ISOLATION AND PURIFICATION OF THE METHYL PARATHION HYDROLASE ENZYME FROM METHYL PARATHION-DEGRADING *PSEUDOMONAS* SP.

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WANPHEN YAMKUNTHONG : ISOLATION AND PURIFICATION OF THE METHYL PARATHION HYDROLASE ENZYME FROM METHYL PARATHION-DEGRADING *PSEUDOMONAS* SP. THESIS ADVISORS : VITHOON VIYANANT, Ph.D., JESDAWAN WICHITWECHKARN, Ph.D., SUKSIRI VICHASRI GRAMS, Dr.rer.nat., BOONSRI JONGSAREEJIT, Ph.D. 147 p. ISBN 974-665-129-3

This study was intended to determine that methyl parathion-degrading bacteria *Pseudomonas stutzeri* used methyl parathion as a carbon source and investigate the metabolism of the methyl parathion hydrolase from this bacterium.

This study was achieved by using Southern hybridization, ion exchange chromatography, gel filtration chromatography, and hydrophobic interaction chromatography. The bacteria hydrolyzed methyl parathion to *p*-nitrophenol, which is the hydrolytic product of methyl parathion, and then degraded *p*-nitrophenol further. A Southern hybridization study indicated that this bacteria does not carry a gene homologous to the *opd* gene and *adp* B gene from the parathion-degrading bacteria *Flavobacterium* sp. ATCC 27551 and the coumaphos-degrading bacteria *Nocardia* sp. strain B-1, respectively. In addition, the expression of the methyl parathion hydrolase is an inducible one and it was found to be a membrane-bound enzyme. The enzyme was purified in 3 steps using Resource S, Sephadex G-100, and Octyl sepharose 4 FF columns. It was purified to approximately 212 fold with a specific activity of about 430 units/mg of protein. After running the partially purified protein from each purification step on an SDS-polyacrylamide gel and performing an activity staining, this enzyme was found to have a molecular weight of approximately 40 kDa. In addition, the enzyme activity was inhibited by DTT but $CuCl_2$ and EDTA had very little effect on the methyl parathion hydrolase activity.

Methyl parathion-degrading bacteria, *Pseudomonas stutzeri*, were found to be capable of using methyl parathion, an organophosphate insecticide as a carbon source. The enzyme was isolated and purified with the aim to use its N-terminal amino acid sequence as information for synthesizing a specific probe to identify and clone its gene in the future.

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วันเพ็ญ แย้มขุนทอง : การศึกษาการแยกบริสุทธิ์ของเอ็นไซม์เมธิลพาราไธออนไฮโดรเลสจากแบคทีเรียที่ย่อยสลายเมธิลพาราไธออน *Pseudomonas* sp. (ISOLATION AND PURIFICATION OF THE METHYL PARATHION HYDROLASE ENZYME FROM METHYL PARATHION-DEGRADING *PSEUDOMONAS* SP.) คณะกรรมการควบคุมวิทยานิพนธ์ : วิฑูรย์ ไวยนันท์, Ph.D., เจษฎาวรรณ วิจิตรเวชการ, Ph.D., สุขศิริ วิชาศรีกรามส์, Dr.rer.nat., บุญศรี จงเสรีจิตต์, Ph.D. 147 หน้า. ISBN 974-665-129-3

เชื้อแบคทีเรียที่ย่อยสลายยาฆ่าแมลงเมธิลพาราไธออน (methyl parathion) ในกลุ่มออร์กาโนฟอสเฟต (organophosphate insecticide) ได้แก่ เชื้อ *Pseudomonas* sp. ซึ่งเชื้อนี้สามารถใช้เมธิลพาราไธออนเป็นแหล่งคาร์บอน โดยสามารถย่อยสลายเมธิลพาราไธออนเป็นพารา-ไนโตรฟีนอล (*p*-nitrophenol) ซึ่งเป็น hydrolytic metabolite ของเมธิลพาราไธออน จากการศึกษาโดยวิธี Southern hybridization พบว่าเชื้อแบคทีเรียที่ย่อยสลายยาฆ่าแมลงเมธิลพาราไธออนชนิดนี้ไม่มียีนที่ homology กับ *opd* ยีน และ *adp* B ยีน ของเชื้อแบคทีเรียที่ย่อยสลายยาฆ่าแมลงพาราไธออน (parathion) และยาฆ่าแมลงคูมาฟอส (coumaphos) ซึ่งเป็นยาฆ่าแมลงในกลุ่มออร์กาโนฟอสเฟต ได้แก่ เชื้อ *Flavobacterium* sp. ATCC 27551 และ *Nocardia* sp. strain B-1 ตามลำดับ นอกจากนี้การแสดงออกของเอ็นไซม์เมธิลพาราไธออนไฮโดรเลส (methyl parathion hydrolase enzyme) ของเชื้อแบคทีเรียนี้เป็นแบบ inducible (inducible expression) และเกาะติดอยู่ที่เมมเบรน (membrane-bound) เอ็นไซม์ชนิดนี้ถูกทำให้บริสุทธิ์โดยใช้ 3 ขั้นตอนคือ ใช้คอลัมน์ Resource S, Sephadex G-100, และ Octyl sepharose 4 FF พบว่า เอ็นไซม์มีความบริสุทธิ์เพิ่มขึ้น 212 เท่า มีค่ากิจกรรมจำเพาะ (specific activity) 430 ยูนิต/มิลลิกรัมของโปรตีน เมื่อนำเอ็นไซม์ที่ได้ไปแยกแถบโปรตีนบน SDS polyacrylamide gel และเมื่อนำไปทำ activity staining จะเกิดแถบของโปรตีนใน SDS polyacrylamide gel พบว่าเอ็นไซม์นี้มีน้ำหนักโมเลกุลประมาณ 40 กิโลดาลตัน นอกจากนี้เอ็นไซม์เมธิลพาราไธออนไฮโดรเลสจะถูกยับยั้งด้วย dithiothreitol (DTT) แต่ cupric chloride ($CuCl_2$) และ ethylenediamine tetraacetic acid (EDTA) จะมีผลต่อกิจกรรมของเอ็นไซม์เพียงเล็กน้อย เอ็นไซม์ชนิดนี้ถูกทำให้บริสุทธิ์เพื่อใช้ในการสังเคราะห์ดีเอ็นเอติดตามในการหายีนของเอ็นไซม์ตัวนี้ต่อไป

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LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
APS	ammonium persulfate
α	alpha
β	beta
BMM	basal mineral medium
BSA	bovine serum albumin
OC	degree celsius
CHL	Chinese hamster lung
CFU/ml	colony forming unit per milliliter
CTAB	cetyltrimethyl ammonium bromide
DIG	digoxeginin
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic
et al.	et alii (and other)
FPLC	fast protein liquid chromatography
γ	Gamma
g	gram
g/cm3	gram per cubic centrimeter
g/m	gram per molar
GFC	gel filtration chromatography

LIST OF ABBREVIATIONS (CONTS)

