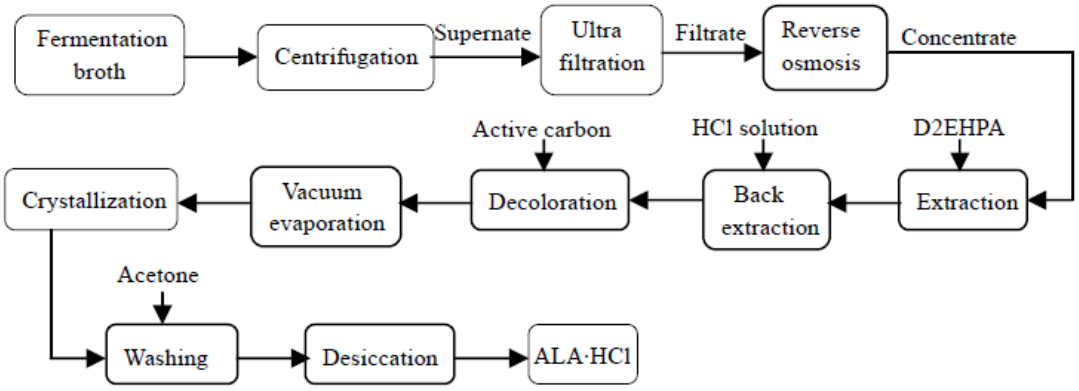



**Figure 2. 11** Process flow chart of 5-ALA separation and purification present by Zhang *et al*. (2010)

## 2.3 Stability of 5-ALA in aqueous solution

The stability of pure 5-ALA in aqueous solution is a critical problem for its use in various fields. Stability of 5-ALA involve changes in pH, concentration, temperature and degree of oxygenation (Donnelly *et al*., 2007). The pure 5-ALA in aqueous solution is unstable under inappropriate environment such as strongly alkaline conditions and high temperature (>50 $^{o}$C). The stock 5-ALA solutions should be prepared at concentration 0.1- 2% at pH 5. At this condition it can be stored up to 1 month at 20 $^{o}$C (De Blois *et al*., 2002).

Novo *et al*. (1996) proposed the degradation mechanism of 5–ALA under alkaline condition when two molecules of 5-ALA were condensed with a nucleophilic attack by a lone pair of the amino group of 5-ALA to a γ-carbonyl of another 5-ALA molecule to form 3,6-dihydropyrazine 2,5-dipropionic acid (DHPY). The DHPY is then oxidized to pyrazine 2,5-diproipionic acid (PY) under aerobic condition. An aqueous solution at lower pH values (< 2) would be required to preserve the 5-ALA stability for long term storage (Figure 2.12).

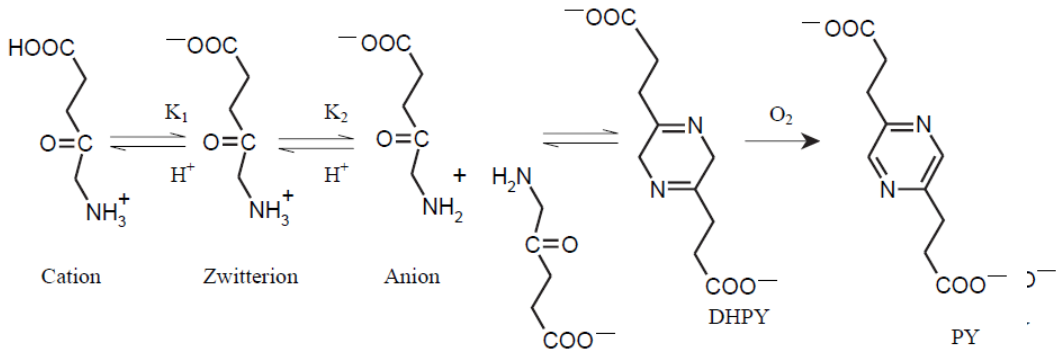



**Figure 2.12** Possible condensation reactions of 5-ALAin aqueous solutions and pH-dependent equilibrium (Novo *et al.*, 1996).

Elfsson *et al*. (1999) demonstrated that the stock solutions of 1% (60 mM) 5-ALA that was incubated at 50°C (pH 2.35), 5-ALA was stable during the whole incubation period (37 days). The half-lives of 1% ALA at pH 4.81 and 7.42 were 257 h and 3.0 h, which followed the second-order decomposition, respectively. The degradation rate increased about 1.5 times with each 10°C rise in temperature at pH 7.53 within the range studied (37–85 °C).

De Blois *et al*. (2002) reported that an initial concentration of 5-ALA equal to or less than 0.5% at pH 5 is stable for at least three months. The 0.1% 5-ALA solutions with pH values between 4 and 8 a pH shown the dependency of ALA

degradation, comprising fast decomposition at pH values higher than 7, whereas at a pH value of 6 or lower the solutions remained within the range of 90–110% of the initial concentration for at least 128 days. When increasing concentrations, the degradation rate is supposed second-order degradation kinetics. The shelf life of 0.1% 5-ALA solution pH 5 under accelerated condition (63°C and 85°C) was 281 days. In addition, the color of 1% 5-ALA solution at pH 5 can be notice. The red color of degradation product intensified from a colorless, light orange to a dark red in 2–21 days when storage at 37 °C, 55°C and 65°C.

The stability of the 5-ALA solution is a main concern that that still needs to be solved before using it in various fields. Therefore, there are some researches proposed the techniques to improve the stability of 5-ALA for long time storage.

Sakiyama *et al.* (2010) studied stabilizing of 5-ALA by adding zinc (II) ion to 5-ALA solution (neutral pH). The zinc (II) ions were bound to amino groups of 5-ALA molecules and as a result, the nucleophilic attack was stopped. Although, stability of 5-ALA in aqueous solution was resolved, but it is still liquid which requires strong container and space for storage.

The form of 5-ALA in commercial presented in the form of a powder, well known as 5-ALA hydrochloride. It is stable and can be stored more than 2 years but under the temperature of -20 $^{o}$C. 5-ALA-hydrochloride is popular to use in medicine. However, it is not widely utilized in agriculture because of high cost of production.

Therefore, in this research will be proposed to stabilize the production 5-ALA in film formation by using biodegradable polymer as material for an alternative choice to be applied in agriculture.

## 2.4 Biopolymers and mechanism of film-forming

### 2.4.1 Biopolymers

Biopolymers are defined as polymers that are derived from renewable resources, as well as biological and fossil based biodegradable polymers. It can be divided into two groups, that biodegradable and non-biodegradable. They can be classified on their origin as being either bio-based, produced from plants, animals, or microorganisms. There are many more non-degradable bio-based biopolymers than there are biodegradable bio-based biopolymers (Niaounakis, 2014).

There are a number of industrial that uses of biopolymers, ranging from coatings and adhesives, and the increased use in blends in the area of bioplastics (Winkworth-Smith and Foster, 2013). In this research will focus on using the natural biodegradable for application in film formation. The biodegradable natural polymers classification is represented in Table 2.4.

**Table 2.4** Classification of biodegradable natural polymers

| **Biodegradable Natural Polymers** | | |
|---|---|---|
| Natural polymer of Plant origin | Natural polymer of animal origin | Natural polymer of microbial origin |
| Polysaccharides (Cyclodextrin, Cellulose, Hemicellulose, Starch, Inulin, Pectin, Glucomannan, Guar gum, Arabinogalactan, Carragenn) | Polysaccharides (Chitosan, Hyaluronan, Chondroitin sulphate) | Polysaccharides (Alginate, dextran) |
| Protein (Soy protein) | Proteins (Collagen, Gelatin, Albumin, Fibrin, Silk fibroin) | Polyamides (Poly-y-glutamate) |
| Polyesters (from higher plants) | Resin (Shellac) | Polyanhydrides (Polyphosphate) |

(Doppalapudi *et al*., 2015)

There are some examples of biodegradable film that are used in cosmetics, pharmaceutical, and food products as following:

Li *et al*. (2006) studied the konjac glucomannan (KGM)–chitosan (CHI) blend film incorporating nisin to improve antimicrobial efficacy. They found that the blend film (KGM/CHI; 8:2 (w/w)) incorporating nisin at 42,000 IU/g can improve antimicrobial activity when compared with konjac glucomannan incorporating nisin.

Huang *et al*. (2015) presented the application of konjac glucomannan (KGM). The $Ca(OH)_2$ treated KGM ($Ca(OH)_2$-KGM) film exhibited more favorable properties of swelling, tensile strength, and elongation compared with the KOH-treated KGM (KOH–KGM) film, and had a suitable water vapor transmission rate. The alkali-treated KGM films exhibit suitable chemical-physical properties for use as wound dressings, and effectively accelerate wound repair.

Petriccione *et al*. (2015) showed that the chitosan film can be used as protective barriers to reduce respiration and transpiration rates and to retard color changes when coating the surface of sweet cherry during postharvest life.



### 2.4.2 Mechanism of film formation

Biopolymers are generally gelatinized to produce film-forming solutions. When the biopolymer dispersed in aqueous solutions, solvent removal is required to achieve solid film formation and control of its properties (Hernandez-Izquierdo and Krochta, 2008). The rate of drying and temperature influence the crystallinity of biopolymer films and mechanical properties**.**

The films can be formed via two steps (Figure 2.13); first, a wet process in which biopolymers are dispersed in a solution casting followed by evaporation of the

solvent. Second, a dry process, relies on the thermo plastic behavior exhibited by d polysaccharides and some proteins at low moisture levels in compression molding and extrusion. For the dry process, heat is applied to the film-forming, increasing the temperature to above the melting point of the film-forming materials to cause them to flow.

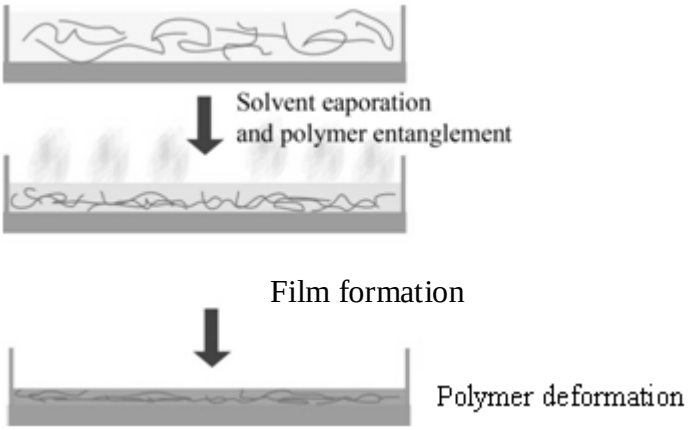



**Figure 2.13** Stage of film formation from aqueous polymer dispersion (modified from (Onions, 1986)

At the early stages of drying, the rate of solvent evaporation is importantly independent of the presence of polymer. The rate of evaporation depends upon on the ratio of surface area to volume of the film, the rate of air flow and the vapor pressure. As the viscosity and $T_g$ (glass transition temperature) increase, free volume decreases, and the rate of solvent evaporation depends on rapidly the solvent molecule scan reach the surface of the film. The rate of solvent loss is controlled by the rate of diffusion of the solvent through the film (Nussinovitch, 2009). Therefore, drying condition is a crucial step to produce film with an acceptable property.

### 2.4.3 Biopolymer film for encapsulation

Biopolymers can be sustainable, carbon natural are always renewable, because they are made from plant materials which can be grown indefinitely. Therefore, the use of biopolymers would create a sustainable industry. The biopolymers in bio-based (starch, pectin, dextran, chitosan, gelatin and glucomannan) are commonly used for coating material in many fields such as agriculture, food industry and medicine as biodegradable plastics (Huang *et al*., 2005), composites and films (Córdoba *et al*., 2008) and surface coatings (Patil *et al*., 2008).

Encapsulation has been widely used in many industrial applications including food, medical cosmetic and agriculture. Encapsulation is a process to entrap core material within a carrier material. The main propose of encapsulation is to protect core materials from environmental conditions (heat and humidity) and controlling compound release (Bansode *et al*., 2010). There are various form to encapsulate compound in dry powder such as tablets, capsules, micro-particles, beads, gel and films (Agnihotri *et al*., 2004).



The type of film for coating or entrapment the substances can be divided as two types including, immediate-release film coatings and modified-release film coatings. The polymers that are used in immediate-release film coating formulations are generally water soluble. The characteristic of polymer are form strong, flexible film, facilitate ease of processing and permit rapid drug or bioactive compound release from dosage forms. Film coatings used for modified-release application can be divided to two main categories, as delayed-release (or enteric) coating and extended-release coatings. The polymer is used for modified-release must have a specific properties for solubility (Qiu and Zhang, 2000).

The release of bioactive agents may be either, diffusion-controlled (diffusion of drug through a rate-controlling barrier/matrix), degradation controlled (chemical or physical breakdown of the matrix leads to bioactive agent release) and via an environmental trigger (change in pH, ionic strength, or pressure tailors the release of the bioactive agent) (Lin and Metters, 2006; Pal *et al*., 2013). The mechanisms of release can be physical or chemical in nature, but always relate form of diffusion. The diffusion is the movement of individual molecules of a substance through a semi permeable barrier from an area of higher concentration to an area of lower concentration (Lin and Metters, 2006). The degradable polymer and systems loaded with the compound of interest, the release will be controlled by the cleavage of the polymer bonds within the network, even though the diffusion of the liberated therapeutic compound may be rate-limiting (Gupta *et al*., 2002; Pal *et al*., 2013). For non-biodegradable systems, release will be diffusion-controlled. On the other hand, the osmotic pressure or matrix swelling effect on compound release (Leong and Langer, 1988; Pal *et al*., 2013).

The delivery systems of swelling-controlled may be start with dry and followed by swelling through absorbing dissolution media. It is showed that same trend of the swelling and the aqueous solvent content within the drug formulation that was increased in the drug dissolution and allowing the drug diffusion through out of the swollen network into the environment. There are numerous examples of food-related biopolymers used in the swelling-controlled-release of therapeutic compounds (Lin and Metters, 2006; Pal *et al*., 2013).

There are various types of biopolymers (starch, pectin, dextran, chitosan, gelatin and glucomannan) that are commonly used for coating material and film. The

biopolymers that are selected to use in this research include konjac glucomanan and chitosan. Because, they are not harmless to use and they are widely used in food, medicine and agriculture.

### 2.4.3.1 Konjac glucomannan

Konjac glucomannan (KGM) extracted from the corm tissues of *Amorphophallus* konjac plant (Nishinari *et al*., 1992). KGM is a high molecular weight polysaccharide, consisting of β-D-glucose and β-D-mannose residues in a molar ratio of 1: 1.6 linked by β-1,4-glycosidic bonds, the acetyl groups along the KGM backbone are located, on average, every 9–19 sugar units at the C-6 position (Figure 2.14) (Nishinari *et al*. 1992; Katsuraya *et al*., 2003).The chemical properties of KGM include non-ionic (neutral), and a good water solubility due to high amount of the hydroxyl group in molecule, make it easy to dissolve in water and to bring about high viscosity and forming thick hydrocolloids even if used at low concentration (Impaprasert *et al*., 2014; Li *et al*., 2006).



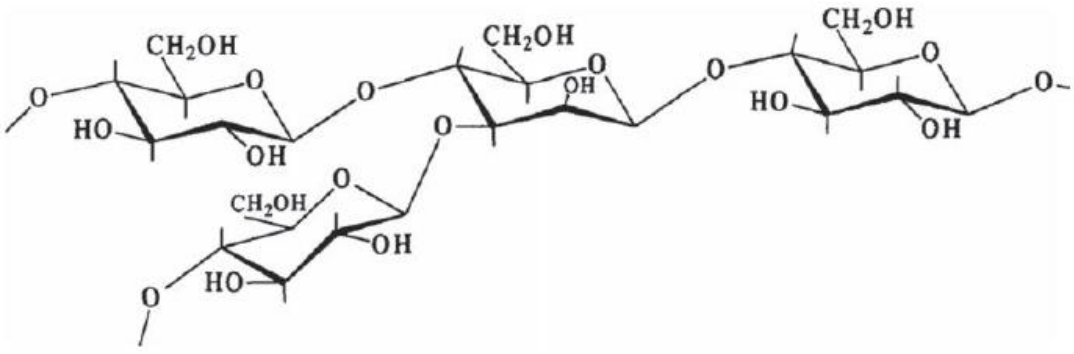



**Figure 2.14** Chemical structure of KGM (Lin *et al*., 2012)

KGM film is formed via intermolecular interactions of the hydrogen bonding and self-entanglement (Cheng *et al*., 2002; Luo *et al*., 2013). KGM film has

good properties such as to exhibit enhanced water vapor barrier when compared to other polysaccharide films (Cheng *et al*., 2002; Huang *et al*., 2002) Moreover, the properties of KGM films may be easily modified by blending with other compatible polymers. The presence of C-O group in KGM structure opens the possibility of combining KGM with other polymers through hydrogen bonding (Xu et al., 2007).

Some research have been carried out to improve the properties of KGM by alkaline treatment for deacetylation (Cheng *et al*., 2002; Li and Xie, 2006). Luo et al. (2013) studied the effect of alkali on deacetylation of KGM. The result found that when the water solubility and the self-aggregation were decreased effect to smaller molecular chains of the expansion degree (Figure 2.15). Moreover, in distilled water, the molecular chain of negative charge density showed larger than molecular chain size in alkali solution. Because of the alkali solution had ability to activate the electrons to move close to the hydroxyl group of the oxygen atom and generated oxy anion. Therefore, the expansion of the molecular chain was restricted by the relation force of the electrostatic repulsion, the ionized molecular chain and hydroxide ion in this solution. This is indicated to counteraction relationship of the hydration of water and alkali inductive effect. The alkali effect acting as an electrolyte and damaged the hydrogen bond of molecular chain of hydration and molecule of water that reflect to the expansion and suppression of molecular chain.

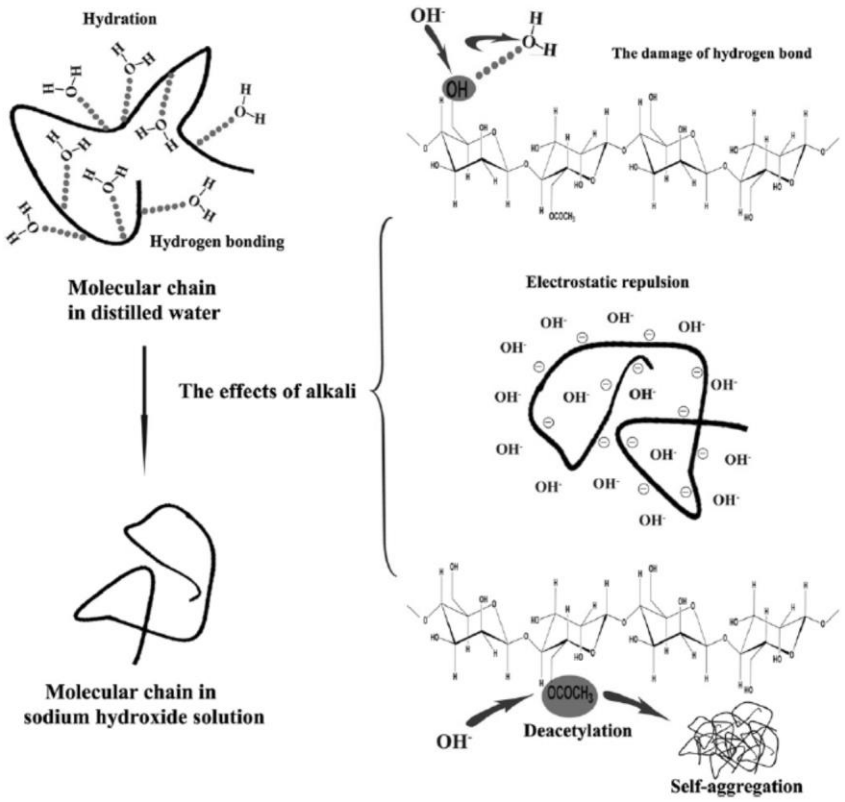



**Figure 2.15** The schematic diagram of the alkaline effects on the molecular chain (Luo *et al*., 2013).

There are many studies showed that interactions of KGM with other biopolymers such as chitosan, sodium alginate, poly (vinylpyrrolidone) and sodium carboxymethyl cellulose have produced films with enhanced tensile strength, breaking elongation, and thermal stability (Xiao *et al*., 2000; Xiao *et al*., 2001). The blending of biopolymers between KGM and chitosan can enhance mechanical properties of the film.