GSH	glutathione
HCl	hydrochloric acid
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
i.e.	id est
IEC	ion exchange chromatography
kb	kilobase
kDa	kilodalton
LB	Luria broth
lb/in^2	pound per square inch
M	molar
min	minute
mg	milligram
mg/kg	milligram per kilogram
ml	milliliter
μl	microliter
μm	micrometer
MES	2-(N-Morpholino)ethane sulphonic acid
mM	millimolar
MP	methyl parathion

LIST OF ABBREVIATIONS (CONTS)

MW	molecular weight
NaP_i	sodium phosphat buffer
$(NH_4)_2SO_4$	ammonium sulfate
nm	nanometer
2-PAM	pralidoxime
PAR	parathion
PNP	*p*-nitrophenol
rpm	revolution per minute
RT	retention time
SCE	sister chromatid exchange
SD	standard deviation
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SSC	sodium trisodium citrate buffer
TCA	trichloro acetic acid
TEMED	N, N, N', N'-tetramethylene-ethylenediamine
Ve	elution volume
Vo	void volume
v/v	volume by volume
w/v	weight by volume
$\overline{X}$	mean value

CHAPTER I

INTRODUCTION

Organophosphate insecticides such as parathion (*O,O*-Diethyl *O*-4-nitrophenylphosphorothioate; PAR) and its methyl analog, methyl parathion (*O,O*-Dimethyl-*O*-4-nitrophenylphosphorothioate; MP) have been used extensively in Thailand and in other countries. These insecticides were introduced to replace the hazardous chlorinated hydrocarbon insecticides (1, 2, 3, 4, 5). In Thailand, import of methyl parathion was the third order among other insecticides and reached to 1,327 tons, which cost 145 million Bath in 1998 (6). Methyl parathion is highly toxic and has been used widely for controlling insects for agricultural and public health purposes (7, 8, 9). The intensive use of methyl parathion has caused enormous environmental contamination and public health problems (10, 11, 12).

The cholinergic sign of methyl parathion poisoning was caused by the accumulation of acethylcholine at nerve endings, muscarinic receptors and central nervous system. Methyl parathion becomes toxic when it is metabolized to methyl paraoxon (1, 5, 10, 13, 14, 15) but atropine can cure this toxin. The oral LD_{50} and skin LD_{50} methyl parathion in rats were 9 mg/kg and 63 mg/kg of body weight, respectively (5, 16). Several studies reported the toxicity of methyl parathion (17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27) and its effects on various organisms (28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39).

Methyl parathion contaminates the environment as a result of disposal of domestic and industrial wastes, accidental spill, and discharge from insecticide

containers, run-off, wash-off, and leaching from the application areas. The contamination of this insecticide was found in soil, water surface, ground water, and suspended sediments. The persistance of methyl parathion in the environment causing accumulation of the chemical in living organisms and food chain (11, 40, 41, 42, 43, 44, 45, 46, 47, 48).

In Thailand, Tayapatchara and Sakultriangtrong *et al.* reported the presence of residual methyl parathion in soil and water in agricultural areas (9, 49, 50). The presence of residual methyl parathion in freshwater fish in central agricultural areas was also reported by Chamraskul and Lertveerasirikul (51).

Methyl parathion can be detoxified by strong alkaline (1 N NaOH) (8, 10, 15), however, the most important route for degradation of most insecticides from the environment is by microbial degradation (16, 52). Organophosphate insecticides are generally degraded by hydrolytic process of microorganisms. Several studies revealed that the first hydrolytic products of microbial degradation of methyl are *p*-nitrophenol (PNP) and dimethylthiophosphoric acid. These products are then further metabolized to other simple compounds (53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70). Several studies indicated that these microorganisms can be used to detoxify agricultural wastes (71, 72, 73, 74, 75).

Although there were many reports of parathion-degrading and methyl parathion-degrading microorganisms isolated both in the form of mixed cultures and pure isolates but a few of them have been studied at the enzymatic and molecular level. These researches studied mainly on parathion hydrolase activity of *Flavobacterium* sp. (ATCC 27551) and *Pseudomonas diminuta* strain MG and the parathion hydrolase gene of these microorganisms (54, 55, 62, 66, 71, 76, 77, 78, 79,

80, 81, 82, 83, 84, 85). There appears to be 2 groups of degradative gene, *opd* and *adp* B genes, which code for enzymes responsible for hydrolyzing organophosphate insecticides (54, 55, 66, 76, 78, 79, 80, 81, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93). Several studies showed the detection of organophosphate insecticide and its changes during bacterial degradation by high performance liquid chromatography (HPLC) (95, 96, 97, 98, 99). However, the activity staining of the methyl parathion hydrolase on SDS-polyacrylamide gel has never been reported.

In Thailand, Keprasertsup reported that *Pseudomonas* sp. isolated from mixed culture used MP for growth in basal mineral medium (BMM). The result of this study indicated that this *Pseudomonas* sp. used MP as a sole source of carbon, degrading it to PNP and also degrading PNP further. The quantification of MP and PNP in the culture was determined by high performance liquid chromatography (HPLC). Recently, *Pseudomonas* sp. was tentatively identified as *Pseudomonas stutzeri* (7).

This *Pseudomonas stutzeri* is an interesting microorganism because it is capable of hydrolyzing MP and is a native microorganism of Thailand. In addition, this bacterium has never been studied for its methyl parathion hydrolase enzyme at the molecular level. This study aims at investigating the metabolism of methyl parathion degradation of this *Pseudomonas* sp. and at studying on the expression and the location in SDS-polyacrylamide gel of methyl parathion hydrolase enzyme. This enzyme was isolated, purified, and determined its molecular weight in SDS-polyacrylamide gel by activity staining. The enzymatic and molecular study of the bacterium native to Thailand will serve as a basic knowledge, which will help for improvement of the methyl parathion hydrolae gene and will eventually lead to an

effective method for decontamination of organophosphate insecticides in the environment in the future.

OBJECTIVES

The objectives of this study are as follow;

1. To study the growth curve of *Pseudomonas stutzeri*, which degrades methyl parathion (MP) and *p*-nitrophenol (PNP) and to analyze MP and PNP, which is the first metabolite of MP hydrolysis, by using high performance liquid chromatography (HPLC) and a spectrophotometer.
2. To identify the nature of methyl parathion hydrolase enzyme of this bacterium whether it is a membrane-bound or expression is inducible or constitutive.
3. To isolate and purify methyl parathion hydrolase enzyme by cation exchange chromatography, gel filtration chromatography, and hydrophobic chromatography.
4. To locate the electrophoretic band of methyl parathion hydrolase enzyme of this bacterium in SDS- polyacrylamide gel using " activity staining" method.
5. To study the effects of reagents on the activity of methyl parathion hydrolase enzyme of this bacterium as determined by spectrophotometric technique at 410 nm.

CHAPTER II

LITERATURE REVIEW

1. Methyl Parathion (MP)

Insecticides are chemical agents used widely for pest control and public health. There are three groups of insecticides, which have been used extensively in Thailand, namely chlorinated hydrocarbons, carbamates, and organophosphate (5).