Ye *et al*. (2006) reported that the suitable ratio between KGM and chitosan was aiming at the highest miscibility and blend homogeneity because of the strong intermolecular hydrogen bonds existing between the amino groups of chitosan and the hydroxyl groups of KGM in the blended films.

### 2.4.3.2 Chitosan

Chitosan, a polymer of 1,4 linked b-D-glucosamine and N-acetyl glucosamine units, is prepared by deacetylation of chitin (Figure 2.16). Chitosan has been proved to be nontoxic, biodegradable, biofunctional, biocompatible and have antimicrobial characteristics (Kim *et a*l., 2011; Tharanathan and Kittur, 2003). The degree of deacetylation has effect on solubility, the distribution of acetyl groups along the main chain, the molecular weight and the nature of the acid used for protonation (Pillai *et al*., 2009). The chitosan is soluble in dilute acid solutions below pH 6.0 due to the presence of amino groups. Moreover, the chitosan molecular weight can also affect the quality of the final film such as elasticity or brittleness (Leceta *et al*., 2013; Park *et al*., 2002).

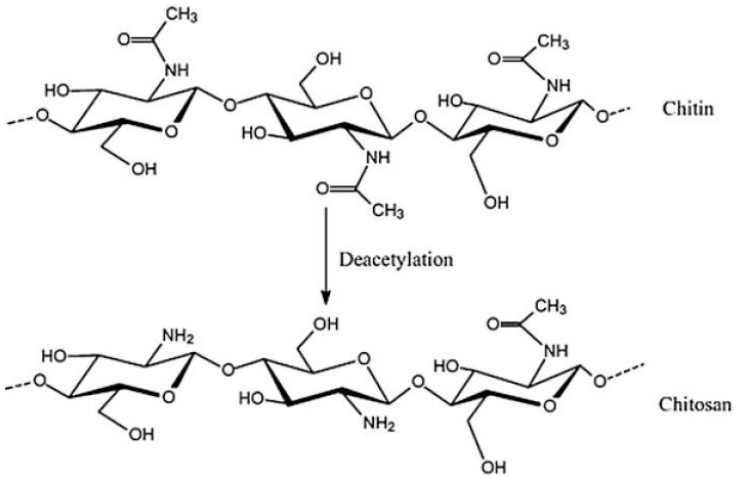



**Figure 2.16** Chemical structure of chitin and chitosan (Kumar, 2000)

Chitosan films obtained by dry phase inversion were prepared from an aqueous solution of chitosan in acetic acid. It have a good film-forming properties as a result of intra- and intermolecular hydrogen bonding (Pillai *et al*., 2009). Chitosan films are biodegradable, biocompatible, flexible, durable, strong, tough and hard to

break, have moderate values of water and oxygen permeability, decrease the respiratory rate of food and also inhibit the microbial growth (Ruiz-Navajas *et al*., 2013). The cationic amino groups on the $C_2$ position of the repeating glucopyranose units of chitosan can interact electrostatically with the anionic groups (usually carboxylic acid groups) of other polyions to form polyelectrolyte complexes(e.g. pectin, alginate, carrageenan, xanthan gum, carboxymethyl cellulose, chondroitin sulphate, dextran sulphate, hyaluronic acid) (Berger *et al*., 2004; Hamman, 2010). There has been a great interest in the protein drug-coating and when KGM was mixed with chitosan, the properties of films were improved (Campos *et al*., 2011; Puttipipatkhachorn *et al*., 2001).

**2.4.3.4 Application of konjac glucomannan and chitosan using in coating materials**

In present, the KGM and chitosan are defined as novel natural polymer materials for coating materials in various fields as follow and shown in Table 2.5.

Puttipipatkhachorn *et al*. (2001) prepared the films from different grades of chitosan varying in molecular weight and degree of deacetylation to study drugrelease from chitosan matrix films. The model acidic and basic drugs as salicylic acid and the ophylline were into cast chitosan films. Interaction between salicylic acid and chitosan, resulting in salicylate formation, whereas not found in theophylline-loaded chitosan films. The sustained release action of salicylic acid and theophylline from the high viscosity chitosan films was suggested that the swelling property.

Wang and He (2002) reported that a kind of alginate (ALG)–konjac glucomannan (KGM)–chitosan beads could be used as controlled release matrix. It

was observed that KGM could be contained within beads, and faintness hydrogen binding and electrostatic interaction existed between ALG and KGM.

Wang *et al*. (2008) presented carboxymethyl konjac glucomannan–chitosan (CKGM–CS) nanocapsules used for immobilizing l-asparaginase. The result showed that the particle size of encapsulation particle ranged from 100–300 nm and the efficiency of encapsulation reached to 68.0% while the concentrations of particle of CKGM nanocapsules and CS nanocapsules particle were 0.01%. In contrast, the immobilized enzyme (free l-asparaginase) was showed increase thermo stability and be favorable to use as acid and alkaline resistance to in the environment.

Yang *et al*. (2009) studied 5-ALA molecule in nano-particles made by chitosan (CNA) with ionic gelation method. CNA particles prepared at pH 7.4 and pH 9 of 5-ALA solutions with a concentration higher than 0.5 mg/ml showed a promising loading efficiency of up to 75%. The result explained that when CNA particles in higher pH 7.4 solution, the positive charge of 5-ALA molecule interact with chitosan with sodium tripolyphosphate while still maintain negative charge. This reaction aims to achieve high loading efficiency. There were more 5-ALA molecules bearing the negative charge that should have strong electrostatic attraction between the two molecules and achieved high loading efficiency.

**Table 2.5** Application of konjac glucomannan film, chitosan film and blend film

| Polymer | Application | References |
|---|---|---|
| Konjac glucomannan | Konjac glucomannan capsule for chronic stomach disease | (Wu and Shen, 2001) |
| | Edible film from konjac powder and application on coating 'Tuptimjun' java apple | (Hongkulsup *et al*., 2010) |
| | Enzyme entrapment | (Wang *et al*., 2008) |
| | Fruit coating | (Supapvanich *et al*., 2012) |
| Chitosan | Chitosan-based micro- and nanoparticles in drug delivery | (Agnihotri *et al*., 2004) |
| | Coating for the protection of amino acids | (Chiang *et al*., 2009) |
| | Encapsulate 5-aminolaevulinic acid to improve the detection of colorectal cancer cells in vivo | (Yang *et al*., 2009) |
| | Nanoparticles and microspheres for the encapsulation of natural antioxidants extracted | (Harris *et al*., 2011) |
| Glucomannan–Chitosan | Novel polyelectrolyte carboxymethylkonjacglucomannan–chitosan nanoparticles for drug delivery. I. Physicochemical characterization of the carboxymethyl konjac glucomannan–chitosan nanoparticles | (Du *et al*., 2005) |

From above researches, the konjac glucomannan and chitosan shown a potentiality for application to coating material. Because, they are non-toxic, can be used in various fields (food medicine and agriculture) and a good properties for protection the substance from environment factor such as selective gas permeability ($CO_2$ and $O_2$), enhanced water vapor barrier. However, most research presented to

used KGM and CHI in the microcapsule but only a few researchers have studied the KGM and CHI in film form for coating material. Therefore, this research is aiming to use KGM and CHI for entrapment the 5-aminolevulinic acid in film to improve the stabilized of 5-ALA in solid condition.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Raw materials

*Rhodobacter sphaeroides* suspension was supplied from Nutrition Co, Ltd. The suspension was centrifuged at 8000 rpm at 4 $^{o}$C for 30 min. The top clear solution (crude 5-ALA solution) was separated and kept under -20 $^{o}$C for further study.

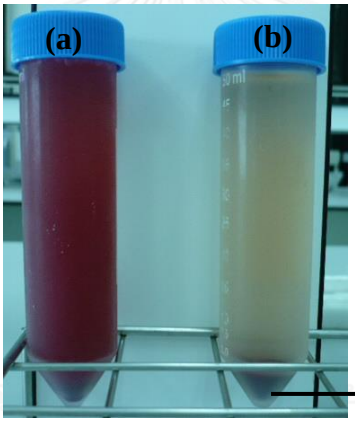



**Figure 3. 1** (a) *Rhodobacter sphaeroides* suspension
(b) The suspension after centrifugation process

Konjac glucomannan (KGM) with a molecular weight ranging from 200-2,000 kDa was purchased from Yunnan Gengyun Konjac Resources Developing Co., Ltd. (Kunming, China).

Chitosan (CHI) 90 % DD, with a molecular weight 60-80 kDa was purchased from Marine Bio resources Co. Ltd (Samutsakorn, Thailand).

### 3.1.2 Chemicals and reagents

**Table 3. 1** Chemicals for 5-ALA determination.

| Name | Company | Country |
|---|---|---|
| 5-aminolevulinic acid hydrocoride 98% | Sigma-Aldrich | USA |
| Fluorescamine | Sigma-Aldrich | USA |
| Trichloroacetic acid (TCA) (HPLC grade) | Merck | Germany |
| Acetonitrile Isocratic (HPLC grade) | Merck | Germany |
| Borate (A.R. grade) | Merck | Germany |
| Sodium hydroxide(A.R. grade) | Ajax Finechem | New Zealand |
| Hydrochloric acid (A.R. grade) | Sigma-Aldrich | USA |
| Acetylacetone (A.R. grade) | SD fine-chem Limited | New Zealand |
| 4-Dimethylaminobenzaldehyde | Ajax Finechem | New Zealand |
| Perchloric acid (A.R. grade) | Sigma-Aldrich | USA |

**Table 3. 2** Chemicals for separation process of 5-ALA using ion exchange chromatography

| Name | Company | Country |
|---|---|---|
| Sodium acetate (A.R. grade) | Ajax Finechem | New Zealand |
| Sodium hydroxide(A.R. grade) | Ajax Finechem | New Zealand |
| Hydrochloric acid(A.R. grade) | Sigma-Aldrich | USA |
| Acetic acid (A.R. grade) | Sigma-Aldrich | USA |

**Table 3. 3** Chemicals for preparation of 5-ALA in to film formation

| Name | Company | Country |
|---|---|---|
| Standard 5-aminolevulinic acid | Sigma-Aldrich | USA |
| Potassium hydroxide | Ajax Finechem | New Zealand |

**Table 3. 4** Apparatus

| **Instruments** | **Model** | **Company, country** |
|---|---|---|
| Electronic balance (4 digital) | BSA 2245 | Sartorius, Germany |
| pH meter | Cyberscan 1000 | Eutech, Singapore |
| Centrifuge | Universal 32R | DJB Labcare, Germany |
| Columns | C16 | Pharmacia biotech, Sweden |
| Adaptors | AC16 | Pharmacia biotech, Sweden |
| Peristaltic Pump | P-1 | Pharmacia biotech, Sweden |
| Fraction collector | RediFrac | Pharmacia biotech, Sweden |
| Water activity meter | - | AquaLab, USA |
| Magnetic stirrer | Steroma G | Steroglass, Italy |
| High-Performance Liquid Chromatography with fluorescence detection (HPLC/FLD) | 1100 series | Agilent, USA |
| Spectrophotometer | M.4001/4 | Genesys™, USA |
| Fourier transform infrared (FT-IR) spectroscopy | Spectrum One | Perkin-Elmer, USA |
| X-ray diffraction (XRD) pattern | D5005 | Siemens AXS, Germany |
| Field emission scanning electron microscope | JSM-7610F | JEOL, Japan |
| Micrometer | Dial Thickness Gauge 7301 | Mitutoyo, Japan |
| Hot air oven | FD 240 | Binder, Germany |

## 3.2 Methods

### 3.2.1 Determination of 5-ALA content from *Rhodobacter sphaeroides* suspension

The crude 5-ALA solution separated from *R. sphaeroides* suspension was taken to determine 5-ALA using colorimetric method (Mauzerall and Granick, 1956) and HPLC-fluorescence method (Namjoshi *et al*. 2007).

#### 3.2.1.1 Colorimetric method

The reaction mixture contained 2 ml of crude 5-ALA solution obtained after centrifugation, 1 mL of 1M sodium acetate buffer (pH 4.67) and 0.5 mL of acetylacetone were added to the mixture. The mixture was then boiled in a water bath (100°C) for 15 min. After cooling, 2 mL of the reaction mixture was mixed with 2 mL of freshly prepared modified Ehrlich's reagent, which contained 1 g of $\rho$-dimethyl-aminobenzaldehyde in 42 ml of glacial acetic acid and 8 ml of 70% perchloric acid. The absorbance of the mixture at 553 nm was measured by spectrophotometer (M.4001/4; GENESYS$^{TM}$, CA) at room temperature. The derivatization scheme between acetylacetone and 5-ALA is shown in Figure 3.2.

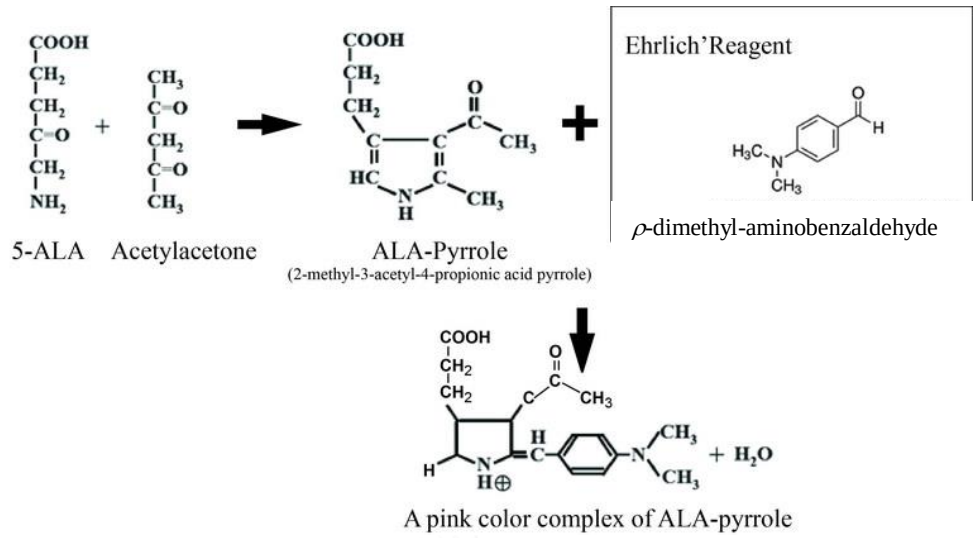



**Figure 3. 2** The derivatization scheme between acetylacetone and 5-ALA

### 3.2.1.2 HPLC-fluorescence method

The condition for running HPLC-fluorescence is shown in table 3.5.

**Table 3. 5** Condition of HPLC-fluorescence method for 5-ALA content determination

| | |
|---|---|
| Column: | Phenomenex Jupiter $C_{18}$ 300 $A^o$(5µm, 150 mm x 4.6 mm) |
| Guard column | wide pore $C_{18}$ |
| Flow Rate: | 1 mL/min |
| Injection volume | 10 µL |
| Detection: | Fluorescence detector |
| Mobile phase | 30% acetonitrile (0.1% TFA) : Water (0.1% TFA) |
| Excitation/emission wavelengths | 395 / 480 nm |
| Retention Time (min) | 9.75 |

**Derivatization and preparation of standard calibration curve**

The derivatization mixture was performed by mixing 200 μL of the standard 5-ALA concentration in range 0.1-6.4 ppm with 200 μL of 0.1% fluorescamine solution and 600 μL of 0.1 M borate buffer. The derivatization of 5-ALA samples was analyzed by reacting with fluorescamine for 20 min at room temperature.

**Determination of 5-ALA content**

The derivatization mixture was performed by mixing 200 μl of the 5-ALA sample with 200 μL of 0.1% fluorescamine solution and 600 μL of 0.1 M borate buffer. The derivatization of 5-ALA content was analyzed by reacting with fluorescamine for 20 min at room temperature. The 20 μl of derivatization of 5-ALA were injected into an Eclipse XDB $C_{18}$ column (5μm, 150 mm × 4.6 mm; Agilent Technology, CA). The elution was performed at a flow rate of 1 mL/min and the excitation/emission wavelengths were 395/480 nm. The mobile phase was30% acetonitrile:70% water (0.1%TFA) with a retention time of 9.24 min for fluorescamine derivatives of 5-ALA (Namjoshi *et al*. 2007). The condition for running HPLC-fluorescence is shown in table 3.5. The derivatization scheme between fluorescamine and 5-ALA is shown in Figure 3.3.

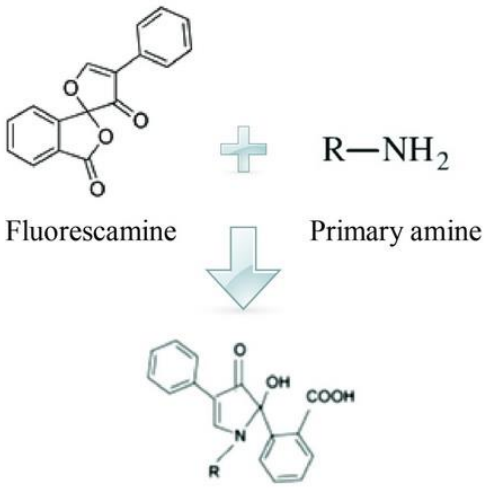



**Figure 3.3** The derivatization scheme between fluorescamine and 5-ALA (Stockert, 2008)

### 3.2.2 Separation of 5-ALA using ion exchange chromatography (IEC)

#### 3.2.2.1 Selection of a suitable pH of eluent

**Resin preparation**

The Dowex 50X8 cation resin (Figure 3.4) was soaked in 4 N NaOH for 24 h. After that, the cation resin was rinsed with deionized water (DI water), and then the cation resin was soaked in 4 N HCl for 6 hours, 1 N HCl for 6 h. and rinsed with DI water. The properties of Dowex 50X8 cation resin is shown in Table 3.6.

**Table 3. 6** Properties of Dowex 50X8 cation resin

| Cation resin | (Dowex 50X8) |
|---|---|
| Free Ion form | $H^+$ |
| Matrix | styrene-divinylbenzene |
| Functional group | Sulfonic acid ($SO_3^-$) |
| Total Exchange Capacity | 1.7 meq/mL |
| Water retention capacity | 50-58 % |
| Particle size | 200-400 mesh |
| pH range | 0-14 |

Source: The Dow Chemical Company

**Figure 3.4** Dowex 50X8 cation resin

The pH of crude 5-ALA solution was adjusted from 7.81 down to 4.5 with conc. HCl, and then it was filtered with 0.45 μm syringe filter (Polyethersulfone :PES). The starting buffer pH was chosen for substances to be bound to the exchanger are charged. The starting pH was at least 1 unit below the isoelectric point of 5-ALA (pI~6.2) for cation exchangers to facilitate adequate binding.

Two gram of cation resin was packed into glass column (0.7 x 30 cm). A gradient elution removes 5-ALA by a gradual increase in ionic strength. Sodium acetate buffer (20-400 mM) pH 4, 4.5, 5 and 5.5, were used as eluent. 0.1 ml of crude 5-ALA solution (pH 4.5) was loaded on the top of column and allowed to drain

through. The eluent was added and the eluate was collected every 2 min (1 fraction = 1 mL). 5-ALA content was determined by colorimetric method. The presence of ALA was confirmed by Thin Layer Chromatography (TLC), performed using butanol: acetic acid: water (12:3:5) and revealed with 0.2 % ninhydrin (Mauzerall and Granick, 1956). The flow of the experiment is showed in Figure 3.5.

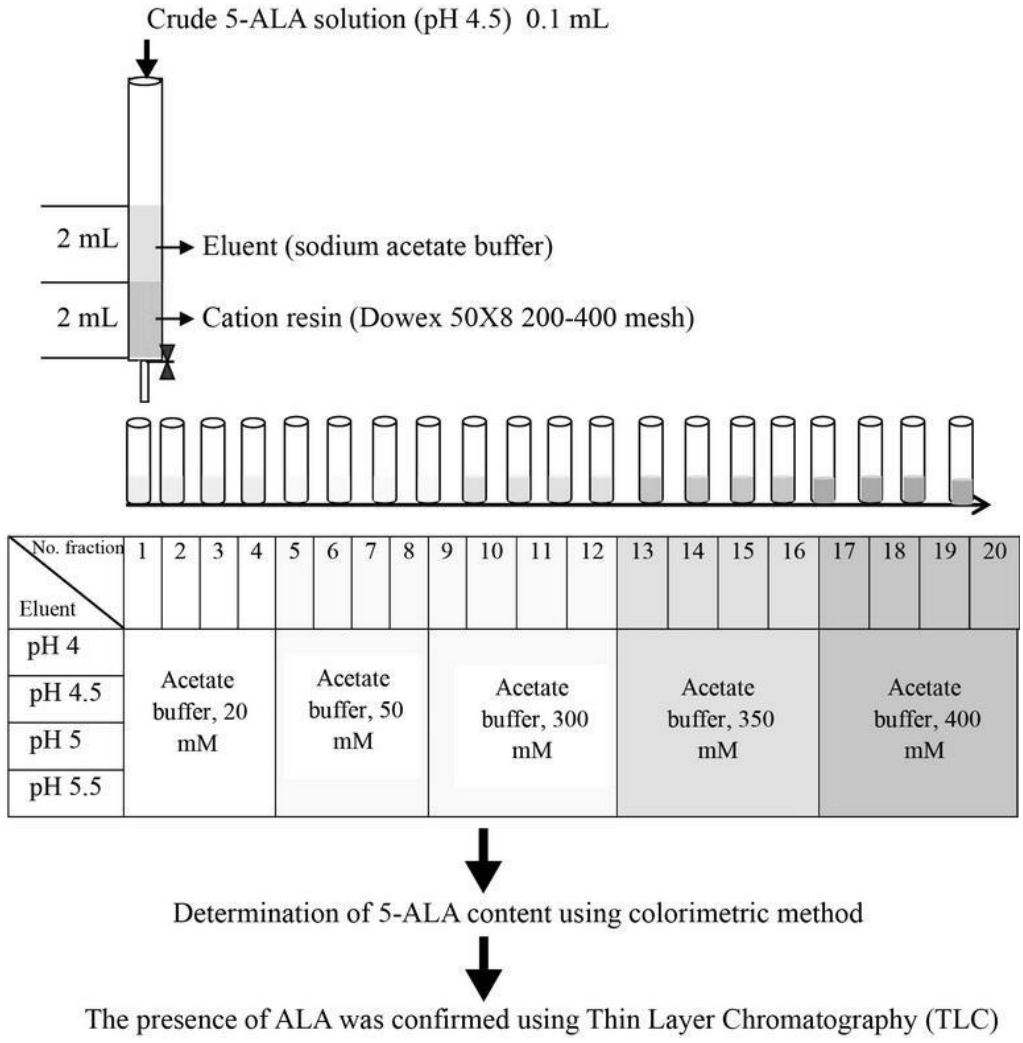



**Figure 3.5** Flow chart for experiment rafting up for selection of suitable pH of eluent

### 3.2.2.2 Selection of sample loading volume

The cation resin was packed into a column (C16/40) with bed volume at 50 mL. The initial volume of crude 5-ALA solution was varied to 10, 15 and 20% of bed volume. Then it was loaded into the column. The fraction was corrected every 5 min up to 30 fractions (flow rate = 1.5 mL/min). The 5-ALA content was determined using colorimetric method and the result was confirmed using HPLC-fluorescence method.

### 3.2.2.3 Elution process

Two type of elution processes were performed by controlling the ionic strength or pH by using a linear or stepwise elution. In this study, the elution process by changing in ionic strength using a linear elution. This process is recommended to use when starting with unknown sample or when a separation of closely spaced protein peaks is a concern (Bollag *et al*., 1996). With increasing the ionic strength, it increases competition of charge group of eluent on ion exchanger and reduces the interaction between ion exchanger and the sample substances. The components of a gradient elution system are shown in Figure 3.6.

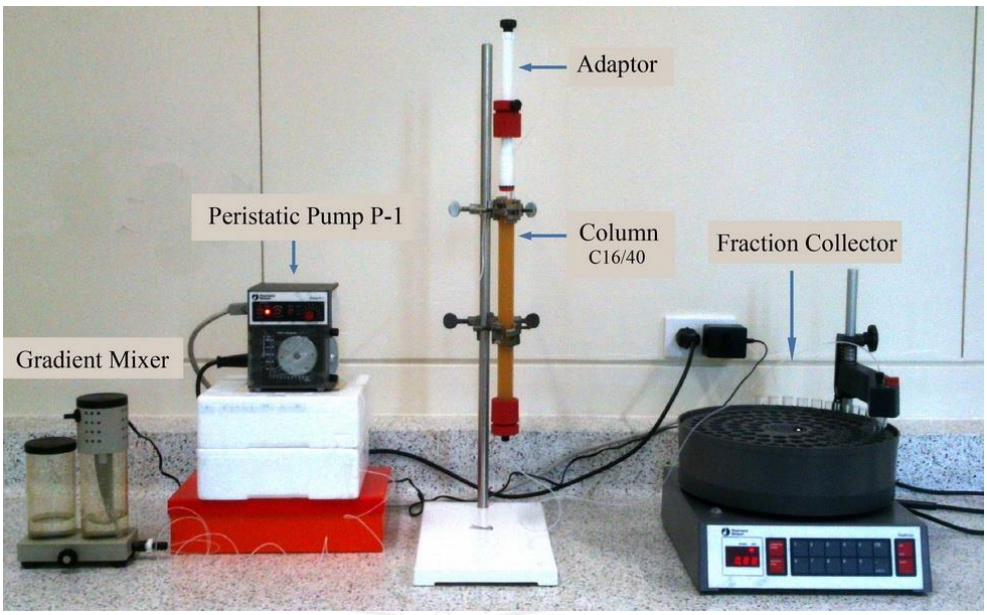



**Figure 3. 6** Auto collecting sample from column by changes in ionic strength using a linear elution.

**Steps for elution process**

The buffer, 0.01 M acetate buffer pH 4.5, was used to adjust pH of column to 4.5 with 3-4 beds volume or until the pH of column was stable. After that the crude 5-ALA solution was loaded at volume of 10% of bed volume. One bed volume of 0.01 M acetate buffer pH 4.5 was loaded into, the column for washing that have no charge or opposite charge with cation resin, and then the gradient elution removes 5-ALA by a gradual increase in ionic strength. Elution gradient started using 0.01 M acetate buffer (pH 4.5) to 1 M acetate buffer (pH 4.5) with gradient mixer. Fraction was collected at every 5 min up to 30 fraction (flow rate = 1.5 mL/min). The column used 2 bed volume of 1 M acetate buffer (pH 4.5) and then deionized water, 4-5 bed volume. The column is re-equilibrate after washing before the next separation by using buffer (0.01 M acetate buffer, pH 4.5) until the pH of column reach to 4.5. The

5-ALA content was determined using colorimetric method and the result was confirmed using HPLC-fluorescence method stated in Section 3.2.1.2.

### 3.2.3 Study of a suitable condition for fabrication of film containing 5-ALA

KGM powder after being swelled in distilled water at room temperature (26± 1ºC) for 3 h. was adjusted to 1% (w/v). Then, 0.05 M KOH was added to the solution at 0.14% (v/v). This mixture (KGOH) was stirred with a magnetic stirrer for 30 min. Chitosan powder was dissolved in 1% acetic acid and the solution was adjusted to 1 % (w/v).

The KGOH solution, chitosan (CHI) and the blend polymers (KGOH/CHI) were mixed at different ratios 100/0, 80/20, 75/25, 50/50, 25/75, 20/80 and 0/100 (% w/w) to make the films. The blend polymers were coded as KGOH, K80C20, K75C25, K50C50, K25C75, K20C80 and CHI, respectively. The KGM was also made into film and used as the reference to compare with the KGOH. After that, 0.2 mg of 5-ALA were added to each of the solutions. The mixture solutions were stirred for 30 min. Finally, the mixed solution with 5-ALA was cast into a clear acrylic (5 cm x 10 cm) plate and dried by hot air oven at 40°C for about 18 h. The finished intact film was peeled off from the acrylic plate. The films without 5-ALA were prepared as described above for comparing with the films containing 5-ALA. All films were kept in a desiccator until further analysis.

#### 3.2.3.1 Determination of the 5-ALA entrapment efficiency

All films containing 5-ALA were dissolved in acetate buffer (pH 5.5) (final volume 50 mL) and shaken at 25 ºC for 1 h. The sample was centrifuged at1000×g for 20 min. The fluorescence derivatization method of 5-ALA was prepared by reaction with fluorescamine (Namjoshi *et al*., 2007). The amount of 5-ALA was determined using HPLC (1100 series, Agilent Technology, CA) equipped with a fluorescence detector (FLD G1321A, Agilent Technology, CA). The HPLC-fluorescence method according to 3.2.1.2.

#### 3.2.3.2 Characterization and determination of properties of films with and without 5-ALA

**Fourier transform infrared (FT-IR) spectroscopy**

The interaction in all films with and without 5-ALA was confirmed using the Fourier transform infrared (FT-IR) spectrometer (model Spectrum One, Perkin-Elmer, Waltham, MA). The films were scanned in range 4000-400 $cm^{-1}$ in transmission mode using a KBr-pellet method. Data were collected by averaging 64 scans with a resolution of 4 $cm^{-1}$ at 25 ºC.

**X-ray diffraction (XRD) pattern**

The X-ray Diffraction (XRD) pattern of the films with and without 5-ALA were examined using an X-ray diffractometer (Bruker AXS Model D8 Discover) equipped with Cu Kα radiation at 40 kV and 40 mA. The diffraction angle ranged from $2\theta = 5^{o}$ to $45^{o}$ at $0.02^{o}$ intervals with a scanning rate of $0.5^{o}$/sec.

**Film thickness and morphology**

The films were conditioned at 25°C and 52% relative humidity (RH) for 48 hours before determination of thickness and morphological observation. Film thickness was measured using a micrometer (Dial Thickness Gauge 7301, Mitutoyo, Tokyo, Japan). Reported thickness was the mean value of seven measurements. The cross-section of the films with and without 5-ALA was characterized using the field emission scanning electron microscope (JSM-7610F, Tokyo, Japan) using an accelerating voltage of 2 kV. All film was evaluated at 2000x and 10000x magnification.

**Water absorption and water solubility of film**

The water absorption of film was determined using the method described by Mahasukhonthachat *et al*. (2010) with modification. The water solubility of film was determined according to Han *et al*. (2012) and Xiao *et al*. (2015) with some modification. The film (0.10 g) was dispersed in distilled water (24.9 mL) with shaker at 25 °C for 1 h. The solution was then centrifuged at 1000×g for 20 min at 25 °C. The sediment was weighed (W1). The supernatant was dried at 100°C and the weight (W2) was measured. The water absorption and solubility were calculated as followed equation.

$$\text{Water absorption ( g water/g dry weight basis)} = \frac{[\text{W1-(W0-W2)}]}{\text{W0-W2}} \quad (1)$$

$$\text{Solubility (\%)} = \frac{\text{W2} \times 100}{\text{W0}} \quad (2)$$

Where W0 is the initial dry weight of the film.

### 3.2.4 Determination of stability of the 5-ALA in film

The film containing 5-ALA was kept in aluminum laminated bag (Figure 3.7) and was stored at -20 ±1, 4±1and 28±1$^{o}$C for 40 days. The sample was taken out to determine 5-ALA content using HPLC-fluorescence method (see section 3.2.3.1).

**Figure 3.7** The films containing 5-ALA was kept in aluminum laminated bag

### 3.2.5 Statistical analysis

All experiments were repeated three times (replicates). Data was shown as mean values with standard deviations. Statistical analysis was carried out using SPSS (version 17.0 software; IBM SPSS, Chicago, IL). Analysis of variance (ANOVA) was performed and Duncan's new multiple range tests was used to determine difference between means ($p \leq 0.05$).

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Determination of 5-ALA content from *Rhodobacter sphaeroides* suspension

The concentration of 5-ALA in crude solution of *R. spaeroides* from five different production lots was determined using colorimetric method and HPLC-fluorescence. The results are shown in Table 4.1.

**Table 4.1** The 5-ALA content in the crude 5-ALA solutions by using colorimetric method and HPLC-fluorescence

| Crude 5-ALA solution | 5-ALA (mg/L) | | Ratio of 5-ALA determined using colorimetric & HPLC |
|---|---|---|---|
| | Colorimetric method | HPLC-fluorescence | |
| Sample 1 | 0.797 ± 0.082 | 0.730 ± 0.001 | 1.09 |
| Sample 2 | 1.513 ± 0.061 | 1.302 ± 0.009 | 1.16 |
| Sample 3 | 1.295 ± 0.082 | 1.130 ± 0.015 | 1.15 |
| Sample 4 | 1.011 ± 0.102 | 0.787 ± 0.014 | 1.28 |
| Sample 5 | 1.009 ± 0.029 | 0.668 ± 0.026 | 1.51 |
| Average | 1.125 ± 0.277 | 0.923± 0.200 | 1.23 |

From the result shown in Table 4.1, the content of 5-ALA from each sample of crude 5-ALA solution was different as the samples were collected from different lots. It was quite clear that the content of 5-ALA determined using colorimetric method was higher than using HPLC-fluorescence method in all samples. The

average ratio of 5-ALA content between colorimetric method and HPLC-fluorescence was 1.23 (Table 4.1).

The principle of colorimetric method proposed by Mauzerall and Granick (1956) is a reaction of acetyl acetone with amino group of 5-ALA to form the ALA-pyrrole (2-methyl-3-acetyl-4-propionic acid pyrrole) at temperature at 100 °C. Then, the ALA-pyrrole reacted with the 4-(dimethylamino) benzaldehyde (DMAB) in the mixture of perchloric acid and acetic acid to form a pink colored compound (Figure 4.1).

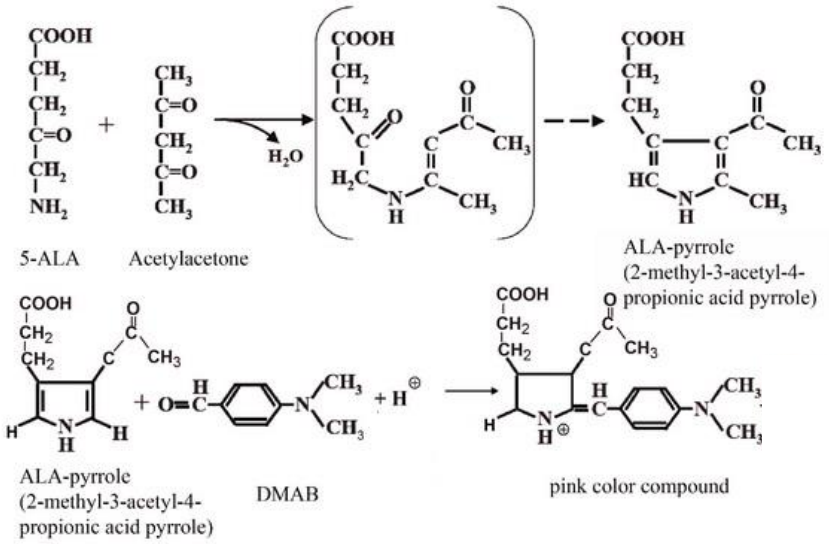



**Figure 4. 1** Reaction scheme for derivatization of 5-ALA using acetyl acetone (Mauzerall and Granick, 1956)

In the production of 5-ALA by *R. sphaeroides*, there are other compounds that was coexisting in a fermentation broth during microbial growth such as glycine, peptides, amino acids, saccharide, organic acids, porphyrin, carotenoid and ingredient of culture medium (Kim *et al*., 2001; Okada *et al*., 2012). Hence, the crude 5-ALA solution, separated from *R. sphaeroides* suspension, may contain other

compounds that may have similar structure like 5-ALA (e.g. glycine, amino acids), and the compounds that consist of pyrrolein the structure (e.g. porphyrin, porphobilinogen). Therefore, in colorimetric method, it is possible that the 5-ALA-like compounds interferences with colorimetric determination. As a result, the 5-ALA content was over estimated (Tomokuni *et al*., 1992).

For the HPLC-fluorescence method, the fluorescamine was used to react with 5-ALA to form fluorescent derivatization of 5-ALA (Namjoshi *et al*., 2007). It was well known that the fluorescamine directly react and specific with primary amines ($R\text{-}NH_2$) in aqueous solution at alkaline pH and room temperature to form the blue-green fluorescent pyrrolinones. Therefore, the 5-AlA content can be determined by comparison it with the standard 5-ALA.When the fluorescamine reacted with secondary amine, it may form non-fluorescent compounds (Figure 4.2) (Martin *et al*., 1988; Namjoshi *et al*., 2007; Stockert *et al*., 2008). Therefore, using the fluorescamine to convert the 5-ALA into the fluorescence derivatization products would be sensitive, rapid, accurate and reproducible method for HPLC-fluorescence (Namjoshi *et al*., 2007). The 5-ALA content can be calculated from the area under a curve. The chromatogram of crude 5-ALA solution compared with standard 5-ALA are shown in Figure 4.3b.

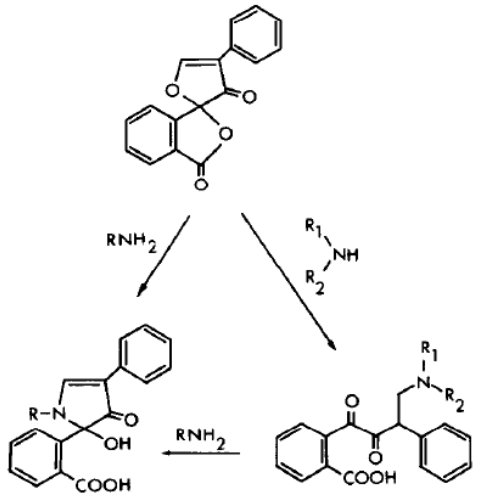



**Figure 4.2** Fluorescamine reaction with primary amines to give pyrrolinones (fluorescent) and with secondary amines to give aminoenones (non-fluorescent) (Martin *et al*., 1988).

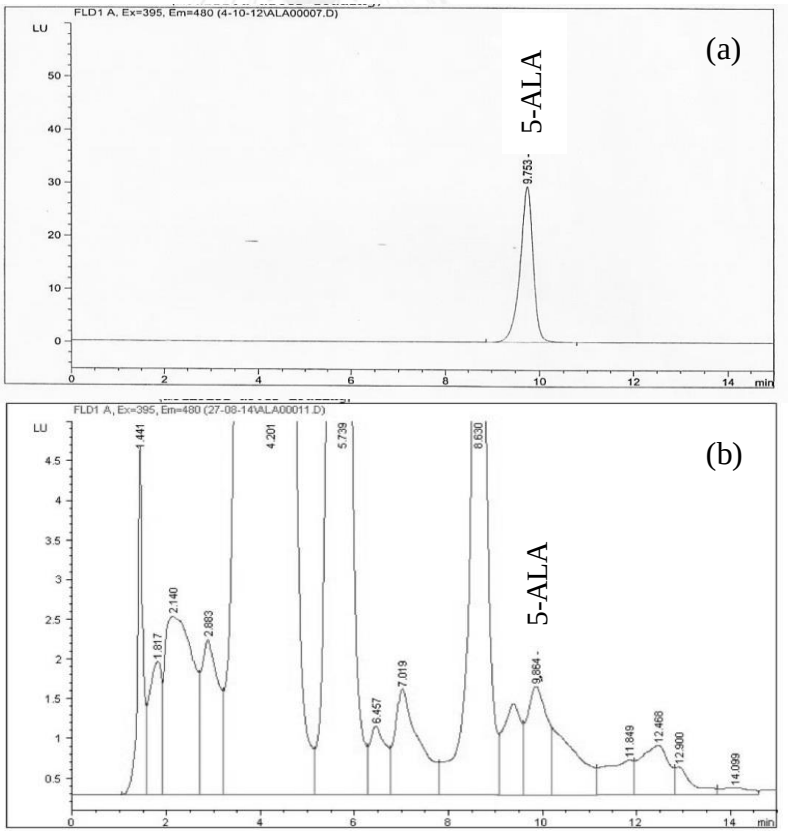



**Figure 4. 3** Chromatogram of (a) Standard 5-aminolevulinic acid

(b) Crude 5-ALA solution

The correlation of the 5-ALA content which determined using colorimetric method and HPLC-fluorescence method in crude 5-ALA solution was reasonable with $r^2$ = 0.8769 (Figure 4.4). It is possible due to the presence of interference of other compounds in crude 5-ALA solution. Although, the HPLC-fluorescence is an accurate and reproducible method but it has a limitation of complication of the apparatus. This reason leads to a favour of using the colorimetric method in many publications (Tomokuni *et al*.,1992; Venosa *et.al*., 2004) as it is convenient and accepted in commercial (Kauppinen *et al*. 2002). In this study, the initial step of separation of 5-ALA by Ion Exchange Chromatography (IEC), the colorimetric method was used to determine the 5-ALA content in the eluate and followed by a confirmation using HPLC-fluorescence in the final step.