Chlorinated hydrocarbons; for example, dichlorodiphenyl trichloroethane (DDT) was the first group, which was used for controlling insect pests and disease vectors. However, because of it persistance in the environment, the intensive use of this compound resulted in enormous problems on environmental contamination (4). Metabolites of this group of insecticides were found to contaminate soil and ground water and were even detected in human (1, 2, 3). The use of this insecticide has been restricted in the United States and in other developed countries. Carbonate and organophosphate insecticides were introduced to replace the hazardous chlorinated hydrocarbon insecticides (4, 16). However, the residuals of these insecticides were also found in soil, water, and flooded soil in the application areas (11, 16, 65).

Ethyl parathion (*O,O* diethyl *O-p*-nitrophenyl phosphorothioate; PAR) and its methyl analogue, methyl parathion (*O,O* dimethyl *O-p*-nitrophenyl phosphorothioate; MP), are a broad-spectrum insecticides and used to be widely use in the agricultural areas. However, the study of U.S. Environmental Protection Agency (EPA) reported that other types of insecticide including MP were used for the replacement of PAR in the United States, (5).

2. Identification of Methyl Parathion

molecular formula: $C_8H_{10}NO_5PS$

molecular structure: as shown in Figure 1

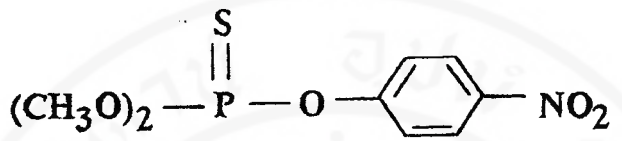

Figure 1. Molecular structure of MP (5).

molecular mass (g/mol): 263.33

common names: methyl parathion; parathion methyl; etc

CAS chemical name: *O,O*-dimethyl *O*-(4-nitro-phenyl)-phosphorothioate

IUPAC systematic name: *O,O*-Dimethyl *O*-4-nitrophenyl phosphorothioate

common synonyms Demethylfenitrothion; dimethyl *para*-nitrophenyl monothiophosphate; *O,O*-dimethyl O-(*para*-nitrophenyl) phosphorothioate; dimethyl *para*-nitrophenyl thionate; dimethyl 4-nitrophenyl phosphorothionate; *O*, *O*-dimethyl *O*-(*para*-nitrophenyl) thionophosphate; dimethyl *para*–nitrophenyl thiophosphate; *O,O*-dimethyl-*O*-(*para*-nitrophenyl) thiophosphate; dimethyl parathion; metaphos; E601; methyl-parathion; 8056 HC; etc.

trade names: A-Gro; Azofos; Bladan M; Divithion; Folidol M; Metacid 50; Metacide; Methyl-E 605; Metafos; Methyl Parathion;

Nitrox; Parapset M-50; Pasatox; Paridol; Thylpar M-50; Toll; Wofatox; Wolfatox; etc.

3. Physical and Chemical Properties of Methyl Parathion

physical state:	pure substance: white crystalline solid or powder
	technical product: amber liquid and similar oil
melting point:	35°-36°C
boiling point:	160°C (pressure at 1 mm of Hg)
vapour pressure:	1.3 mPa at 20°C
density/ specific gravity:	1.358 at 20°C (g/cm^3)
water solubility:	55-60 mg/litre at 25°C
nonaqueous solubility:	soluble in ethanol, organic solvents, and acetone
odor:	like rotten eggs or garlic
other properties:	hydrolyses and isomerized easily
stability:	stable at pH 7 and in acid solvents at room temperature (7, 8, 10, 15)

4. Toxicity of Methyl Parathion

The toxicity of an organophosphate insecticide is exerted onto animals by inhibiting acetylcholinesterase (AChE). Acetylcholine is a neurotransmission agent which involes in chemical transmission of nerve impulse and needs to be destroyed by AChE afterward. Consequently, the disruption of nervous activity is caused by accumulation of acetylcholine at nerve ending in nervous tissue and other effected organs.

4.1 Signs and Symptoms

Signs and symptoms of acute poisoning by MP are predictable from their biochemical mechanism of action. They are divided into (i) muscarinic effects, (ii) nicotinic effects, and (iii) central nervous system effects.

(i) Muscarinic effects are symptoms due to the stimulation of structures invaded by the parasymphathetic nervous system. Muscarinic receptors for acetylcholine are found primarily in smooth muscles, the heart, and exocrine glands. Therefore, signs and symptoms that result from the stimulation of these receptors include tightness in the chest, wheezing due to broncho-constriction, and increased bronchial secretions. Increased salivation and lacrimation, increased sweating, increased gastrointestinal tone and peristalsis result in nausea, vomiting, abdominal cramp, diarrhoea, tenesmus and involuntary defecation. Brandycardia that leads to heart block, hypotension, and frequent and involuntary urination is due to contraction of smooth muscle of the bladder, and constriction of the pupils. These effects can be antagonized by atropine.

(ii) Nicotinic effects are the result of the accumulation of acetylcholine at the motor nerve endings at skeletal muscle and autonomic ganglia.

Muscular effects include easy fatigue and mild weakness followed by involuntary twitching, scattered fasciculation and cramps, muscular weakness that affects the muscles of respiration and contributes to dyspnea and cyanosis.

Nicotinic actions at autonomic ganglia, in severe intoxication, mask some of the muscarinic effects. Thus, tachycardia may result from the stimulation of sympathetic ganglia to overcome the usual bradycardia due to muscarinic action on

the heart. Pallor, elevation of blood pressure, and hyperglycemia also reflect nicotinic action on sympathetic ganglia. These nicotinic symptoms can be treated by oximes.

(iii) Central nervous system effects are due to the accumulation of acetylcholine in the central nervous system and direct action on its elements. Symptoms may include tension, anxiety, restlessness, insomnia, headache, emotional instability and neurosis, apathy, and confusion. Slurred speech, tremor, generalized weakness, ataxia, convulsion, depression of respiratory and circulatory centers, and coma are other central nervous system effects. These symptoms are controlled by atropine.

Death is due to asphyxiation because of respiratory failure. Contributing factors are the muscarinic actions of broncho-constriction and increased bronchial secretions, nicotinic action leading to paralysis of the respiratory muscle, and the central nervous system action of depression and paralysis of the respiratory center. The symptoms caused by exposure of MP are usually rapid, within generally one to five days. In fatal untreated poisoning, death usually occurs within 24 hours (1, 5, 14).

4.2 Metabolisms of Methyl Parathion

Metabolism of MP can be divided into activation and inactivation reactions.

4.2.1 Activation Reaction

The activation reaction occurs in the liver and involves the replacement of sulphur by oxygen through an oxidation mechanism. The oxygen analog, methyl paraoxon is the inhibitor of cholinesterase, which may occur within minutes following the administration of MP. The conversion is called a desulfuration (Figure 2).

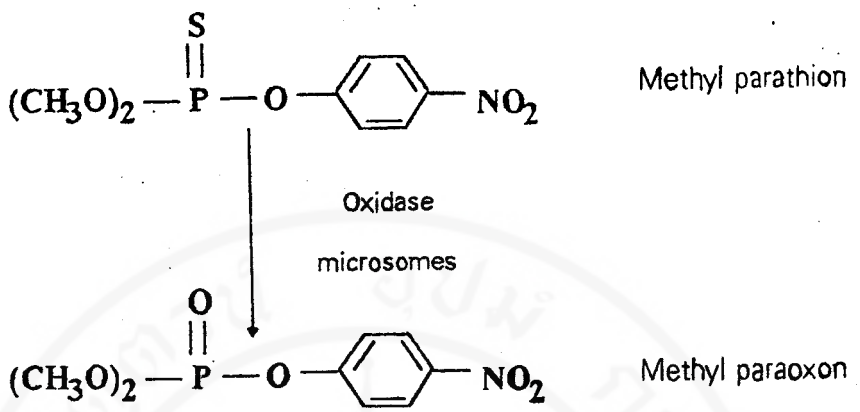

Figure 2. The activation reaction of methyl parathion (desulfuration).

This reaction is catabolized by cytochrom P-450 dependent monooxygenase that belongs to the group of NADPH-dependent mixed function oxidase, located in microsome of the liver. Methyl paraoxon is also necessary for the normal action of MP as cholinesterase inhibitor (Reaction I, Figure 3). The desulfurase activity of the liver is normally high enough to convert MP to methyl paraoxon at an extremely rapid rate. This activity not only occurs in a liver but also in lung and brain.

4.2.2 Inactivation Reaction

In addition to an oxo (= O) group involving anticholinesterase activity, metabolic modification of the alkyl and aryl substituents can also influence the anticholinesterase activity. Reactions II, III, IV, and V (Figure 3) are enzymatic detoxification reactions that yield products, which cannot inhibit AChE.

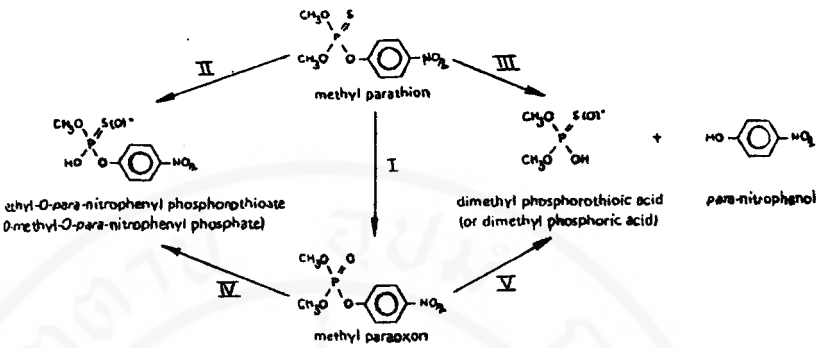

Figure 3. Metabolism of methyl parathion in rodents. Methyl paraoxon may be metabolized via the same pathways as methyl parathion resulting in the oxygen analog, indicated by the presence of $(O)^{\bullet}$ in the figure (15).

I. Toxification: metabolic formation of oxon.

II, IV. Detoxification: glutathione dependent alkyl transferase.

III, V. Detoxification: glutathione dependent aryl transferase.

Reaction V is catalyzed by methyl paraoxonase (esterase). This enzyme is widely distributed among several tissues in rats and other mammals. Methyl paraoxon is detoxified by hydrolysis.

The aryl-phosphorus bond can be cleaved (Reaction IV) prior to oxidation of methyl parathion to methyl paraoxon. A microsomal enzyme catalyses this reaction.

Oxidative dealkylation (Reaction II and IV) also occurs with the dimethyl analogs. MP is preferentially monodealkylated by another system, using glutathione (GSH) alkyltransferase. Glutathione-dependent alkylation occurs in the liver cells and the products of this reaction are S–methyl glutathione and corresponding desmethyl

phosphate compound. This reaction has been termed phosphoric acid triester-glutathione s-alkyltransferase (1, 10, 13, 14, 15).

MP and methyl paraoxon are mainly detoxified by conjugation with GSH. Detoxification is achieved by degradation reactions that involve either demethylation or dearylation. The resulting desmethyl compounds and dimethyl phosphoric acids are essential nontoxic (7, 10). These detoxification reactions are due to the glutathione-dependent alkyl and aryl transferase; the reaction products are *O*-methyl-*O*-*p*-nitrophenyl phosphorothioate or dimethyl phosphorothioic acid and *p*-nitrophenol (PNP). In addition, hydrolysis of methyl paraoxon by tissue arylesterase may occur. Thus, it is possible to follow an exposure to MP by measuring the urinary excretion of PNP (10).

The metabolism of MP is so complicated that the net effect of hepatic biotransformation of MP by intact liver can be either activation or detoxification depending on the relative rates of formation and detoxification of methyl paraoxon within the liver.

4.2.3 Acetylcholinesterase Inhibition and Reversal

Acetylcholine forms during the transmission of nerve impulses in the body includes the central nervous system, and it must be hydrolyzed by AChE to prevent excessive stimulation of the nerve receptors. Methyl paraoxon is the actual inhibitor of AChE, which is reacting at the esteratic site of the enzyme, which the ester linkage of acetylcholine normally combines. This is called the esteratic site of the enzyme. The reaction between MP and AChE, one of the ester group leaves the molecule and a dialkyl group of MP becomes attached to a sarine molecule of the enzyme, and

divided into two parts: PNP and phosphorylated AChE. Phosphorylated AChE may be reversed or aging finally and PNP is excreted via urine, as shown in Figure 4.

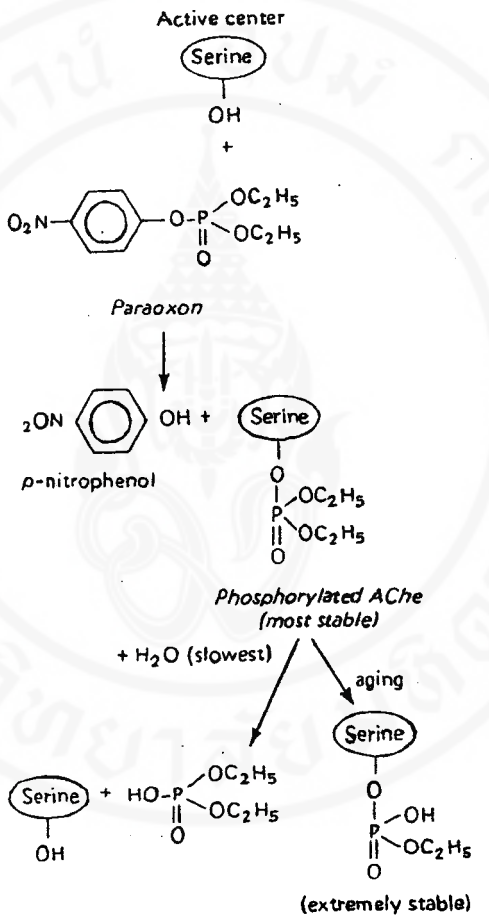

Figure 4. Reactions between the acetylcholine esterase and methyl paraoxon (7, 10).