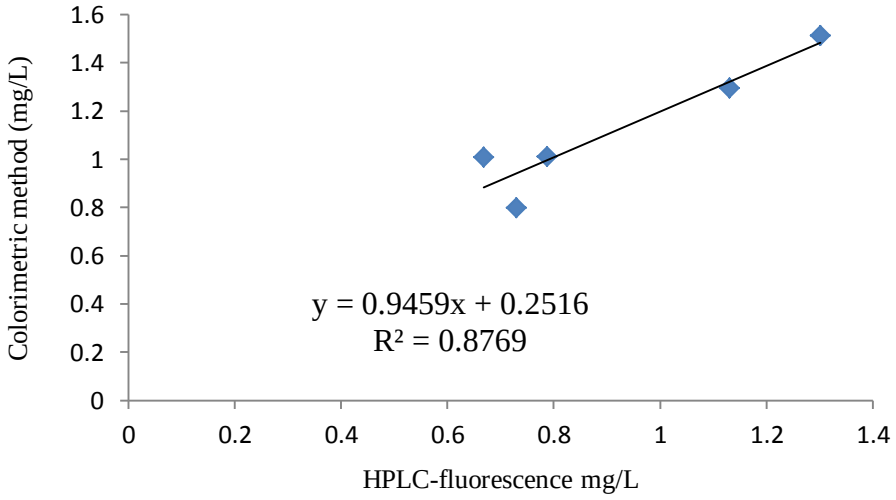



**Figure 4. 4** Comparison the 5-ALA content was determined using colorimetric method and HPLC-fluorescence method in crude 5-ALA solution.

## 4.2 Separation of 5-ALA using ion exchange chromatography

Ion exchange chromatography (IEC) is a powerful purification technique and the most widely used in the protein purification. The design of an optimum IEC for purification of target molecule depends on the numerous parameters including stationary phase (type of ion-exchange group, ion-exchange capacity etc.), elution buffer (type, pH concentration, etc.), type of elution (ionic or pH), mode of elution (isocratic, gradient, or stepwise) and operating variables (flow rate, sample loading, etc.). Separation of protein using IEC is depending on the charge of surface amino acids. Thus, the isoelectric point (pI) or acid dissociation constant (pKa) of charged molecules and the condition of elution buffer are important parameters in determination of the behavior of an ion exchange separation (Bolag *et al*. 1996; Hardin *et al*. 2009).

In this study, the IEC was used to separate the 5-ALA from the crude 5-ALA solution. The acid dissociation constant of 5-ALA were: $pKa_1$ = 4.05 and $pK_2$ = 8.3, and the zwitterions is the major species present in the pH range 5-7.5. In addition, the stability of 5-ALA is must be considered. It is stable under acidic condition (pH $\leq$ 5.5). At pH 5, about 10% of the 5-ALA molecules are cations whereas, at pH 7.3, about 10% of the 5-ALA molecules are anions (Novo *et al*. 1996; Bunke *et al*. 1999). Hence, the crude 5-ALA solution was adjusted the pH down to 4.5 in order to made the 5-ALA a completely positive charge. Therefore, the cation resin was selected and the sodium acetate buffer was used as elution buffer (pKa = 4.76; pH interval 3.7-5.6). Crude 5-ALA solution consists many unknown compounds (Figure 4.3). Therefore, changing in ionic strength using a linear elution was selected as the elution process

(Bolag *et al*. 1996; GE Healthcare, 2010). From the above condition, the parameters in separation process of 5-ALA from crude 5-ALA is as follows:

### 4.2.1 Selection of a suitable pH of elution buffer (eluent)

Sodium acetate buffer at pH 4.0, 4.5, 5.0 and 5.5, concentration of 20 – 400 mM was used an eluent. The colorimetric method was used to determine the 5-ALA in eluate and to confirm its purity with Thin Layer Chromatography (TLC). An ion exchange chromatography column with cation resin ($H^+$ form) was used for separation process.

Figure 4.5 shows the absorbance of each fraction using sodium acetate buffer (pH 4) as an eluent. The target molecule started to elute when the concentration of buffer reached 300 mM, and it still be eluted when the concentration of the eluent increased to 400 mM, which up to 10 fractions were collected. The purity of each fraction was determined using TLC. The presence of 5- ALA in eluate was confirmed by a reaction of 5-ALA with ninhydrin. The product of the reaction is yellow pigment. The purple color spot can be seen in all fractions which is indicated the impurity.

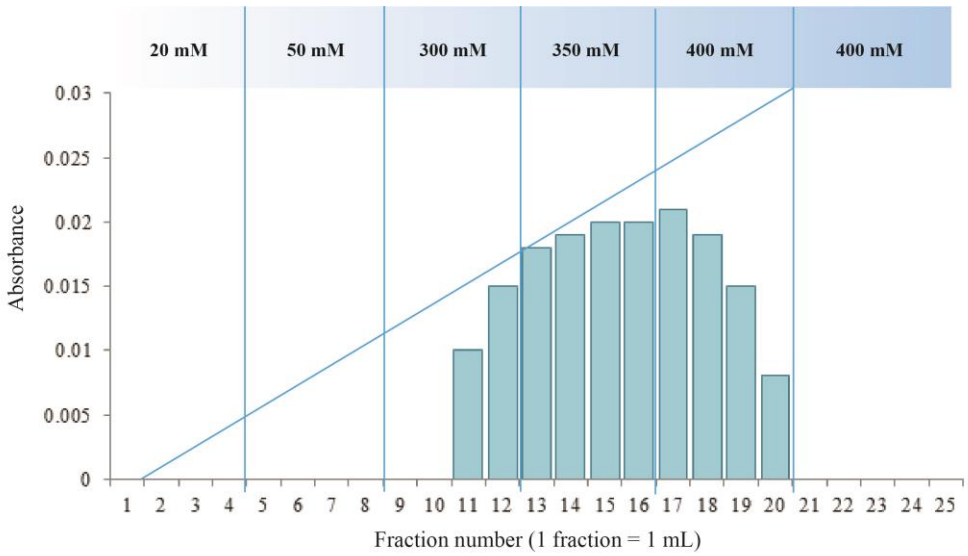



**Figure 4.5** The absorbance at 553 nm of each fraction eluted using sodium acetate buffer 20 mM, 50 mM, 300 mM, 350 mM and 400 mM (pH 4) as eluent.

When using sodium acetate buffer at pH 4.5 as eluent, the target molecules started to elute when the concentration of buffer reached 350 mM and all the fraction was collected up to 6 fractions. The impurity was not found in all fractions when it was determined the purity by TLC. The absorbance of each fraction using sodium acetate buffer at pH 4.5 is shown in Figure 4.6 and the result of TLC is displayed in Figure 4.7. When sodium acetate buffer at pH 5.0 and 5.5 were used as an eluent, the 5-ALA was not found in all collected fractions.

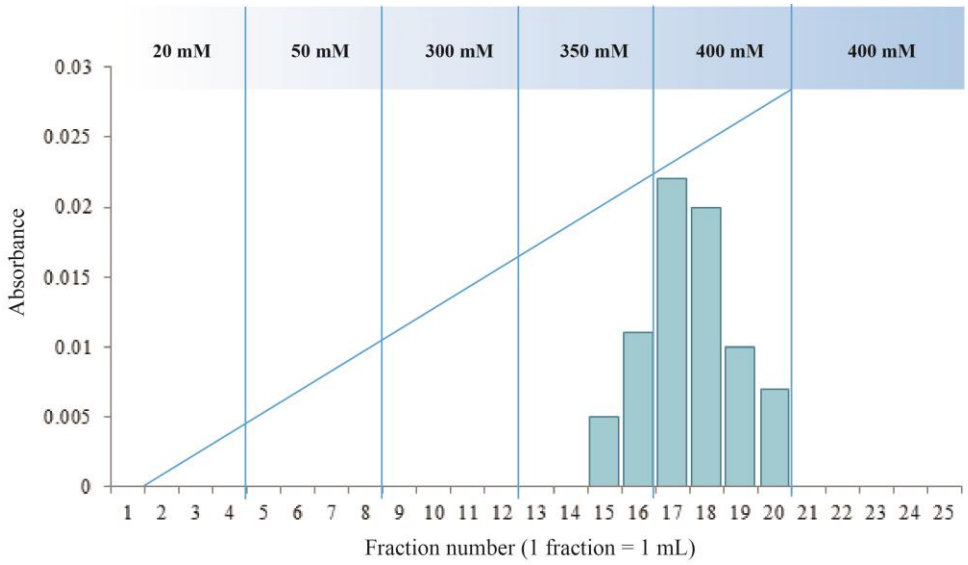



**Figure 4.6** The absorbance at 553 nm of each fraction eluted using sodium acetate buffer 20 mM, 50 mM, 300 mM, 350 mM and 400 mM (pH 4.5) as eluent.

The pH of eluent can affect the protein of interest in a mode of operation (bind-and-elute) with the ion exchange matrix (Ahamed *et al*. 2008). The crude 5-ALA solution was adjusted to pH 4.5, when loaded into the column, the 5-ALA molecules were retained in IEC by tight electrostatic interaction with cation resin. Therefore, 5-ALA was not found in the eluate when using sodium acetate buffer at pH 5.0 and 5.5 as an eluent.

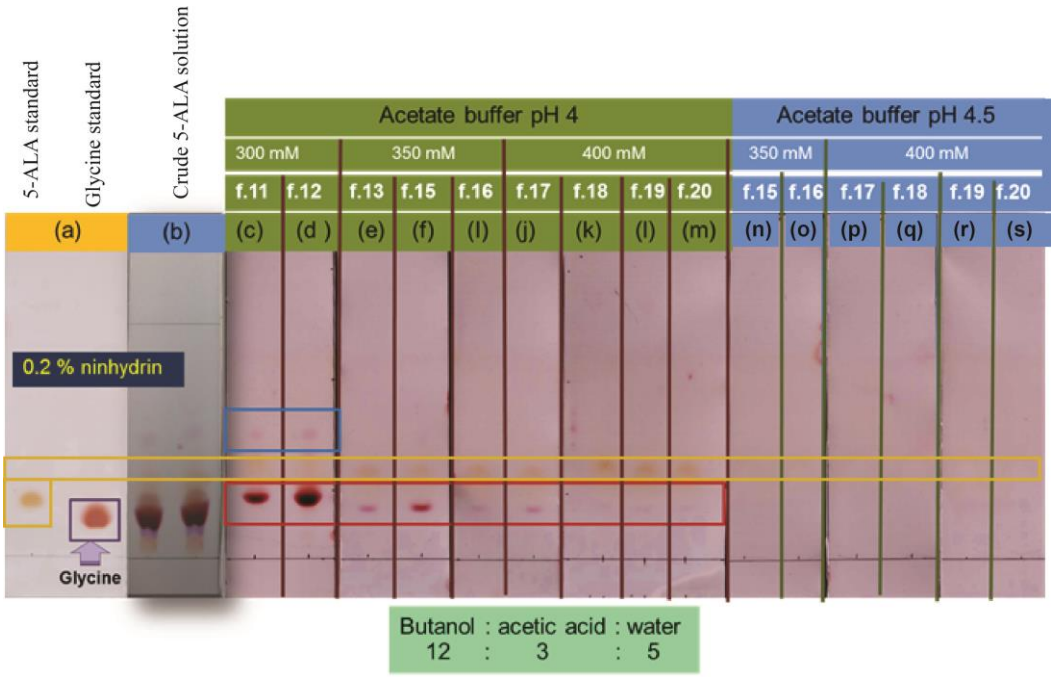



**Figure 4.7** TLC of (a) standard of 5-ALA and glycine (b) crude 5-ALA solution (c-m) eluate (fraction 12-20) using acetate buffer (pH 4) as eluent, (n-s) eluate (fraction 15-20) using acetate buffer (pH 4.5) as eluent.

The results showed that, the suitable pH value of eluent was 4.5. Since there is no impurity found at this condition. In addition, the range of eluate showing the target molecule was narrow. As a result, it is easy to collect the fraction. Mauzerall and Granick (1956) proposed to separate 5-ALA from urine by ion exchange chromatography. They achieved the separation of 5-ALA by using sodium acetate buffer at pH 4.76. Di Venosa *et al*. (2004) published a method for separation 5-ALA from ALA derivative solution by using IEC. They found that the percentage of 5-ALA that was eluted in this condition was 90 ± 4% when using sodium acetate buffer pH 4.8.

### 4.2.2 Selection of sample loading volume

The bed volume (BV) of column (C16/40) was fixed as 50 mL and the initial volume of crude 5-ALA solution was loaded into the column as 10, 15 and 20 % of bed volume. After collection of each fraction to determined 5-ALA by colorimetric method, the absorbance profile at 553 nm of each condition is shown in Figure 4.8.

Figure 4.8a shows the elution pattern profile after applying gradient elution when loading crude 5-ALA solution for 10% of bed volume (5 mL). The target molecules started to elute during gradient elution step at 0.49 M. These results indicated that target molecules were adsorbed in the column with cation resin and they were eluted when the ionic strength of elution buffer was gradually increasing (0.49-1M).

When increasing the ratio between initial volume of crude 5-ALA and bed volume (BV) of column as 15% (7.5 mL): BV 50 mL and 20% (10 mL): BV 50 mL, the result is shown in Figure 4.8b and 4.8c, respectively. The gradient elution was found that the target molecules were started to elute in washing step using 0.01 M acetate buffer. During gradient elution step, the target molecules can be found in the wide range of fraction which required a long time to collect sample.

In washing step, the target molecules were eluted. It may due to the excess molecules cannot be adsorbed with the cation resin which indicated that the initial volume of crude 5-ALA solution at 15% and 20% was overloaded the capacity of resin.

Therefore, the suitable ratio between initial volume of crude 5-ALA and bed volume (BV) of column is 10% of bed volume and it is used for the next step of elution process.

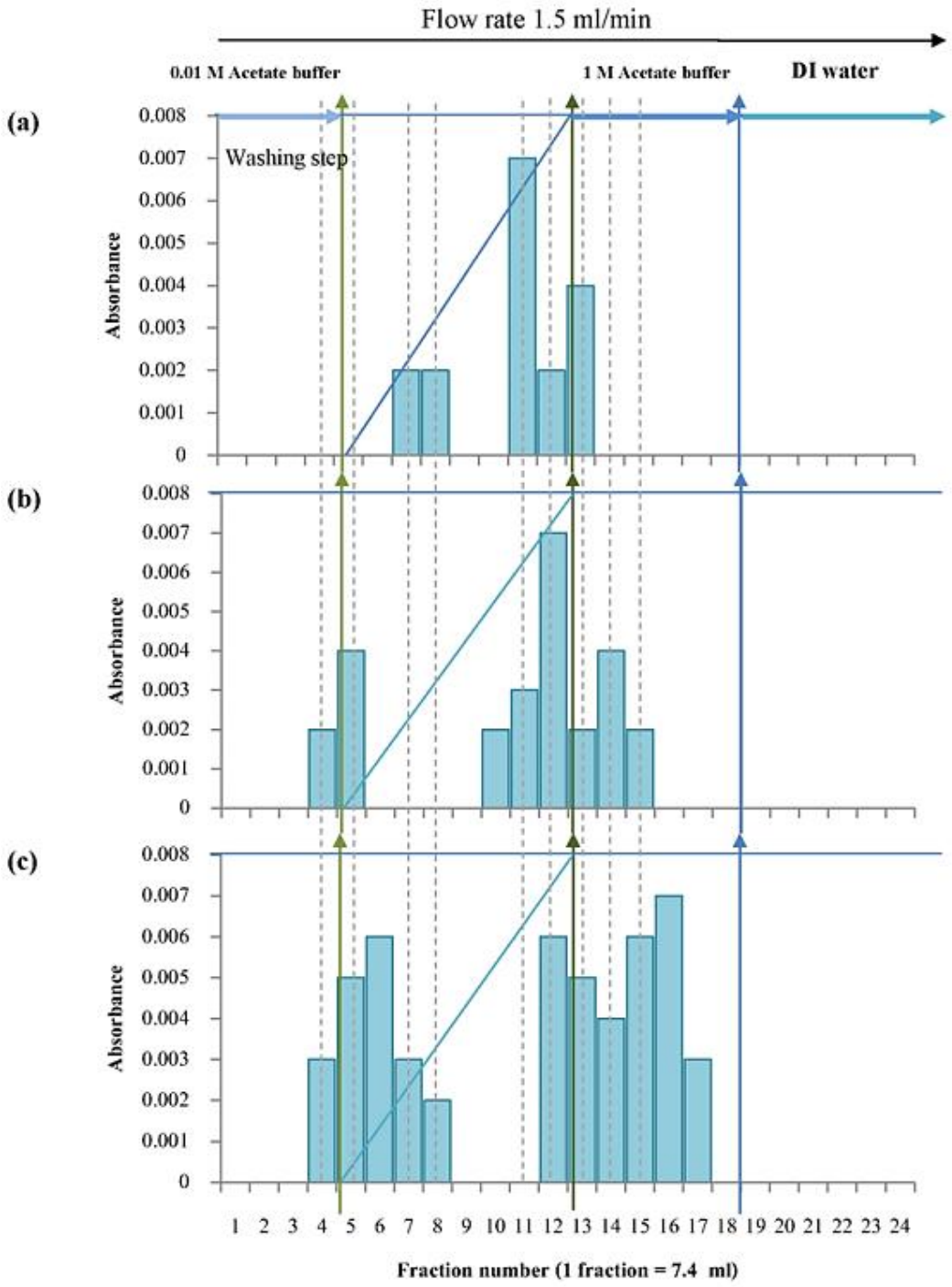



**Figure 4.8** Elution pattern with gradient profile and absorbance at 553 nm of fractions ;bed volume (BV) = 50 mL. Crude 5-ALA solution was loaded into the column as (a) 10% of bed volume (b) 15% of bed volume and (c) 20% of bed volume.

### 4.2.3 The elution process

From the previous experiment, the appropriate condition for separation the 5-ALA from crude 5-ALA solution is using sodium acetate buffer pH 4.5 as an eluent and the initial volume of crude 5-ALA solution was loaded into the column as 10% of bed volume (BV=50 mL: 5 mL of sample). The linear gradient elution was performed by changing in ionic strength during the elution process. In this experiment were divide in 3 parts including;

First, standard 5-ALA solution (conc. 5 mg/L) were used to evaluate and justify the separation efficiency of the elution process. The standard 5-ALA solution was used to test capability of resin for adsorption and condition for eluting the 5-ALA molecule. The amount of standard 5-ALA was 10% of bed volume (5 mL: BV 50 mL) when loaded. The absorbance profile of each collected fraction after gradient elution is displayed in Figure 4.9a. The 5-ALA was not found in the first step of elution. This absence of 5-ALA indicated that it was well adsorbed into cation resin. When starting gradient elution, the 5-ALA was eluted continuously when the concentration of sodium acetate buffer was gradually increased up to 0.92 M. The total percentage of recovery of standard 5-ALA in this condition was ~78 % which was confirmed using HPLC-fluorescence.

Second, the crude 5-ALA solution (conc.0.787mg/L) was loaded at 10% bed volume. The absorbance profile of each collected fraction after gradient elution of the crude 5-ALA solution is shown in Figure 4.9b. It was found that the target molecule was eluted in four groups and the presence of 5-ALA was found in each fraction. This presence of 5-ALA was confirmed using HPLC-fluorescence. The 5-ALA was found in fraction number 12 and 13 at concentration of eluent as 0.4-0.5 M. Although, the

HPLC-fluorescence can measure the concentration of 5-ALA in eluate of crude 5-ALA solution, but the 5-ALA concentration after elution process was very low and not worth collecting (~$2.24\times10^{-4}$ mg). The concentration of 5-ALA that was produced from *R. sphaeroides* is quite low (conc.0.923 mg/L) which was not suitable for separation by gradient elution in IEC. Therefore, the 5-ALA that produced from microbial perhaps requires a further step of concentration before loading into the elution process. The 5-ALA productivity from microbial production can be considered to improve by adding levulinic acid (LA) to the medium to inhibit the $\sigma$-aminolevulinic acid dehydratase (ALAD) during extracellular formation of 5-ALA by *R. sphaeroides* (Noparatnaraporn *et al*., 2000). In addition, the metabolic engineering may be one of the alternatives for improving 5-ALA production (Choi *et al*., 2004; Wang *et al*., 2006) . Therefore, in the next part, the standard 5-ALA was spiked into crude 5-ALA solution to increase the quantity of 5-ALA to be sufficient for evaluation the elution process.