1. Phosphorylation of the enzyme.

2. Reactivation reaction.

3. "Aging"

In reaction 2 (reactivation), spontaneous reversal of enzyme inhibitor occurs by hydrolyzing dimethyl phosphorylated cholinesterase. The dimethyl phosphorylated cholinesterase recovers extremely slow. Induced recovery of enzyme inhibitor can occur by certain chemicals that will accelerate the hydrolysis of the dimethyl phosphorylated cholinesterase, and hence accelerates the regeneration of active AChE.

In reaction 3 (aging) involves cleavage of an R-O-P- bond with the loss of R and the formation of a charged monosubstituted phosphoric acid residue still attached to protein. The reaction is called "aging".

The most successful compounds for detoxifying AChE inhibitor are oxime derivatives, and the best known of these compounds is 2-pyridine aldoxime methiodide (2-PAM, pralidoxime) which is a part of the standard therapy of organophosphate insecticide poisoning. In addition to the fact that 2-PAM accelerates the dephosphorylation of AChE, it can also enhance the direct hydrolysis of the active inhibitor at physiologic pH (Figure 5). The effectiveness of 2-PAM in reversing AChE inhibition *in vivo* depends upon its early administration following poisoning, because the "aged" phosphorylated enzyme is irreversible by the oxime (1, 10, 13, 14, 15).

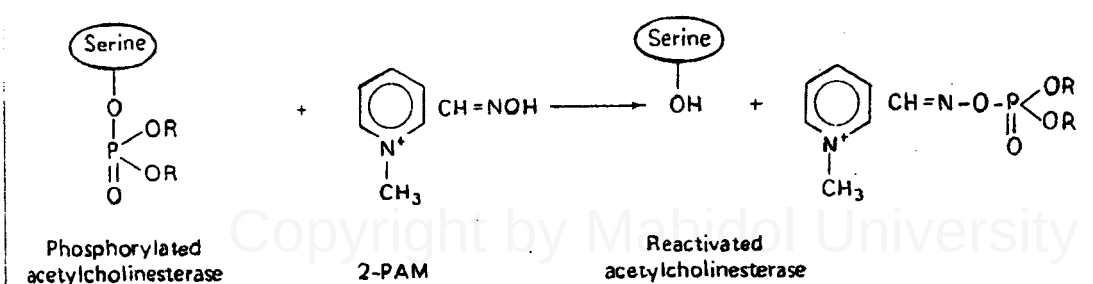

Figure 5. Relationship between phosphorylated acetylcholinesterase and pralidoxime.

4.3 Treatment of MP Poisoning

Methyl parathion produces rapid poisoning, and if in sufficient doses, the patient will be dead. In very severe case, the treatment should include (i) artificial respiration, (ii) atropine sulfate, 2 to 4 mg intravenously as soon as cyanosis is overcome, (iii) slow administration of 2-PAM, 1g intravenous after treating a patient with atropine. In case of contamination of skin or eyes, it should be washed with an alkaline soap, which will not only remove MP but will also hydrolyse the phosphate ester (14).

4.4 Genotoxicity

Several studies reported the genotoxicity of MP. In 1984, Rashid *et al.* investigated the systems used for detection of back mutation and DNA-damage in *Salmonella typhimurium* and *Escherichia coli*. They found that MP was mutagenic to *S. typhimurium* strain TA 100 after activated with rat liver and cytosolic enzymes. Moreover, MP was effective in inducing DNA damage to *S. typhimurium* strain AT 1538. This strain lacks excision repair when compared to the strain TA 1978 that is proficient in excision repair mechanism (17).

Arroyo *et al.* treated *Vicia faba* root tips for two hours with 0.5, 0.75, 1.0, and 2.0 μg/ml (part per million: ppm) of MP. The results showed that the concentrations from 0.5 to 1.0 ppm, MP was effective in inducing sister chromatid exchanges (SCE). At 2.0 ppm, the tissues were damaged and the mitotic index was diminished (18). Ahmed *et al.* reported that MP induced different types of chromosomal aberrations such as micronuclei, single and multiple bridge formation, and laggard chromosome (19).

Mathew *et al.* tested MP genotoxicity in Swiss albino mice using sperm abnormality assay. The results showed that the percentage of abnormal sperms were increased in MP-treated animals when compared to that of distilled water controls at various time intervals. The different types of abnormal sperms observed were amorphous, hookless, banana, folded and double-headed/tails. This research may be concluded that MP causes germ cell mutation and sperm shape abnormalities in mice (20).

In 1993, Ni *et al.* tested mutagenecity of MP with micronucleus assay system both in mouse marrow cells *in vivo* with multi-intraperitoneal administration and in cultured Chinese hamster lung (CHL) cells *in vitro*. It was found that MP induced micronucleus in CHL cell *in vitro* but not in mouse marrow cells *in vivo* (21). Chen *et al.* were successful in indicating sister chromatid exchanges (SCE) and cell cycle delay in Chinese hamsters cell line V79 and in human lymphoid cell line B35 M with MP (22). Moreover, Sobti *et al.* also found that MP increased SCE in human lymphoid cell line LAZ-007 (23).

4.5 Teratogenicity

Several studies reported the teratogenicity of MP. In 1984, Varnagy *et al.* studied teratogenic effects of MP (Wofatox-50 EC) on pheasant embryos by injecting 0.1 ml of the aqueous emulsion Wofatox-50-EC into the air space of embryonated eggs. They found certain morphological changes of eggs: primary hypoplasia or atrophy developed in the cervical musculature, and accompanied by lardosis, scolosis of the cervical spine, and cyllosis (24). Moreover, Varnagy *et al.* injected 0.1 ml of MP (Wofatox-50EC) into the embryonated eggs of hens and pheasants at the 12th day

of incubation. The results showed that MP caused a diminished body mass, a high incidence of developmental malformations (the anomalies of cervical lordosis, scoliosis, and cyllosis), and embryonic moralities at higher dose levels (hen: 4%, pheasant: 2%). The incidence of abnormally developed fetuses was sporadic at lower doses (hen: 0.4%, pheasant: 0.2%) (25).

Deli *et al.* treated chicken eggs with 0.4% MP solution for 4 or 8 days. They found that MP significantly decreased the content of α-actinin, α-, β-, and γ-tubulin in cervical muscle of the embryos. The data suggested that the muscle-damaging effects of MP might be related to the decrease in tissue content of cytoskeletal protein (26).

4.6 Carcinogenicity

In 1979, National Cancer Institute (NCI) in USA studied carcinogenecity of MP in mice. Six-week-old female B6C3F1 mice and male mice received dietary food containing either 62.5 or 125 mg MP/kg. Untreated groups were used as a control. It was found that 80-86% of them were still alive at the end of the study. There was no significant increase in tumor rates (27).