Third, the elution process of the crude 5-ALA solution spiked with 5-ALA standard (conc. of 5-ALA as 19.31 mg/L) was loaded to column at 10% bed volume. The result was shown in Figure 4.9c. The 5-ALA was eluted in two groups at the concentration of eluent was 0.5-0.7 M and the concentration of eluent was 1 M. The percentage of recovery of 5-ALA from crude 5-ALA solution was around 43%. The above result indicated the lower limit of concentration of 5-ALA that this condition be able to separate 5-ALA from the crude 5-ALA solution. It was noticed that when the pH of crude 5-ALA solution was adjusted to 7.09, the 5-ALA content was 28.30 mg/L, determined by HPLC- fluorescence and when the pH of crude solution was reduced down to 4.5, the 5-ALA content was decreased to 19.31 mg/L. In the

preparation of the crude 5-ALA solution before loading into the column, the pH of solution was adjusted down to 4.5 to make the 5-ALA molecule become positive charge. In the crude solution also have some molecules that perhaps exhibit negative charge and then bind to molecule of 5-ALA resulting in reduction of 5-ALA that can be eluted out from the column. When using standard 5-ALA, the concentration of 5-ALA that was eluted out was the highest due to the purity of standard 5-ALA solution. The absence of impurities may bind to 5-ALA resulting in higher recovery yield.

Therefore, for the further study the additional step before IEC separation process should be considered for removal of impurities that could reduce the efficiency of IEC separation process. Moreover, the concentration of crude 5-ALA should be increased prior the IEC separation process. The method of concentration such as freeze concentration or low pressure concentration should be applied to increase recovery of 5-ALA from crude solution.

Hence, for the study of entrapment of 5-ALA in biodegradable film, the standard 5-ALA was used. This is because of high purity of the standard 5-ALA and the interaction between film biopolymers and 5-ALA molecule can be clearly investigated and explained without interferences.

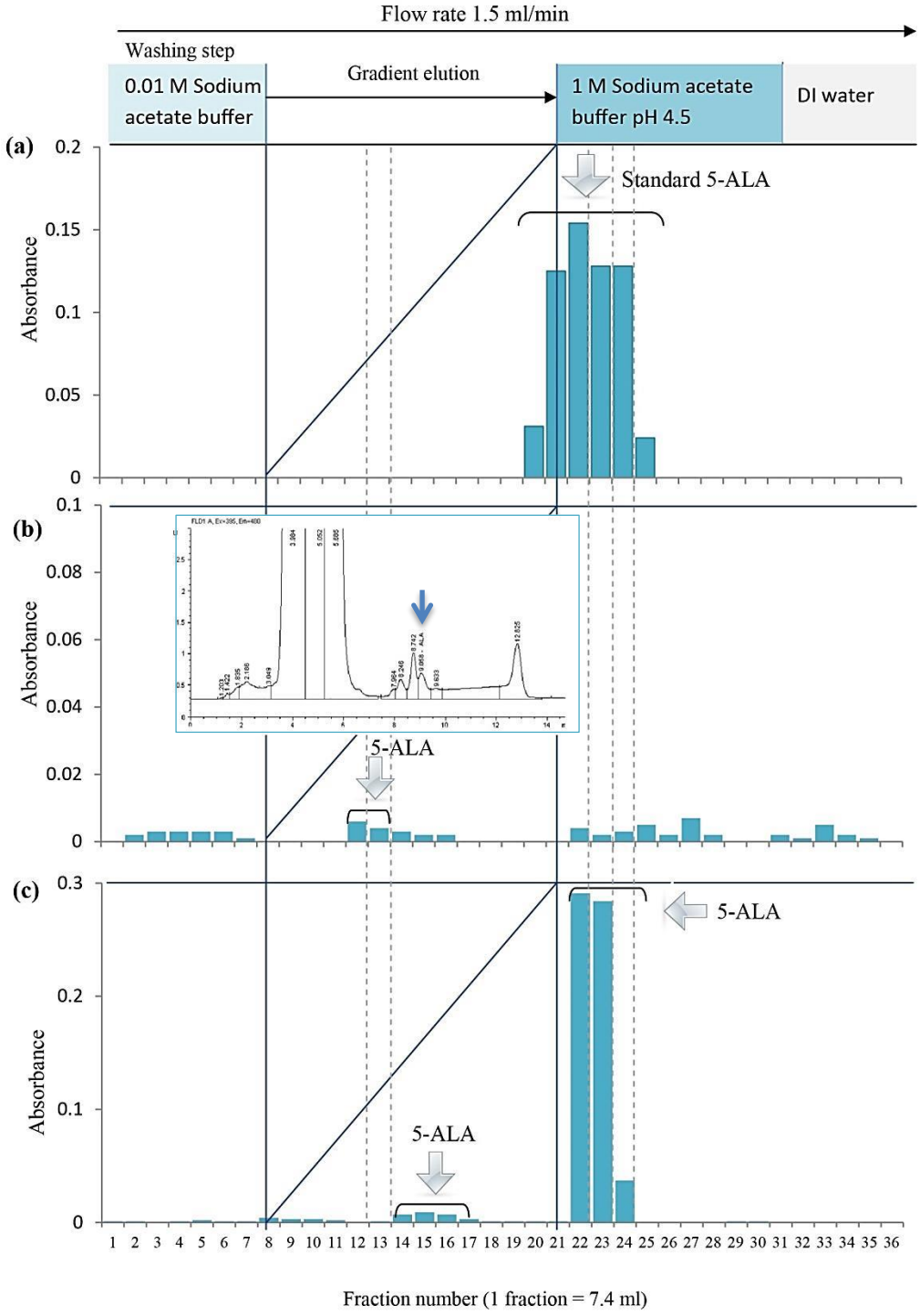



**Figure 4.9** The elution pattern with gradient profile and absorbance at 553 nm of fractions of (a) standard 5-ALA (b) crude 5-ALA solution and (c) crude 5-ALA solution were spiked the standard 5-ALA.

### 4.3 Films containing 5-ALA

Films containing 5-ALA were made from Konjac glucomannan (KGM), KGM added KOH (KGOH), chitosan (CHI) and the blend polymers (KGOH/CHI) mixed at different ratios 100/0, 80/20, 75/25, 50/50, 25/75, 20/80 and 0/100 (% w/w). The blend polymers were coded as KGOH, K80C20, K75C25, K50C50, K25C75, K20C80 and CHI respectively. The KGM was also cast into film and studied as the reference to compare with the KGOH. The standard 5-ALA was added to the film during film preparation. After that, film containing 5-ALA were determined for entrapment efficiency, characterization and properties of films and stability of the 5-ALA in film as follows:

#### 4.3.1 Entrapment efficiency of 5-aminolevulinic acid under films

The entrapment efficiency of 5-ALA under film was determined using HPLC-fluorescence. The results are shown in Table 4.2.

**Table 4. 2** Entrapment efficiency of 5-ALA under film condition

| Sample | Polymer ratios (% w/w) | | | Initial content of 5-ALA in film (mg) | Entrapment efficiency (%) |
|---|---|---|---|---|---|
| | KGM 1% | KGOH 1% | CHI1% | | |
| 1 KGM | 100 | - | - | 0.2 | $55.7^{d}\pm$ 0.730 |
| 2 KGOH | - | 100 | - | 0.2 | $58.3^{c}\pm$ 0.360 |
| 3 K80C20 | - | 80 | 20 | 0.2 | $43.4^{e}\pm$ 0.910 |
| 4 K75C25 | - | 75 | 25 | 0.2 | $41.0^{f}\pm$ 0.550 |
| 5 K50C50 | - | 50 | 50 | 0.2 | $44.1^{e}\pm$ 0.550 |
| 6 K25C75 | - | 25 | 75 | 0.2 | $65.9^{a}\pm$ 0.370 |
| 7 K20C80 | - | 20 | 80 | 0.2 | $59.5^{bc}\pm$ 0.920 |
| 8 CHI | - | - | 100 | 0.2 | $60.3^{b}\pm$ 0.180 |

Values are the averages $\pm$ standard deviations
Different small letter (a-f) indicates significant differences between all samples ($p \leq 0.05$)

Table 4.2 shows entrapment efficiency of 5-ALA in films at different ratios of biopolymers. Entrapment efficiency was measured by determining the amount of 5-ALA release into solution after dissolving film in buffer (pH 5.5).

A single biopolymer, KGM film, could entrap 5-ALA by ~ 56%. When the KGM was treated with KOH, entrapment efficiency of 5-ALA increased up to 58.33%. Since alkali resulted in the deacetylation of the structure of KGM, the presence of acetyl group in the structure of the KGM obstructed the interaction between KGM and 5-ALA. Therefore, the deacetylation process can promote the interaction of KGM with 5-ALA and other polymers (Alonso-Sande *et al*. 2009). It has been reported that the pH in the range 9-10 was necessary for gelation and film formation of KGM (Cheng *et al*., 2002). Therefore, the KGOH film had the ability to entrap 5-ALA better than KGM films.

The 5-ALA was entrapped in CHI film up to 60.29%, higher than the amount of 5-ALA in KGM and KGOH films. This may be due to the interaction between amino group ($NH_2$) of CHI and carboxyl group (-COOH) of 5-ALA (El-Ghaffar and Hashem, 2010). The pH of CHI solution was 3.12. As CHI powder dissolved in the solution at low pH (< 6) the amino group in CHI structure get protonated and shows a positive charge (Dash, *et al*. 2011). When mixing KGOH and CHI at different ratios, maintaining a larger amount of CHI than KGOH resulted in increasing entrapment efficiency. Particularly, at the ratio of 25:75 (%w/w) of the two polymers (KGOH/CHI) in the blend showed highest percentage of entrapped 5-ALA (65.9%). Therefore, the blended film at suitable ratio between KGOH/CHI could entrap 5-ALA better than using only KGOH or CHI.

The entrapment efficiency was about 60%. This value was measured via solubility of 5-ALA after dissolving film in buffer at pH 5.5. The low value in entrapment efficiency might be because 5-ALA forms covalent bond with the structure of polymer. Thus, 5-ALA cannot be released when film is dissolved in buffer solution. However, when this film is applied in agriculture, the enzyme from microorganism can digest the covalent bond, resulting in the release of 5-ALA.

### 4.3.2 Characterization and properties of films with and without 5-ALA

All films with and without 5-ALA were characterized and possible mechanism of 5-ALA entrapment in film was explained.

#### 4.3.2.1 Fourier transforms infrared (FT-IR) spectroscopy

The postulated interaction between 5-ALA and biopolymer in film was confirmed using Fourier transforms infrared (FT-IR) spectroscopy. The film samples with and without 5-ALA were investigated. The results of the analysis by FT-IR are shows in Table 4.3 and Figure 4.10. The FT-IR spectrum indicated that the 5-ALA should be entrapped in the matrix of the KGM film since there was no difference between the KGM spectrum with and without 5-ALA. The presence of hydroxyl group (–OH), stretching of carbonyl of acetyl group, and stretching of C-O of the associate hydroxyl group were indicated by the absorption bands at 3428-3434 $cm^{-1}$, 1730 $cm^{-1}$ and 1640-1642 $cm^{-1}$, respectively (Li *et al.*, 2015; Pan *et al.*, 2011). The absorption band at 1428 $cm^{-1}$ and 1381 $cm^{-1}$ were assigned to stretching of –CH and $CH_3$ deformation, respectively (Lambert *et al.*, 1987).

When adding 5-ALA to the KGM, it can be observed that the absorption band around1730 $cm^{-1}$ shifted to a lower wave number around 1724 $cm^{-1}$. The absorption

band around 1724 $cm^{-1}$ in the KGM with 5-ALA was assigned to the associated C=O group in the 5-ALA structure (Chen *et al*., 2003). There is a possibility that the potassium hydroxide (KOH) was added to the KGM solution and, as a result, the acetyl group in a structure of KGM was eliminated which was shown in the FT-IR spectrum. The absorption band of acetyl groups around 1730 $cm^{-1}$ in KGOH film was smaller than the KGM. One can presume that the alkali caused the deacetylation in KGM (Cheng *et al*., 2002; Herranz *et al*., 2012). Adding 5-ALA to the KGOH solution (pH 9) resulted in5-ALA being in the anion form and the amino group of 5-ALA became deprotonated. The KGOH film had a new absorption band at 1572 $cm^{-1}$ which was assigned to the amino group of 5-ALA deformation (Amide II), but this band was not found in KGM film with5-ALA. Therefore, one can postulate that the presence of amide II in KGOH film with 5-ALA resulted in the entrapment of 5-ALA under the interaction between the N-H of 5-ALA and C=O in acetyl group of KGOH structure. Moreover, KGOH is a high molecular weight polymer, as a result, KGOH film was complex. Therefore, it was possible that 5-ALA would be entrapped in KGOH film matrix.

For CHI films, the main peak can be seen at the absorption band of 3434 $cm^{-1}$, assigned to the stretching of amino group bonded to–OH, suggesting intermolecular hydrogen bonding of chitosan molecules (Nunthanid *et al*., 2001; Ye *et al*., 2006). The absorption bands at 1562 $cm^{-1}$, 1410 $cm^{-1}$and 1075 $cm^{-1}$ were assigned to bending absorption band of amide II, C-N stretch and $C_6$-OH stretching of C–O–C, respectively (Balau *et al*., 2004; Lambert *et al*., 1987). In the FT-IR spectrum of CHI film with 5-ALA, it can be observed that the absorption band at 1562 $cm^{-1}$ and 1410 $cm^{-1}$had sharper peaks. It was found that 5-ALA was entrapped under secondary

amide where the amino group of CHI interacted with carboxyl group of 5-ALA. The interaction of chitosan and 5-ALA is similar to previous research on chitosan amino acid condensation products (El-Ghaffar and Hashem, 2010).

The FT-IR spectrum of the polymers from KGOH and CHI at different ratios is shown Figure 4.10 (c-f). As the proportion of chitosan increased, the absorption bands around 1730 $cm^{-1}$ and 1381 $cm^{-1}$ disappeared. The stretching of intramolecular hydrogen bond at 1642 $cm^{-1}$ in KGOH coupled and shifted to a lower wave number, around 1562 $cm^{-1}$, suggesting that new hydrogen bonding occurred in the blended films (Ye *et al*.2006). The absorption band about 1567-1562 and 1410 $cm^{-1}$ of the blended films with 5-ALA increased with respect to the films without 5-ALA. When CHI was increased in the matrix, with 5-ALA incorporated, the absorption band was about 1638 $cm^{-1}$, shifting to a lower wave number and disappeared in the pure CHI film. The schematic diagram of FT-IR spectrum of film is described in Figure 4.11. It is possible that 5-ALA was entrapped under two possible mechanisms. The first possible mechanism is interaction of secondary amide between the amino group of CHI and carboxyl group of 5-ALA. The K75C25 had the proportion of CHI higher than KGOH, the pH value of the blended polymer solution was reduced to 3.27, resulting to protonation of the amino group of CHI and 5-ALA. It is possible the amino group of CHI and carboxyl group of 5-ALA were adducted, therefore a secondary amide was presence. The second possible mechanism, the 5-ALA molecule was entrapped within complexity of KGOH. Due to, the high molecular weight of KGOH and a rich number of hydroxyl group in KGOH chain which causing the interaction between the KGOH and CHI through hydrogen bonding between amino group of CHI and hydroxyl group of KGOH. As well as the complexity of KGOH

polymer chain could provide space within the network of blended film. As a result, the blended film (KGOH/CHI: 25/75) can retain more molecules of 5-ALA than other treatment. However, this proposed hypothesis for the second mechanism should be continued in further study.

**Table 4. 3** Assignments of FT-IR characteristic absorption peaks of films without and with 5-ALA made from native KGM, KGOH, CHI and blend films between KGOH and CHI in different ratios (%w/w).

| **Characteristic peak ($cm^{-1}$)** | 5-ALA | **Samples** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | KGM | KGOH | K80C20 | K75C25 | K50C50 | K25C75 | K20C80 | CHI |
| O-H ; stretching of N-H bonded to -OH | - | 3428 | 3434 | 3434 | 3434 | 3434 | 3433 | 3434 | 3434 |
| | 5-ALA | 3434 | 3428 | 3434 | 3434 | 3433 | 3433 | 3434 | 3434 |
| C-H of methyl | - | 2922 | 2922 | 2921 | 2920 | 2921 | 2922 | 2924 | 2925 |
| | 5-ALA | 2918 | 2918 | 2920 | 2920 | 2919 | 2927 | 2920 | 2927 |
| | - | 2889 | 2889 | 2888 | 2886 | 2885 | 2883 | 2882 | 2880 |
| | 5-ALA | 2890 | 2890 | 2889 | 2887 | 2887 | 2886 | 2886 | 2884 |
| C=O | - | 1730 | 1730 | 1720 | 1717 | - | - | - | - |
| | 5-ALA | 1724 | 1727 | 1717 | 1715 | - | - | - | - |
| N-H-R1 | - | - | - | 1567 | 1567 | 1565 | 1565 | 1564 | 1562 |
| | 5-ALA | - | 1572 | 1567 | 1567 | 1565 | 1562 | 1562 | 1562 |
| C-H bending in $CH_3$ | | 1428 | 1428 | - | - | - | - | - | - |
| | 5-ALA | - | - | - | - | - | - | - | - |
| C-N stretch | | - | - | 1419 | 1412 | 1411 | 1410 | 1411 | 1410 |
| | 5-ALA | 1418 | 1417 | 1412 | 1411 | 1410 | 1411 | 1409 | 1409 |
| $CH_3$ deformation | - | 1381 | 1380 | 1382 | 1381 | - | - | - | - |
| | 5-ALA | 1379 | 1379 | 1382 | 1382 | - | - | - | - |
| $C_6$-OH stretching of C–O–C | - | 1062 | 1062 | 1064 | 1064 | 1067 | 1070 | 1071 | 1075 |
| | 5-ALA | 1063 | 1063 | 1066 | 1065 | 1067 | 1072 | 1072 | 1075 |
| | - | 1027 | 1027 | 1027 | 1029 | 1030 | 1031 | 1031 | - |
| | 5-ALA | 1027 | 1027 | 1028 | 1029 | 1028 | - | - | - |

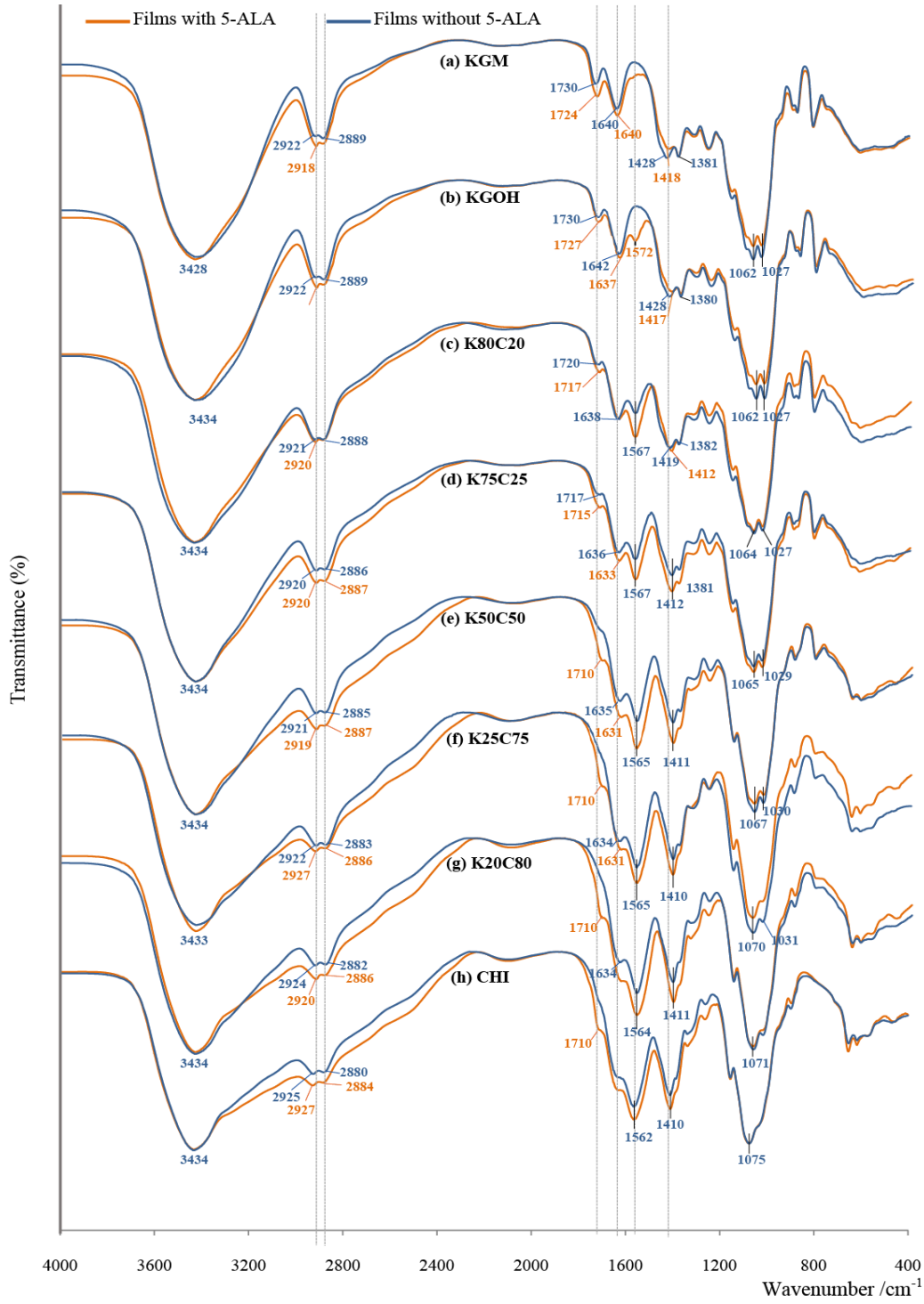



**Figure 4. 10** FT-IR spectra of films without and with 5-ALA made from (a) native KGM, (b) KGOH, (c) K80C20, (d) K75C25, (e) K50C50, (f) K25C75, (g) K20C80 and (h) CHI.

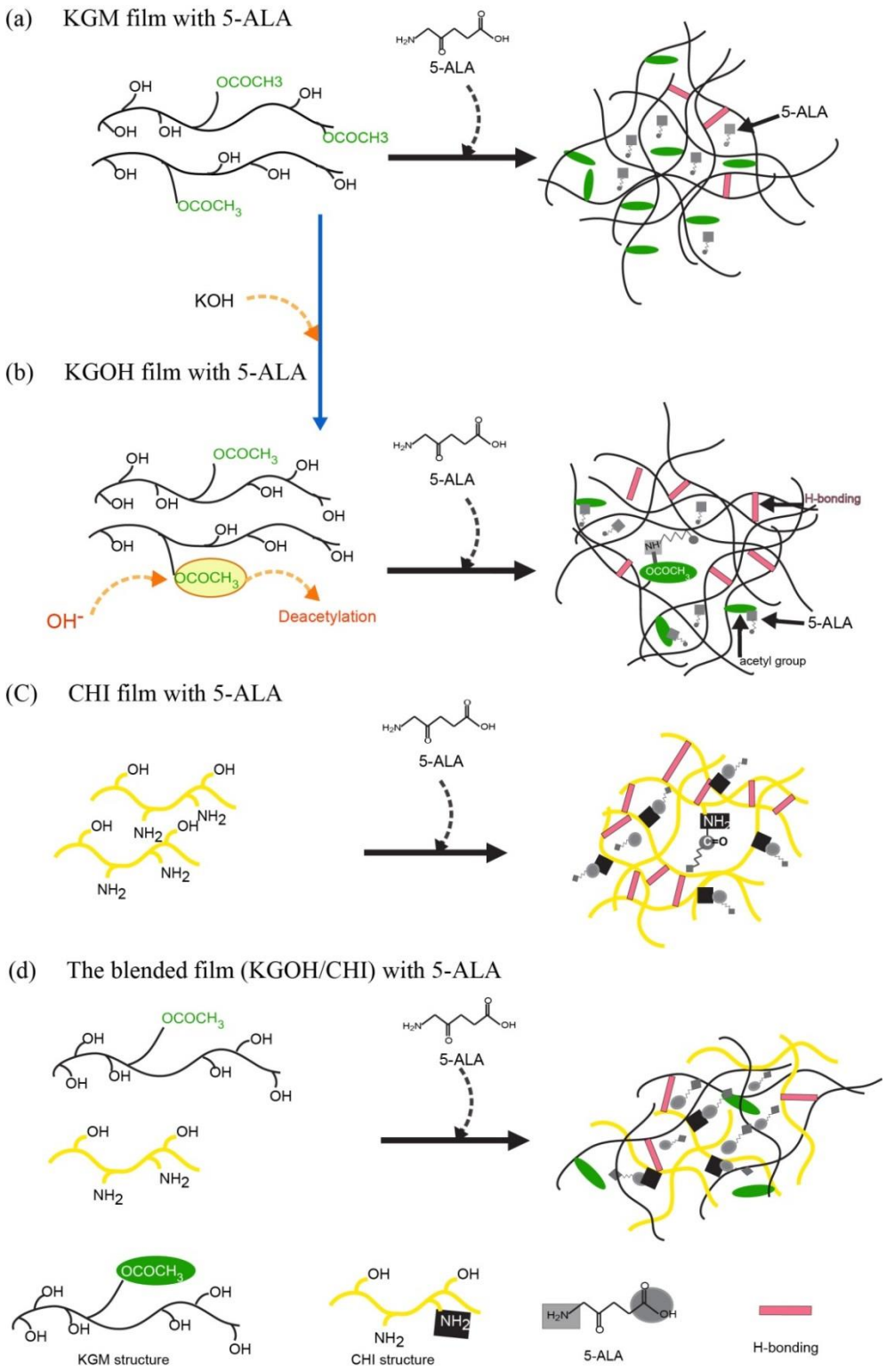



**Figure 4.11** The possible mechanisms for entrapment (a) 5-ALA in (b) KGOH films**,** (c) CHI film and (d) the blend films between KGOH and CHI

#### 4.3.2.2 X-ray diffraction (XRD) pattern

The X-ray Diffraction (XRD) pattern of the films with and without 5-ALA were examined using an X-ray diffractometer. Figure 4.12 shows XRD curve of all films with and without 5-ALA.The degree of crystallinity of the films in various conditions is show in Table 4.4. The structures of KGM and KGOH films show an amorphous state. Generally, pure KGM film is highly amorphous with low crystallinity (Cheng *et al*., 2002). While adding 5-ALA to KGOH showed a low crystalline stage (degree of crystallinity ~ 0.11 %), the XRD pattern of KGOH film with 5-ALA at 2θ of 9.50, 14.96 and 19.22º indicated that the KGOH film with 5-ALA seem to interact between amino group of 5-ALA and complexity of KGOH structure.

The blend films without 5-ALA showed an amorphous form when KGOH content was increased and the crystalline state was only found in blend film especially in K20C80 (crystallinity = 2.69%). Adding 5-ALA into the blend films resulted in higher crystalline state in K25C75 (crystallinity = 4.11%) and K20C80 (crystallinity = 9.81%) when compared to films without 5-ALA. The CHI film without 5-ALA had 1.56% degree of crystallinity. However, when adding 5-ALA, the degree of crystallinity increased to 19.73%. Several sharp peaks were observed in the XRD pattern of CHI film with 5-ALA at 2θ of 8.61, 11.56 and 18.65º indicating the presence of interaction between CHI and 5-ALA. From the XRD pattern, it can be seen that adding 5-ALA into KGM film did not change the structure of film because the KGM had high number of acetyl group; therefore it disrupted interaction between KGM and 5-ALA. When adding 5-ALA into KGOH, CHI and the blended films with a high proportion of CHI, it can be seen that the structure of the films had increased in

crystallinity compared with film without 5-ALA. The increase in crystalinity showed the interaction between 5-ALA and KGOH, CHI, K75C25 and K80C20 films.

**Table 4. 4** Crystalline state and the thickness of films without and with 5-ALA.

| Sample | % Crystallinity | | Thickness of films (μm) | |
|---|---|---|---|---|
| | Without 5- ALA | Adding of 0.2 mg 5-ALA | Without 5- ALA | Adding of 0.2 mg 5-ALA |
| KGM | Amorphous | Amorphous | 24.8 ± 0.002 | 24.3 ± 0.002 |
| KGOH | Amorphous | 0.11% ± 0.01 | $34.8^{a}$ ±0.003 | $28.2^{b}$± 0.005 |
| K80C20 | Amorphous | Amorphous | $35.0^{a}$±0.003 | $25.1^{b}$ ± 0.007 |
| K75C25 | Amorphous | Amorphous | 36.5 ± 0.005 | 36.3 ± 0.006 |
| K50C50 | Amorphous | Amorphous | $33.0^{b}$ ± 0.004 | $40.7^{a}$ ± 0.003 |
| K25C75 | Amorphous | 4.11 % ± 0.36 | 36.5 ±0.003 | 39.8 ± 0.005 |
| K20C80 | 2.69% ± 0.24 | 9.87% ± 0.36 | 42.8 ±0.007 | 41.6 ± 0.005 |
| CHI | 1.56% ± 0.15 | 19.73% ± 0.18 | $42.9^{a}$ ± 0.004 | $34.3^{b}$± 0.004 |

Values are the averages + standard deviations

Different small letter (a-b) indicates significant differences between all samples ($p < 0.05$)

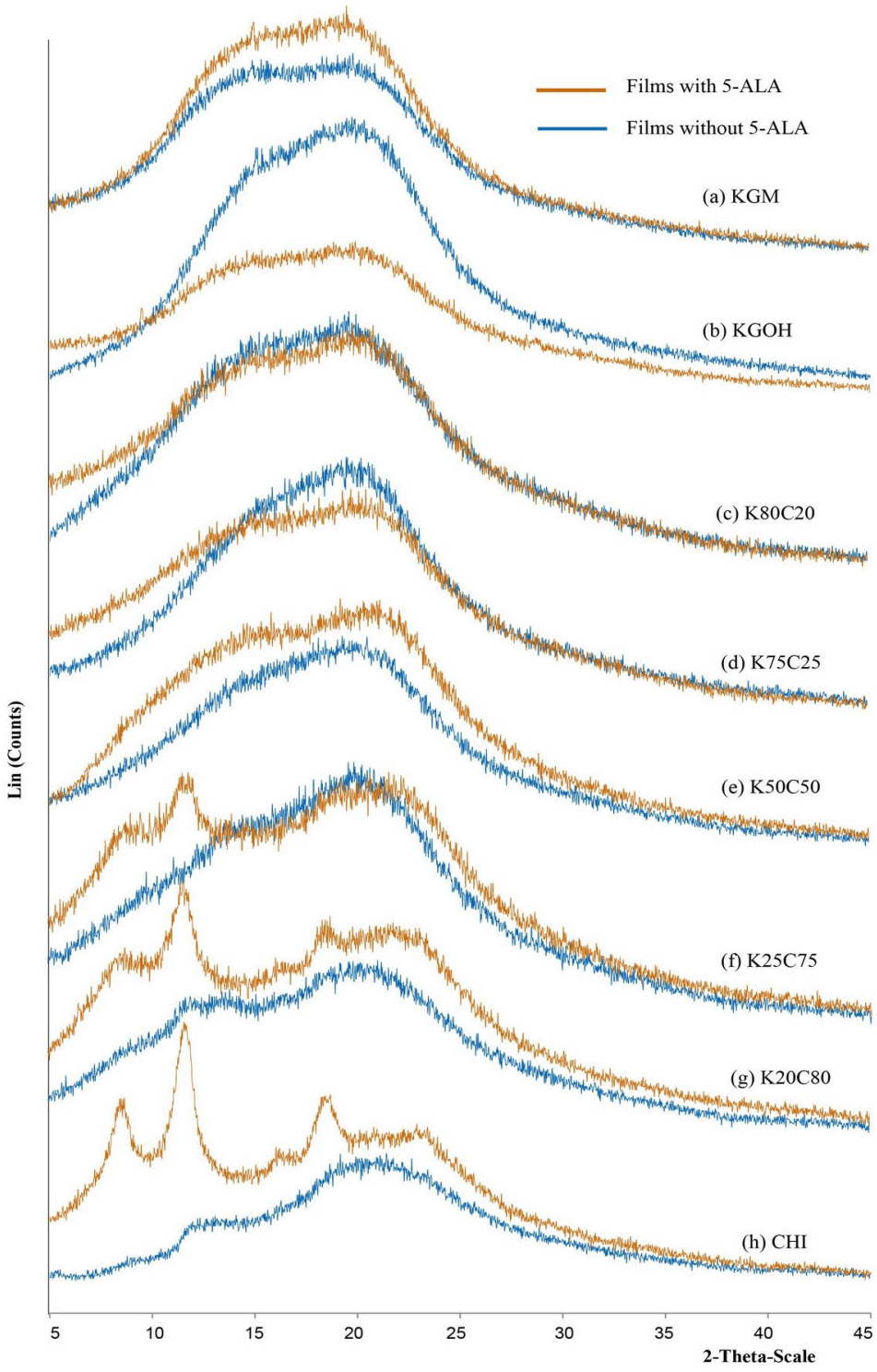



**Figure 4.12** The X-ray diffraction patterns of the films with and without 5-ALA made from (a) KGM, (b) KGOH, (c) K80C20, (d) K75C25, (e) K50C50, (f) K25C75, (g) K20C80 and (h) CHI.

#### 4.3.2.3 Film thickness and morphology of films

The thickness of all films (with and without 5-ALA) is shown in Table 4.4. The thickness of CHI film was smaller than that of KGM and KGOH films. A significant proportion of CHI in the blended films increased their thickness. The addition of 5-ALA resulted in a decrease in thickness in all films. However, the thickness of films showed no relationship with entrapment efficiency (Table 4.4).

The field emission scanning electron microscopic image (FE-SEM) of cross sections of the films at a magnification of 2,000× and 10,000× are shown in Figure 4.12. The surface of KGM film and KGOH film without 5-ALA was rougher than the CHI and blended films (Figure 4.13a and 4.13c). The blended films exhibited a rougher structure with increased proportion of KGOH. Since KGOH has a high molecular weight, when it was combined with CHI to form films in which the proportion of KGOH was higher than CHI, the structure of the composite film was amorphous and the cross section surface of the film was rough.

CHI and the blended films where the proportion of CHI was higher than KGOH showed a smooth cross section surface. Since CHI has low molecular weight and high percentage of deacetylation (DD>95%), crystallinity was higher, resulted from H-bonding formation.

As for the films with 5-ALA, the morphology of KGM, CHI and the blended film K50C50, K25C75, K20C80 was smoother than without 5-ALA. Although, the surface cross section of film KGM with 5-ALA seemed to be smoother than film KGM alone, the structure of film KGM with 5-ALA was amorphous, since 5-ALA did not interact with KGM film. On the other hand, the KGOH and the K25C75 films with 5-ALA seemed to have a rough cross section surface, although the structure was

high in crystallinity. This may be due to the interaction of the 5-ALA molecule with film KGOH as well as the blened film.

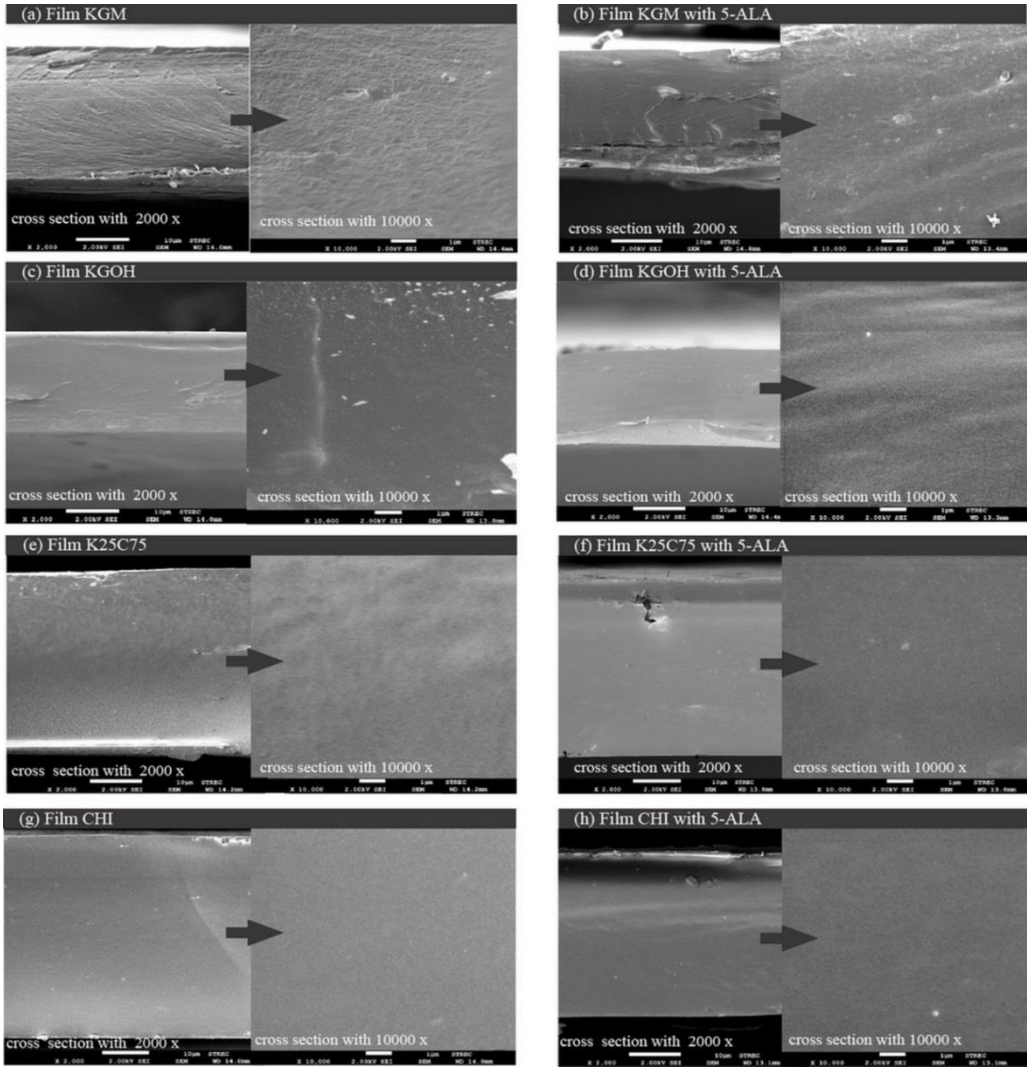



**Figure 4.13** FE-SEM micrographs of the films made from (a) KGM, (b) KGM with 5-ALA, (c) KGOH, (d) KGOH with 5-ALA (e) K25C75,(f) K25C75 with 5-ALA (g) CHI and (h) CHI with 5-ALA

#### 4.3.2.4 Water absorption and water solubility of films

The application of films containing 5-ALA in agriculture and other fields can be used by dissolving the film into water and, as a result, 5-ALA is being released. The water absorption and water solubility of films containing 5-ALA is shown in Table 4.5.

**Table 4. 5** The water absorption and water solubility of films containing 5-ALA

| Films containing 5-ALA | Distilled water | |
|---|---|---|
| | Water absorption (g water/g dry weight basis) | Water solubility (%) |
| 1. KGM | 54.9± 1.85 | 15.1 ± 1.08 |
| 2. KGOH | 79.6± 3.52 | 23.2 ± 0.87 |
| 3. K75C25 | 69.5± 3.06 | 8.0 ± 0.12 |
| 4. K50C50 | 36.8± 0.29 | 82.2 ± 1.12 |
| 5. K25C75 | 43.4±3.81 | 79.8 ± 1.01 |
| 6. CHI | 182.2 ± 2.73 | 5.7 ± 0.51 |



From Table 4.5, after dissolving the all films containing 5-ALA in distilled water. It was found that all films was able to dissolve in distilled water, Although, the KGM and KGOH is a soluble substance, when they are in the form of film the percentage of water solubility was low. This is because the interaction of H-bonding between structure of KGM (Cheng *et al*., 2002; Luo *et al*., 2013). The 5-ALA is a water soluble substance. The possible mechanism of releasing 5-ALA in KGM, KGOH film is that water was absorbed in the structure of film and some parts of the structure was soluble due to a rich of hydroxyl group in structure (Li and Xie, 2006), hence releasing 5-ALA. The CHI and K75C25 films were poor water soluble (~5 and ~8%, respectively) but the CHI film had high absorption of water. The 5-ALA within

chitosan film was released due to water diffusion in chitosan matrix KGOH, CHI and the blend polymers (KGOH/CHI: 25/75(%w/w)) biodegradable film containing 5-ALA could entrap 5-ALA higher than other conditions (entrapment efficiency as 58.3, 60.3 and 65.9 %, respectively). Therefore, they were selected to study the stability of 5-ALA in next step.