5. Effects on Organisms in the Environment

5.1 Microorganisms

In 1983, Portier *et al.* tested the effect of MP (1.5 or 5 mg/liter) on the reproduction of aquatic microorganisms from drainage basins. In bacteria and Actinomycetes, MP had a positive effect on the development. In fungi and yeast, slight negative effects were found that were related to the test condition rather than to the concentration of MP (28).

Bhunia *et al.* cultured *Nostoc muscorum*, which is a major nitrogen-forming organism, with MP at 5, 10, 20, or 35 mg/liter. Only the highest concentration significantly reduced the growth of the cells in culture. However, the chlorophyll-a contents of the cultures were reduced at 5 mg/liter and substantially reduced at 10 mg/liter (29).

5.2 Aquatic Animals

LC_{50} values of more than 1 mg/liter have been found for some freshwater biota (mollusks, fish, and amphibians). Insect sensitivity to MP depends not only on the species but also on the life stage. In general, instar I larvae are more affected than instar IV larvae. In 1978, Appearson *et al.* shown that larvae might develop a resistance to MP. Both freshwater and marine crustaceans are sensitive to MP with EC_{50} values ranging from 0.002 to 0.05 mg/liter (30).

Reddy *et al.* tested the exposure of freshwater crab (*Oziotelhpusa senex senex*) to sublethal levels at the concentration of 0.1-1 mg/liter of MP. The complete inhibition of molt, a delay in the beginning of molt, or a decrease in the percentage of molting animals were the result of the toxicity of MP. A decrease in the carbohydrate content and increase in acid phosphatase activity in both the hepatopancreases and muscle also occurred (31, 32, 33).

Crossland studied the effects of toxic chemicals on the productivity of freshwater ecosystems. He applied MP at concentration 10 and 40 μg/liter to invertebrates and found that the MP treatment had significant effects on the population densities of larva may flies, damsel flies, chironomids, *Daphia longispina*, and fish. (34).

Ghosh *et al.* treated the catfish (*Channa punctatus*) with 52μg /liter of MP resulted in the elevation of serum triiodothyronine (T_3) as well as the depression of brain AChE activity. Moreover, this low dose of MP also impaired the regulation of gonadal function by gonadotropic hormone and gonadotropin-releasing hormone in *Channa punctatus* (35, 36)

5.3 Terrestrial Organisms

Ratter and Franson examined toxicological effects of MP at concentration 0.375 to 3 mg/kg in American kestrel (*Fasco sparverius*). A dose-dependent inhibition of brain and plasma cholinesterase, hyperglycemia, and elevated corticosterone concentration was observed at 3 mg MP per kg of body weight (37).

In 1987, Deshpande and Swamy tested the exposure of sorghum seeds to MP for 1 h before germination and found that it resulted in the accumulation of proline in the seedlings and reduction in growth. Residues of MP in the soil also influenced seed germination and seedling growth (38).

Bennett *et al.* examined the effects of dietary exposure to MP at concentration 400 mg/kg on eggs laying and incubation in mallard ducks (*Anas platyrhynchos*). They found that the number of hatching per nest were 61%, 43%, and 58% decrease in egg laying, early incubation, and late incubation, respectively (39).

6. Entry and Contamination in Environment

Methyl parathion disperses into the environment by direct and indirect routes. The direct route includes the use of MP for controlling insects by spraying, disposal of domestic and industrial wastes such as those from insecticide manufacturers, and

disposal of excessive residues. The indirect route includes drifting from aerial or ground application and movement via wind, water, and soil erosion, run-off, wash-off, leaching from treated area, and accidental spill (11, 40, 41).

Several studies have been reported on the contamination of MP in the environment (11, 40). In 1972, Carey *et al.* found that the contamination of residual MP in soil in agricultural area of South Dakota was 0.01 mg/kg (42). Midwest Research Institute (1975) reported the concentrations of residual MP in soil in agricultural area were 0.09-1.9 mg/kg (43). The U.S. Environmental Protection Agency (1988) listed MP to be the hazardous chemical in soil (44).

In 1977, Pfaender *et al.* reported the concentrations of MP in water samples from the Cape Fear River Basin in North Carolina at peak period in dissolved fraction and particulate-associated fractions were 468 ng/liter and 123 ng/liter, respectively (45). Marcus *et al.* reported the concentrations of organophosphate insecticides from a wastewater of manufacturer, which produced PAR, to be 2 mg/liter in pre-treatment water, 3.2 mg/liter in mid-treatment water, and less than 0.004 mg/liter in post-treatment water (46). Lenardon *et al.* reported some contamination of MP in water and suspended sediments from the Parana River in Argentina (47). Moreover, Cooper found MP in major watershed components of Moon Lake in Mississippi and also found MP in fish tissues. The result of this study showed that MP persisted in the environment long enough until it accumulated in living organism and food chain (48).

In Thailand, Tayapatchara reported the concentrations of residual MP in soil and water in agricultural areas as 0.475 ppm and 0.68 ppm, respectively (9). In 1991, Sakultriengtrong *et al.* found that the concentrations of residual MP in soil and water in pomelo and tangerine orchards were 0.001-0.172 ppm and 0.018-16.3 part per

billion (ppb), respectively (49, 50). In the same year, Chamraskul and Lertveerasirikul also reported that the concentration of residual MP in freshwater fish in central agricultural areas of Thailand were about 0.001-0.398 ppm (51).

7. Degradation of MP and other Organophosphate Insecticides by Microorganisms

The most important route for degradation of several insecticides from the environment is microbial degradation (16, 52). Many groups of microorganism have been found to degrade the compounds in different habitats and circumstances. The reactions involved in the metabolism of organophosphate insecticides by organisms are hydrolysis, reduction, and oxidation.

i) Hydrolysis reactions: Organophosphate insecticides containing either the P=O (phosphate) or the P=S (phosphorothioate) groups are generally degraded by hydrolytic processes of microorganisms. The hydrolytic products of MP are PNP and dimethylthiophosphoric acid (7, 10).

ii) Reductive reactions: The reduction of the nitro group to amine has been reported during the metabolism of organophosphate insecticides by microorganisms. The reductive product is aminomethyl-parathion (7, 10).

iii) Oxidative reactions: The oxidation reactions are more important in degrading organophosphate insecticides in higher organisms than in microorganisms because of the lack of a defined mixed-function oxidase system in microorganisms (10, 11, 53). The oxidative product is methyl paraoxon in higher organisms.

7.1 Pathways of MP in Microorganisms

The essential pathways of MP metabolism in microorganisms are hydrolysis and reduction (Figure 6). The hydrolysis of MP occurs at the nitrophenyl (C-O-P) bond. The hydrolysis products of MP are PNP and dimethylthiophosphoric acid (54, 55, 56, 57). Further metabolism of PNP release nitrite and hydroquinone or *p*-nitrocatechol (58). PNP may probably be reduced to *p*-aminophenol in anaerobic system.

The reduction of the nitro group of MP forms aminomethyl-parathion. Further hydrolysis of PNP yield *p*-aminophenol and dimethylthiophosphoric acid. The reduction of MP often occurs under lower oxygen condition or higher organism matter or yeast extract condition (56, 59, 60).