## 4.4 Determination of stability of the 5-ALA in film

Films containing 5-ALA made from KGOH, CHI and blend film (K25C75) was selected to study the stability of 5-ALA in film. All films were kept in aluminum laminated bag and stored at 3 different temperatures (-20 ±1, 4±1 and 28±1$^{o}$C) for 40 days. The sample was taken out to determine 5-ALA content using HPLC-fluorescence.

### 4.4.1 Quantity of 5-aminolevulinic acid content in film form during storage

The film KGOH, K25C75 and CHI containing 5-ALA as ~0.139 mg, ~0.150 mg and ~0.141 mg, respectively were stored at -20, 4 and 28 °C, the remaining content of 5-ALA in all films was monitored during 40 days. The crude 5-ALA solution was stored at -20 and 28 °C for comparative study with the film containing 5-ALA. The result is shown in Figure 4.13.

Figure 4.14, the crude 5-ALA solution was stored at -20 °C for 30 days, the 5-ALA content remained more than 80% and the 5-ALA content in film KGOH, CHI and K25C75 were remained more than 90% after storage for 40 days (Figure 4.14a). The temperature at -20 °C is a recommended temperature for storage of the standard 5-ALA-HCl in industry.

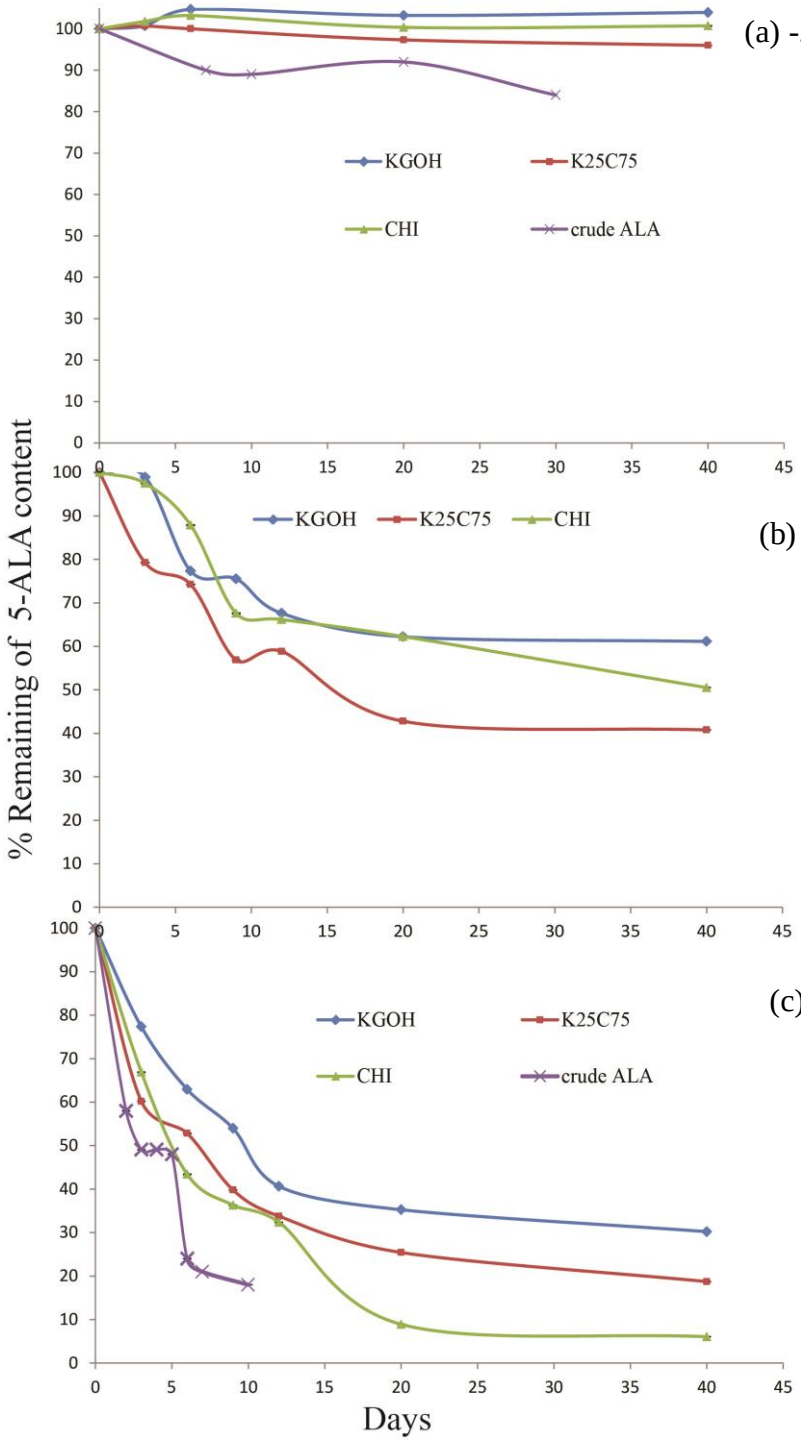



**Figure 4.14** Remaining of 5-ALA content in films at (a) -20± 1 °C (b) 4 ± 1 °C (c) 28 ± 1 °C

When the film containing 5-ALA was stored at 4 °C, the remaining of 5-ALA content is shown in Figure 4.14b. It was found that the 5-ALA content in film KGOH was decreased rapidly within 10 days which could be possible that the 5-ALA was entrapped in matrix of film KGOH that was degraded as it was less stable. The 5-ALA content was still left in film KGOH more than 50 % after 40 days of storage. For film CHI and K25C75, the 5-ALA content was decreased rapidly within 10 days as well as KGOH film. After 40 days, the remaining of 5-ALA in CHI and K25C75 film were remained as ~50 % and ~ 40%, respectively. Although, the blend film (K25C75) showed a highest efficiency for entrapment 5-ALA (~ 65.9%), but during storage the blend film at 4 °C and 28 °C, the 5-ALA content in blend film were declined rapidly indicating that most 5-ALA molecules may be entrapped in matrix of film and the structure of blend film cannot protect the 5-ALA from thermal degradation. While KGOH film can retained the 5-ALA more than 50% when storage at 4 °C for 40 day. It was possible that the interaction between the N-H of 5-ALA and C=O in acetyl group of KGOH structure can stable 5-ALA molecules better than CHI and K25C75 film, respectively. Under alkali condition of KGOH solution (pH 9), the 5-ALA was entrapped under film KGOH with the interaction between the N-H of 5-ALA and C=O in acetyl group of KGOH structure in which this condition the amino group of the 5-ALA is deprotonated. The amino group of the 5-ALA is able to react with the ketone group (Novo *et al.*, 1996) that should have a strong electrostatic attraction between the 5-ALA molecules and acetyl group of KGOH, as a result the KGOH film can retain 5-ALA more than 50% which higher than CHI and K25C75 film.

At 28 °C, the 5-ALA content in crude 5-ALA solution declined rapidly within 10 days. The 5-ALA content in all films was declined rapidly within 20 days.

After storage for 40 days, the remaining of 5-ALA content in KGOH, K25C75 and CHI film as ~30 %, ~20 % and <10%, respectively (Figure 4.14c). In CHI film, the 5-ALA was entrapped in film under secondary amide indicating the interaction between amino group of CHI and carboxyl group of 5-ALA had a lower efficiency to entrapped 5-ALA than KGOH film. Moreover, the blend film (K25C75) showed a same effect as CHI film due to high proportion of CHI in structure. The first and the second order kinetic of all reduction of 5-ALA at 4 and 28 °C showed in Table 4.6.

**Table 4. 6** The kinetic reduction of 5-ALA in KGOH, K25C75 and CHI film storage at 4 and 28 °C

| Temperature (°C) | Film | Reaction rate constant (k) | | $R^2$ | |
|---|---|---|---|---|---|
| | | First order | Second order | First order | Second order |
| 4 °C | KGOH | $1.2\times10^{-2}$ | $2.0\times10^{-4}$ | 0.661 | 0.705 |
| | K25C75 | $2.1\times10^{-2}$ | $4.0\times10^{-4}$ | 0.766 | 0.827 |
| | CHI | $1.7\times10^{-2}$ | $2.0\times10^{-4}$ | 0.820 | 0.884 |
| 28 °C | KGOH | $2.8\times10^{-2}$ | $6.0\times10^{-4}$ | 0.769 | 0.865 |
| | K25C75 | $3.7\times10^{-2}$ | $1.1\times10^{-3}$ | 0.886 | 0.958 |
| | CHI | $7.1\times10^{-2}$ | $4.2\times10^{-3}$ | 0.816 | 0.935 |

From the Table 4.6, the results confirm that reduction of 5-ALA at different treatment and storage temperatures follows the second-order reaction. The reaction rate constant (k) of the reduction of 5-ALA content in KGOH film was lower than that of 5-ALA in K25C75 and CHI film. Since the 5-ALA was entrapped in KGOH film under the interaction between the N-H of 5-ALA and C=O in acetyl group of KGOH structure, the degradation of 5-ALA in KGOH film is slower than that 5-ALA in K25C75 and CHI film. The k of reduction of 5-ALA content in K25C75 and CHI

film were higher than the constant of reaction in KGOH film, it is suggesting that the 5-ALA could be entrapped in the matrix of film which is prone to degrade. The second-order kinetic of the 5-ALA content in KGOH, K25C75 and CHI film during storage at 4 °C and 28 °C are showed in Figure 4.15 and 4.16, respectively

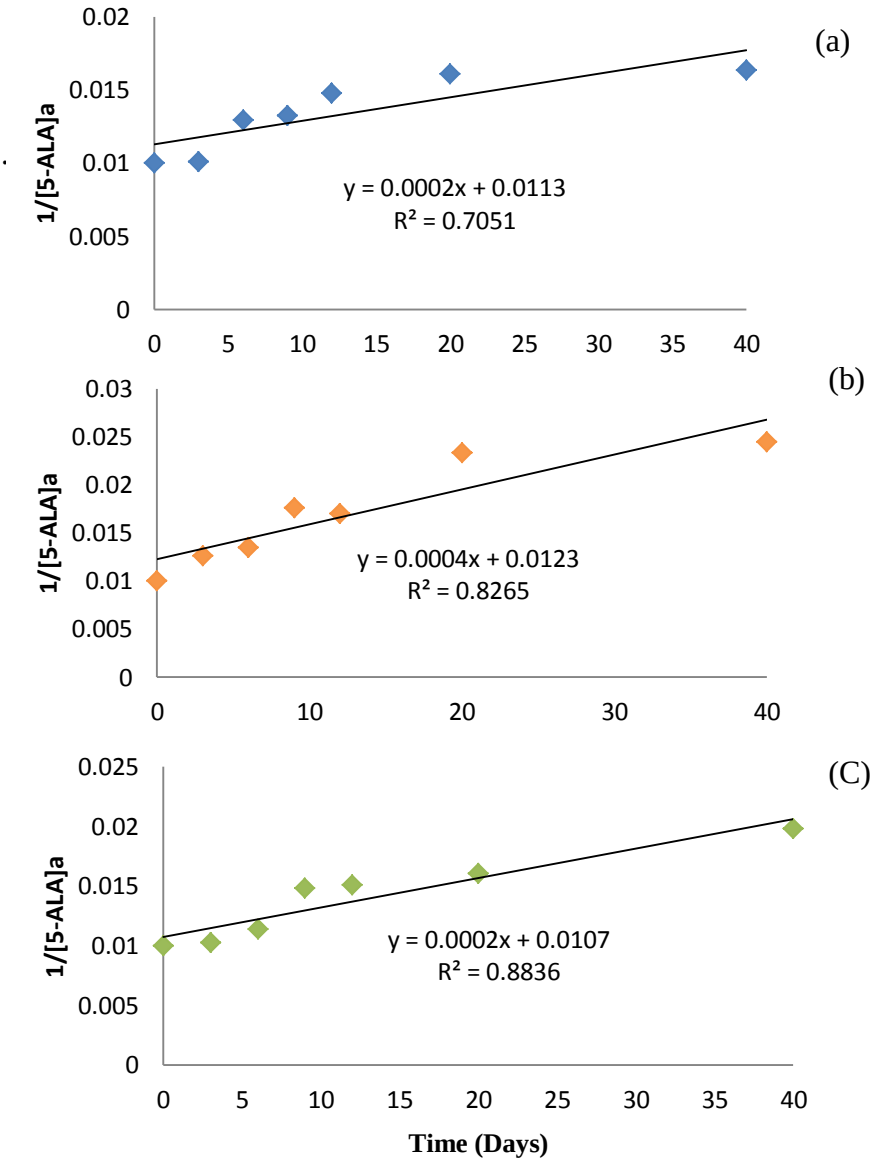



**Figure 4.15** Changing in the 5-ALA content during storage at 4 °C (a) KGM (b) K25C75 and (c) CHI

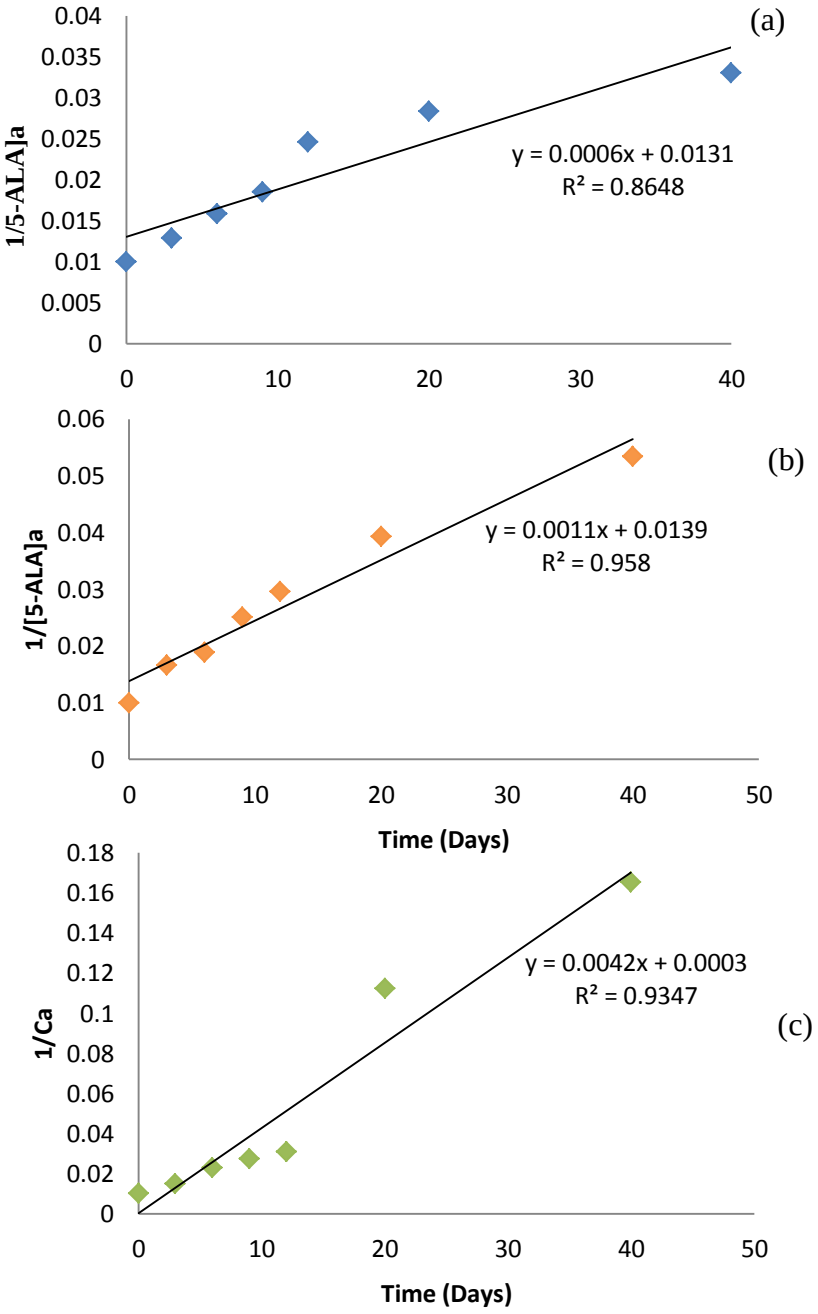



**Figure 4.16** Changing in the 5-ALA content during storage at 28 °C (a) KGM, (b) K25C75 and (c) CHI

# CHAPTER 5

# CONCLUSIONS

The *Rhodobacter sphaeroides* suspension was centrifuged for separating the suspension containing 5-aminolevulinic acid (crude 5-ALA solution) and cell microbial. Crude 5-ALA solution was determined the 5-ALA content by using HPLC-fluorescence is an accurate method for using to determine the 5-ALA content in crude 5-ALA solution.

The crude 5-ALA solution was adjust the pH down to 4.5 which show a completely positive charge before separation by cation exchange chromatography, the sodium acetate buffer pH 4.5 was used as elution buffer. The initial volume of crude 5-ALA was loaded into column as 10% of bed volume. The changing in ionic strength using a linear elution was selected as the elution process. Low concentration of 5-ALA in crude solution (conc.0.723 mg/L) is not suitable for separation by gradient elution in IEC. Spiking with 5 –ALA standard to increase the concentration of 5-ALA in crude 5-ALA solution (19.31 mg/L). The mixed solution was eluted using the condition as above, resulting in the percentage of recovery of 5-ALA becoming ~ 43%.

Films containing 5-ALA were made from konjac glucomannan (KGM), KGM were added KOH (KGOH), chitosan (CHI) and the blend polymers (KGOH/CHI) were mixed at different ratios 100/0, 80/20, 75/25, 50/50, 25/75, 20/80 and 0/100 (% w/w) to make the films. At the ratio of 25:75 (%w/w) of the two polymers (KGOH/CHI) in the blend showed highest percentage of entrapped 5-ALA

(~65.89%), followed by film CHI (~60.3%), K20C80 (59.5%) and KGOH (58.3%), respectively.

The possible mechanisms under which5-ALA can be entrapped under composite film made from KGM, KGM in alkali solution (KGOH), CHI and the blended films between KGOH and CHI include matrix network of the KGM film itself, deacetylation on the KGM structure, the presence of secondary amide between the N-H of 5-ALA and the C=O in acetyl group of KGOH structure. Furthermore, 5-ALA was entrapped in the CHI film under secondary amide, with the interaction between amino group of CHI and carboxyl group of 5-ALA. A suitable KGOH/CHI film blend was 25:75 (%w/w), showing the highest entrapment efficiency. The two possible theories for entrapment are: firstly, secondary amide in the interaction between the amino group of CHI and carboxyl group of 5-ALA, and secondly, the interaction between the 5-ALA molecule and the complexity of structure of KGOH. Therefore, a KGOH/CHI film could be used as a high performance blended film for entrapment of 5-ALA and the proportion between the biopolymer and the amount of 5-ALA must be considered for entrapment process. Stabilizing 5-ALA in a film may be an alternative way to preserve 5-ALA for further usage. The product would have the potential to be developed for use in the agricultural producers and industries.

The possible mechanism of releasing 5-ALA in KGM, KGOH film that is the water were absorbed in structure of film and some parts of structure was solubility, as a result releasing of the 5-ALA. The possible mechanism of releasing 5-ALA in CHI film is due to water diffusion in chitosan matrix.

When storage film KGOH, K25C75 and CHI containing 5-ALA at -20, 4 and 28 °C, the high temperature has effect on stability of 5-ALA in all films. When storage

all film at -20 °C for 40 day, all film can retain the 5-ALA molecule more than 90%. At 4 °C after 40 days, the 5-ALA content in film KGOH were retained more than 50% which it can retained the 5-ALA molecule better than film CHI (the remaining of 5-ALA content as ~50%) and K25C75 (the remaining of 5-ALA content as < 50%), respectively. At 28 °C, after storage for 40 days, the remaining of 5-ALA content in film KGOH, K25C75 and CHI as ~30 %, ~20 % and <10%, respectively. Although, the blend film (K25C75) shown a highest efficiency for 5-ALA entrapment, but when storage within temperature range 4 °C and 28 °C, the 5-ALA content was declined rapidly. While film KGOH can retained the 5-ALA more than 50% when storage at 4 °C for 40 day. From the experiment shown that KGM treated with alkali has a potential to use for 5-ALA entrapment more than CHI and blend polymer.