The oxidative reaction of MP is not found in microbial system but it is an important reaction in higher organisms.

Lal, and Munnecke and Heish showed the proposed pathway of microbial degradation of PAR and MP (11, 53). Sharmila *et al.* studied the degradation of MP in soil and found that hydrolysis was the major pathway in flooded and non-flooded soil (56).

7.2 Microbial Degradation

In 1972 and 1973, Sethunathan and Yoshida showed that PAR could be degraded to PNP by *Flavobacterium* sp. which was isolated from diazinon-amended flooded rice soil (61, 62). Siddaramappa *et al.* isolated a *Bacillus* sp. and a *Pseudomonas* sp. from parathion-amended flooded alluvial soil and found that these microorganisms hydrolyzed PAR to nitrite (63).

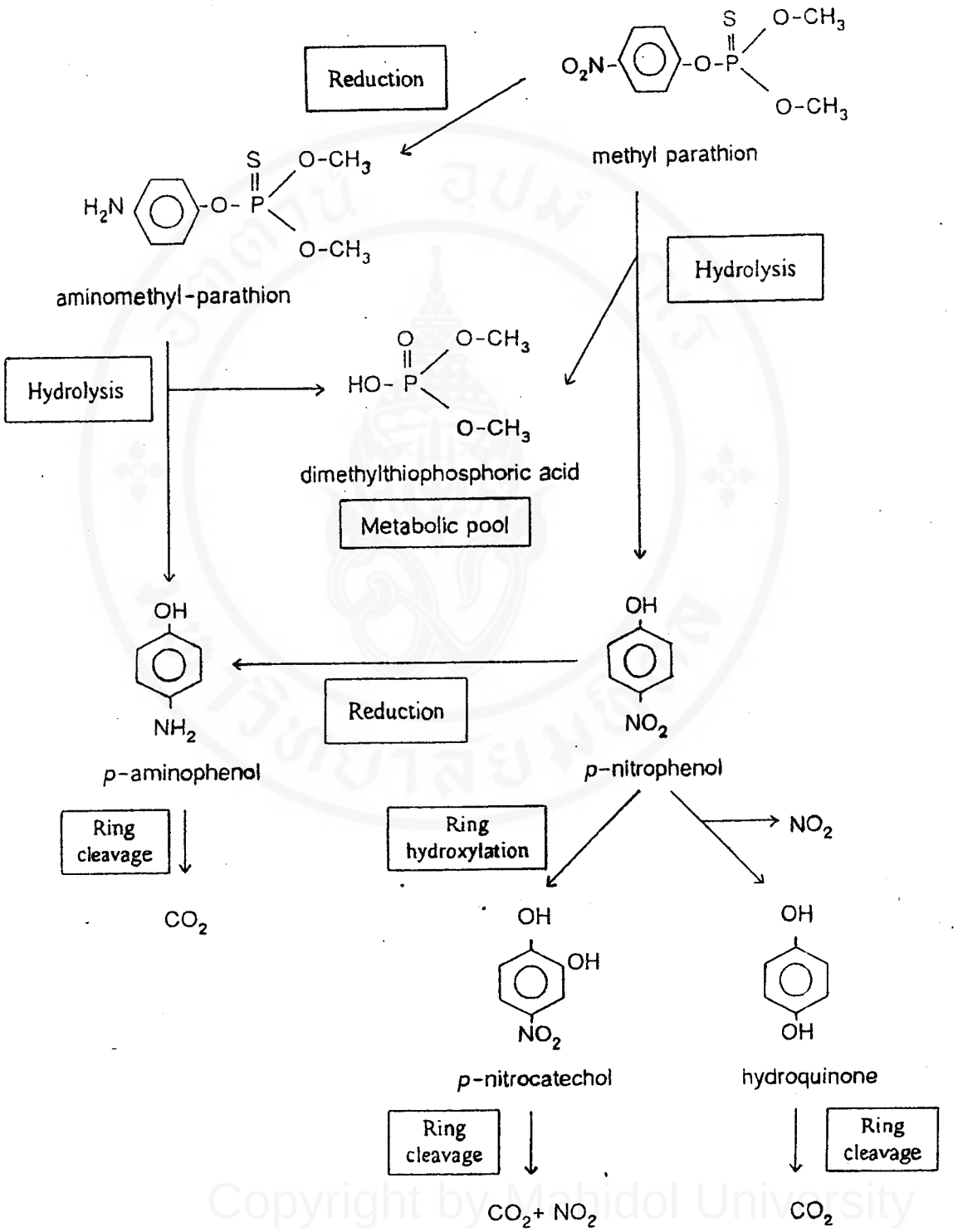

Figure 6. Proposed pathways of methyl parathion in microorganism (7, 11).

Munnecke and Heish obtained a *Pseudomonas* sp. from a parathion hydrolyzing enrichment culture. The organism utilized PAR as carbon source and formed nitrite via hydroquinone metabolism (58). Rossenberg and Alexander isolated *Pseudomonas* strains from soil and wastewater and found that the bacteria hydrolyzed PAR to PNP (64). Gorder and Lichtenstein showed that PAR could be utilized as sole carbon source by microorganisms, which were isolated from pesticide-treated soil (65). Serdar *et al.* reported that a *Pseudomonas diminuta* strain MG hydrolyzed PAR and showed that the parathion hydrolysis was related to the indigenous plasmid pCMS1 (66).

In 1981, Adhya *et al.* found that *Pseudomonas* sp. (ATCC 29353) which was isolated from a flooded soil amended with PAR, hydrolyzed PAR, MP, and diazinon to PNP. Further metabolism of PNP released nitrite in glucose-free medium but in glucose amended medium, PNP could be reduced to *p*-aminophenol. *Flavobacterium* sp. (ATCC 27551), which was isolated from a flooded rice field treated with diazinon, hydrolyzed both PAR and MP to PNP but did not metabolized PNP further (54). Chaudhry *et al.* isolated two mixed bacterial cultures that utilized both PAR and MP as a carbon source. One member of the mixed cultures, a *Pseudomonas* sp. hydrolyzed both PAR and MP to PNP but required glucose or other carbon source for growth (55).

In 1983, Ou *et al.* found the effect of soil-water tension on MP degradation, they reported that decrease in soil water tension could also decrease oxygen tension which resulted in an increase in anaerobic microbial activity and a reduction of PNP to *p*-aminophenol by microorganisms (59). Wolfe *et al.* reported that MP was reduced

to aminomethyl-parathion rapidly in anaerobic sediment systems in the laboratory (67).

In 1989, Ou and Sharma reported that a mixed bacterial culture isolated from soil degraded MP only in the presence of a carbon source such as glucose or yeast extract. *Bacillus* sp. isolated from this mixed culture utilized MP as a carbon source and hydrolyzed MP to PNP rapidly. *Pseudomonas* sp. isolated from the mixed culture lost its capacity to degrade MP but they could hydrolyzed PNP and used it as a carbon source (57).