## Recommendations for further research

In present study, separation of 5-ALA from microbial production and forming biodegradable film which containing 5-ALA still have some limitations l and thus further research should be focusing on the following points:

1. Improving the preparation of the crude 5-ALA solution before loading into the column. This is to reduce the competition between impurities and the 5-ALA when absorption to the resin may affect the yield of 5-ALA after elution. The improvement may clarify the required concentration of 5-ALA and determine the compounds in crude 5-ALA solution in order to develop a method to eliminate them
2. The control of release mechanism of 5-ALA in KGOH film, blended film between KGOH and CHI and CHI film should be further investigated. This could be done by releasing them in culture medium for use in tissue culture (e.g. orchids).
3. To study application of films containing 5-ALA in various areas of agriculture in order determine their properties.

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# APPENDIX

# APPENDIX A
# Analysis procedures

## A.1 Ion exchange chromatography system

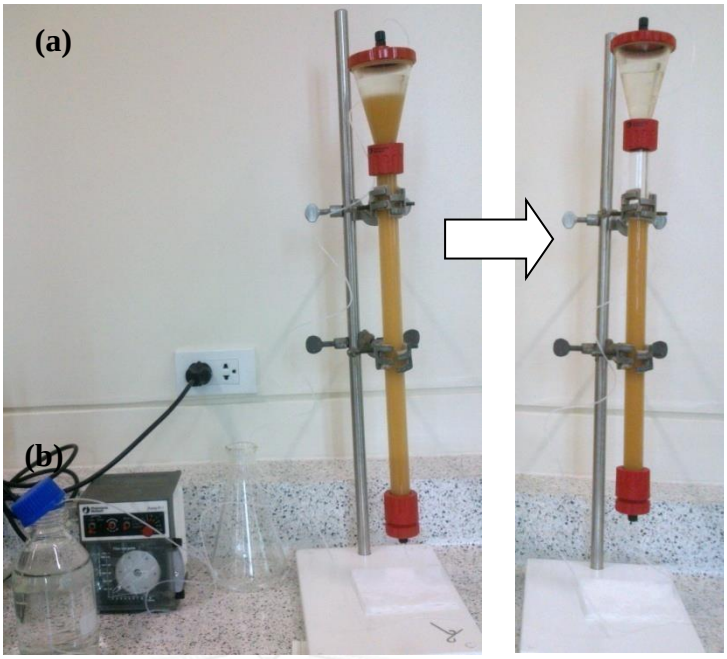
**A.1.1** Preparation and packing the column

Resin preparation

The Dowex cation resin was soaked in 4 N NaOH for 24 hours. After that, the cation resin was rinsed with DI water, and then the cation resin was soak in 4 N HCl for 6 hours, 1 N HCl for 6 hours and rinsed with DI water. The cation resin was placed into glass columns.

Packing the column

The resin was packed in the column at the temperature at which it will be used. Make sure the column is not damaged and that all parts are really clean. Attach the packing reservoir tightly and mount the column vertically on a stand. The column glass tube was wetted with eluent living a few centrimeters of fluid in the bottom. The resin suspension was poured carefully down the wall of the column using a glass rod. All the resin was poured into the reservoir to the top with buffer. Pack the resin in the column until the resin has reached a constant height(Figure A-1a). Then, removing the packing reservoir and using one adaptor instead. Adaptor (Figure A-1b) was wetted by drawing water through it, making sure no air bubbles are trapped under the net. This is best done by submerging the plunger in a beaker of water and attaching the tubing to a pump. Close the tubing with a stopper when all air bubbles have been removed. Insert the adaptor to the top of the column far enough to give the desired bed height. When running the column, the flow rate do not exceed according to the guide column. Equilibrate the column with two bed volume of start buffer.



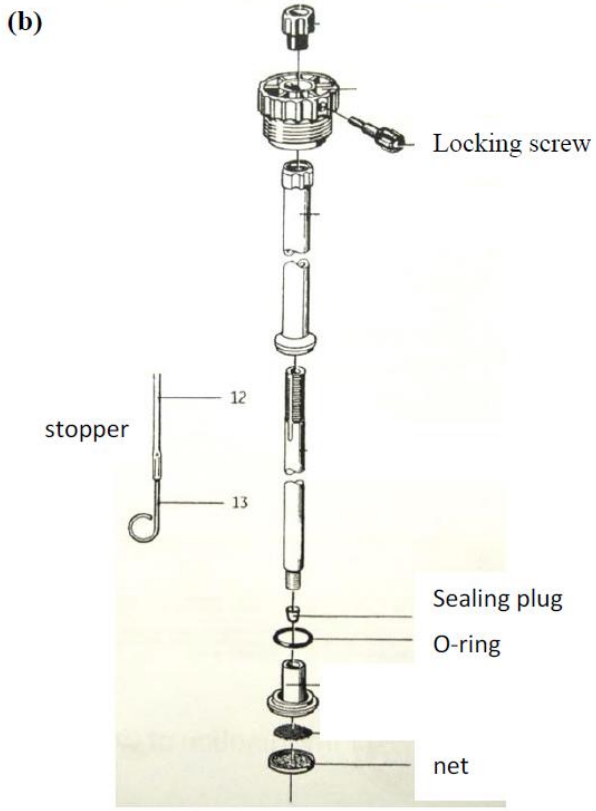




**Figure A- 1** (a) Packing column (b) composition of adaptor

**A.1.2** Preparation 10 mM sodium acetate buffer pH 4.5

Prepare the sodium acetate buffer (10 mM) by dissolving sodium acetate trihydrate ($CH_3COONa·3H2O$) 1.36 g in 800 ml deionized water and adjust to pH 4.5 with glacial acetic acid and dilute to 1 L.

**A.1.3** Preparation 1 M sodium acetate buffer pH 4.5

Prepare the sodium acetate buffer (1 M) by dissolving sodium acetate trihydrate ($CH_3COONa·3H2O$) 136.08 g in 800 ml deionized water and adjust to pH 4.5 with glacial acetic acid and dilute to 1 L.

**A.2 Determination of 5-ALA content using colorimetric method**

The reaction mixture contained 2 ml of the crude 5-ALA solution obtained after centrifugation, 1 ml of 1M sodium acetate buffer (pH 4.67) and 0.5 ml of acetylacetone were added to the mixture. The mixture was then boiled in a water bath (100°C) for 15 min. After cooling, 2 ml of the reaction mixture was mixed with 2 ml of modified Ehrlich's reagent. The absorbance of the mixture at 553 nm was measured by spectrophotometer at room temperature.

**A.2.1** Preparation of 1 M sodium acetate buffer (pH 4.67)

Prepare the sodium acetate buffer (1 M) by dissolving sodium acetate trihydrate ($CH_3COONa·3H2O$) 136.08 g in 800 ml distilled water and adjust to pH 4.67 with glacial acetic acid and dilute to 1 L.

**A.2.2** Preparation of modified Ehrlich's reagent

1 g of p-dimethyl-aminobenzaldehyde dissolved in 30 ml of glacial acetic acid and 8 ml of 70% perchloric acid and the solution is diruted to 50 ml with acetic acid. The reagent is unstable and should be used on the day it is made.

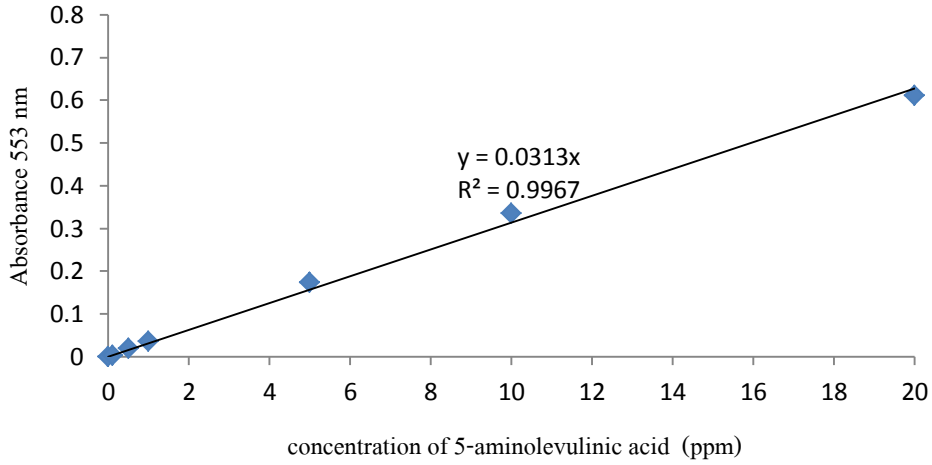



**Figure A- 2** 5-ALA standard curve for determination of 5-ALA content using colorimetric method

## A.3 Determination of 5-ALA content using HPLC-fluorescence

### A.3.1 Derivatization and preparation of standard calibration curve

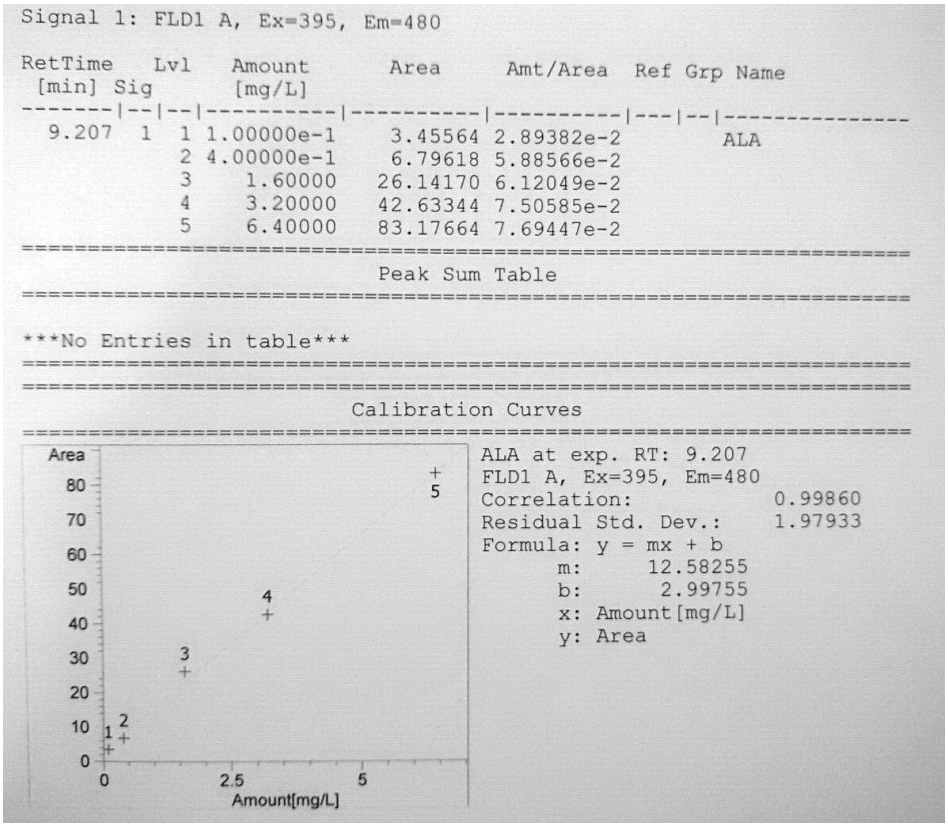
The derivatization mixture was performed by mixing 200 µl of the standard 5-ALA concentration in range 0.1-6.4 ppm with 200 µl of 0.1% fluorescamine solution and 600 µl of 0.1 M borate buffer. The derivatization of 5-ALA samples was analyzed by reacting with fluorescamine for 20 min at room temperature.



| | |
|---|---|
| Column: | Phenomenex Jupiter $C_{18}$ 300 $A^{o}$ (5µm, 150 mm x 4.6 mm) |
| Guard column | wide pore $C_{18}$ |
| Flow Rate: | 1 ml/min |
| Injection volume | 10 µl |
| Detection: | Fluorescence detector |
| Mobile phase | 30% acetonitrile (0.1% TFA) : Water (0.1% TFA) |
| Excitation/emission wavelengths | 395 / 480 nm |
| Retention Time (min) | 9.75 |

### A.3.2 Preparation of 0.1 M borate buffer pH 9

Prepare the stock borate buffer solution (0.1 mol/L) by dissolving 1.24 g boric acid ($H_3BO_3$) in 200 mL deionized water and adjust to pH 9 with 1 M NaOH and dilute to 1 L with deionized water.



Signal 1: FLD1 A, Ex=395, Em=480

| RetTime [min] | Sig | Lvl | Amount [mg/L] | Area | Amt/Area | Ref Grp | Name |
|---|---|---|---|---|---|---|---|
| 9.207 | 1 | 1 | 1.00000e-1 | 3.45564 | 2.89382e-2 | | ALA |
| | | 2 | 4.00000e-1 | 6.79618 | 5.88566e-2 | | |
| | | 3 | 1.60000 | 26.14170 | 6.12049e-2 | | |
| | | 4 | 3.20000 | 42.63344 | 7.50585e-2 | | |
| | | 5 | 6.40000 | 83.17664 | 7.69447e-2 | | |

Peak Sum Table

***No Entries in table***

Calibration Curves



ALA at exp. RT: 9.207
FLD1 A, Ex=395, Em=480
Correlation: 0.99860
Residual Std. Dev.: 1.97933
Formula: y = mx + b
m: 12.58255
b: 2.99755
x: Amount[mg/L]
y: Area

**Figure A- 3** Standard calibration curve of 5-ALA standard

# APPENDIX B
## Raw Data

## B.1 Elution process

**Table B- 1** The absorbance of eluate after gradient elution of crude 5-ALA solution, measurement at 553 nm

| Fraction no. | Absorbance 553 nm | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| 1 | 0 | 0 | 0 |
| 2 | 0.002 | 0 | 0.002 |
| 3 | 0.003 | 0.003 | 0 |
| 4 | 0.003 | 0.003 | 0.003 |
| 5 | 0.003 | 0 | 0.004 |
| 6 | 0.003 | 0.003 | 0 |
| 7 | 0.001 | 0.002 | 0 |
| 8 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 |
| 11 | 0 | 0 | 0 |
| 12 | 0.007 | 0.005 | 0.006 |
| 13 | 0.004 | 0.006 | 0.004 |
| 14 | 0.003 | 0.003 | 0.002 |
| 15 | 0.002 | 0.002 | 0.002 |
| 16 | 0.002 | 0.001 | 0.003 |
| 17 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 |
| 21 | 0 | 0 | 0.001 |
| 22 | 0.002 | 0.003 | 0.004 |
| 23 | 0.002 | 0.003 | 0.004 |
| 24 | 0.003 | 0.002 | 0.001 |
| 25 | 0.001 | 0.002 | 0.002 |
| 26 | 0.002 | 0.001 | 0.003 |
| 27 | 0.001 | 0.001 | 0.002 |
| 28 | 0 | 0 | 0.001 |
| 29 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 |
| 31 | 0 | 0 | 0 |
| 32 | 0 | 0.002 | 0 |
| 33 | 0 | 0.001 | 0 |
| 34 | 0 | 0 | 0 |
| 35 | 0 | 0 | 0 |
| 36 | 0 | 0 | 0 |
| 37 | 0 | 0 | 0 |
| 38 | 0 | 0 | 0 |
| 39 | 0 | 0 | 0 |
| 40 | 0 | 0 | 0 |

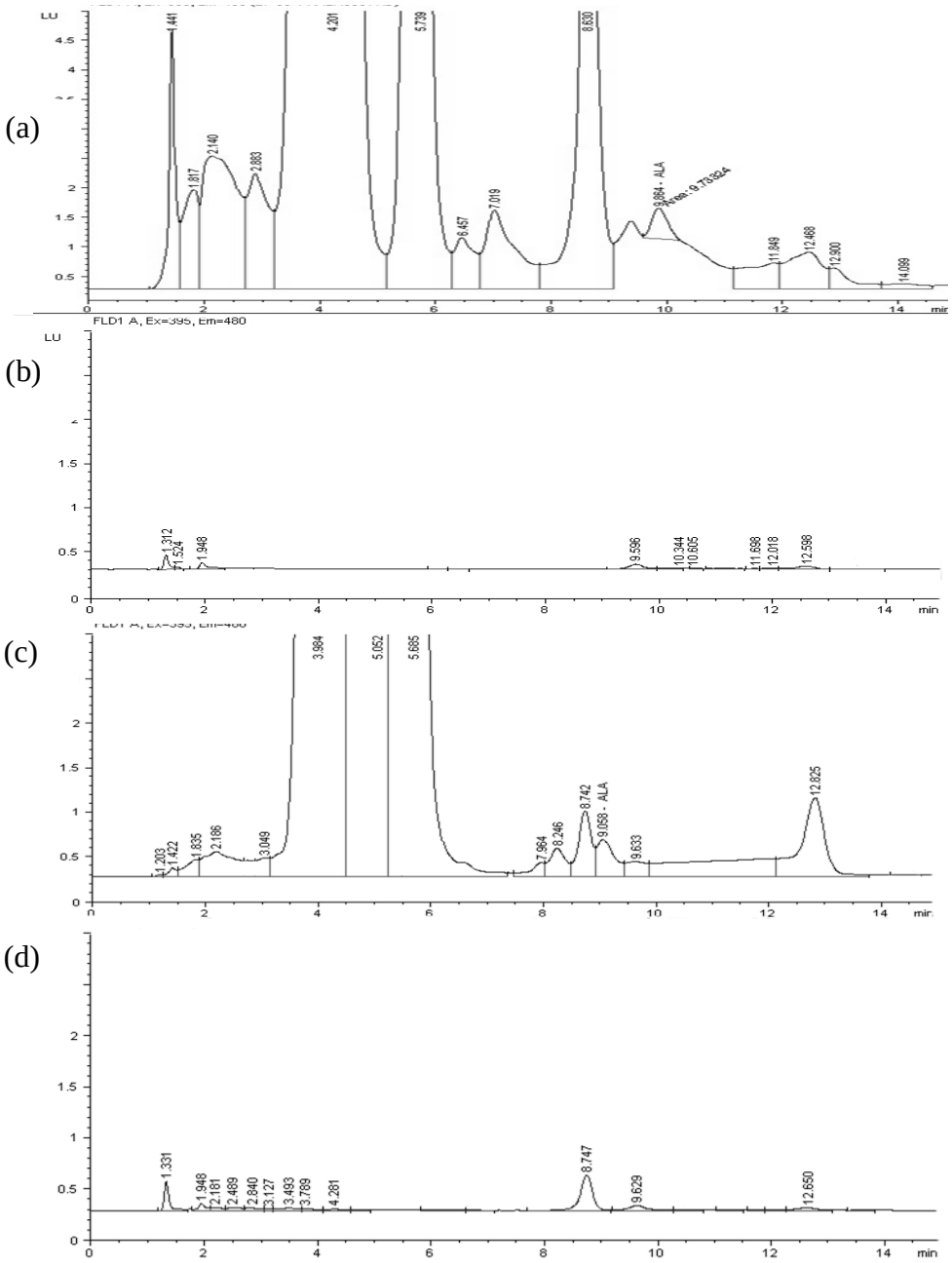



**Figure B- 1** (a) Crude 5-ALA solution, chromatogram of eluate after gradient elution of crude 5-ALA solution by using HPLC-fluorescence (b) fraction no. 2-6 (c) fraction no. 12-13 (d) fraction no. 22-25

# VITA

Miss Phattanit Tripetch was born on December 2, 1980 in Singburi, Thailand. She got her B.Sc. degree in Environmental Technology from King Mongkut's University of Technology North Bangkok in 2002 and M.Sc. in Natural Resources Management from King Mongkut's University of Technology Thonburi in 2005.

List of publication

Tripetch, P., Borompichaichartkul, C. and Srzednicki, G. 2013. Separation Process of 5-Aminolevulinic Acid from Rhodobacter spaeroides for Increasing value of Agricultural Product by Ion Exchange Chromatography. The 2nd Asia Pacific Symposium on Postharvest Research Education and Extension (APS2012). Acta Horticulturae, 1011, 265-271. DOI:10.17660/ActaHortic.2013.1011.32.

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Tripetch, P., Borompichaichartkul, C., Duangmal, K. and Srzednicki, G. 2016. A novel of entrapment of 5-aminolevulinic acid under edible composite film of konjac glucomannan and chitosan. Engineering in Life Science.

List of Conference

Tripetch, P., Borompichaichartkul, C. and Srzednicki, G. 2014. Entrapment of 5-Aminolevulinic Acid under konjac glucomannan and chitosan blend film. The 3rd Asia Pacific Symposium on Postharvest Research, Education and Extemsion (APS2014), 9-11 December 2014. Victory Hotel, Ho Chi Minh City, Vietnam.