Sharmila *et al.* isolated mixed bacterial cultures from flood alluvial and laterite soils, and tested for their ability to degrade MP in a mineral salt medium in the presence and absence of yeast extract. Methyl parathion was hydrolyzed to PNP by mixed culture from alluvial soil in the absence of yeast extract and reached to aminomethyl-parathion in its presence. The mixed culture from laterite soil hydrolyzed MP to PNP only in the presence of yeast extract (60). In the same year, Cortez *et al.* isolated *Pseudomonas* sp. strain 50541 from pesticides waste disposal site. This strain was found to oxidize both MP and PNP rapidly and completely without the accumulation of intermediate metabolites. The complete metabolisms are so called "mineralization" (68).

Rani and Lalithakumari (1994) found that a *Pseudomonas putida* utilized MP as a sole carbon source and/or phosphorus source and hydrolyzed MP to PNP. Then PNP was hydrolyzed to hydroquinone and 1, 2, 4-benzenetriol and, finally, 1, 2, 4-benzenetriol was degraded to maleyl acetate by benzenetriol oxygenase (69).

In 1995, Samara and Siddavattani reported *Flavobacterium balustinum* isolated from an agricultural area in India to hydrolyzed MP to PNP. The degradation of MP was related to the indigenous plasmid, which is 86 kilobase (kb) long (70).

These and other studies clearly indicated that PAR and MP are generally hydrolyzed to PNP and diethylthiophosphoric acid before further metabolized to other more simple compounds. These findings suggested that the hydrolysis of PAR and MP and subsequent degradation of PNP and diethylthiophosphoric acid by soil microorganisms may be responsible for the rapid removal of these compounds from the environment.

7.3 Application and Usage of Organophosphate Insecticides-Degrading Microorganisms

In 1976, Munnecke demonstrated the application of using cell-free bacterial extracts possessing parathion hydrolase activity to degrade various organophosphate insecticides at a rate of 416 nmole/min of protein. This occurred more rapidly than chemical hydrolysis by 0.1 N sodium hydroxide (NaOH) (71).

In 1987, Karns *et al.* used a *Flavobacterium* sp. (ATCC 27551) producing parathion hydrolase in both laboratory and pilot-scale projects for degrading aqueous wastes containing high concentration of another organophosphate insecticide, coumaphos. The hydrolytic product was degraded by ozone and completely mineralized by indigenous microorganisms in a soil column (72).

Recently, there were studies on the application of parathion hydrolase enzyme from certain microorganisms in laboratory scale and field trial for decontaminating the organophosphate insecticide coumaphos. Moreover, these

microorganisms were used to degrade and detoxified a coumaphos in cattle-dipping vats, which disperse along the US-Mexico border. These cattle-dipping vats are used in a tick eradication program designed to prevent the re-introduction of cattle fever into the United States through ticks on cattle imported from Mexico. Each cattle-dipping vat contains so high a concentration of coumaphos (1,650 mg/liter) that they have become one of the environmental problems.

In 1995, Karns *et al.* found that parathion hydrolase enzyme could degrade and detoxify the coumaphos waste in field trial (73). Besides Mulbry *et al.* used parathion hydrolase enzyme both in a laboratory scale and a field scale for degrading a coumaphos that contaminated in soil and water (74, 75).

7.4 Organophosphate Hydrolase Enzyme

Although there were many reports of parathion-degrading and methyl parathion-degrading microorganisms isolated from mixed cultures and pure isolates, but only few of these bacteria have been studied at the enzymatic and molecular level. Detailed studies have focused on the hydrolase activity of *Flavobacterium* sp. (ATCC 27551) (54, 62, 76) and *P. diminuta* strain MG (66). Both strains constitutively produce membrane-associated enzymes that are active against related organophosphates and show broad pH and temperature optima (55, 71, 77). From biochemical and genetic studies it has been shown that the two enzymes are identical (78, 79, 80).

In an attempt to determine the spread of this enzyme, as well as to characterize unrelated parathion hydrolases. Shelton and Somich isolated and characterized coumaphos-metabolizing bacteria. Three morphologically distinct

bacteria (designated B-1, B-2, and B-3) that metabolized coumaphos were isolated from enrichment cultures that were initiated from problem vat dip material. It was found that coumaphos was hydrolyzed to diethylthiophosphoric acid and chlorferon by all three isolates but only B-1 produced additional metabolites. Strain B-1 use chlorferon as a carbon source at low concentration (100 μg/ml), while at the concentration of 400 μg/ml of chlorferon inhibited growth completely. Meanwhile, the growth of B-2 and B-3 were inhibited by 100 μg/ml of chlorferon. These data suggested that, although B-1, B-2, and B-3 are responsible for the primary degradation of coumaphos, other organisms in the enrichment culture might play a secondary role to degrade and remove the inhibitory products of coumaphos metabolism (81).

Mulbry and Karns characterized parathion hydrolases from three bacteria isolated from different locations and found that the hydrolase from a *Flavobacterium* sp. was membrane-bound with a single subunit of 35 kilodalton (kDa) in size. Sulfhydryl reagents such as dithiothreitol (DTT) and metal salts such as copper chloride ($CuCl_2$) inhibited this enzyme. The hydrolase from the SC strain was also membrane-bound. It is inhibited by DTT but is stimulated by $CuCl_2$. It is composed of identical four subunits of 67 kDa in size. On the other hand, the hydrolase enzyme from *Norcadia* sp. strain B-1 was found in bacterial cytosol. It is composed of a single subunit of about 43 kDa and was stimulated and inhibited by DTT and $CuCl_2$, respectively (82).

These microorganisms produce organophosphate hydrolases, which hydrolyze organophosphate AChE inhibitors such as organophosphate insecticide. This group of microbial enzymes are sometimes called phosphotriesterase (83) or

organophosphorus acid anhydrase (OPA anhydrase) (84) or organophosphorus hydrolase (OPH) (85) depending on the substrates.

Moreover, Mulbry *et al.* showed the degradation of organophosphate insecticide coumaphos in contaminated soils and in liquid waste (86).

7.5 Organophosphate Hydrolase Gene

In bacteria, genes encoding degradative enzymes are often plasmid encoded, which range in size from a few to several hundred kilobases of DNA (87, 88). Initial study on parathion-hydrolyzing *P. diminuta* strain MG showed that the mitomycin C-cured bacteria lost parathion hydrolase-activity, and hence the enzyme was plasmid-born. This enzyme was found to be encoded by the pCMS1 plasmid (66). Similarly, a plasmid pPDL2 from *Flavobacterium* sp. (ATCC 27551) was found to be involved in the hydrolysis of PAR and its hydrolase activity was lost when treated the cells with streptomycin (54, 76).

The parathion hydrolase gene (organophosphate-degrading gene; *opd* gene) from both strains have been cloned, transformed, and sequenced into other bacterial host. The *opd* gene fragment of pCMS1 plasmid was cloned and introduced into other Gram-negative bacterial hosts such as *Escherichia coli*. The *opd* gene was found to be very poorly expressed under the control of its own promoter (79, 80, 86). The cloned gene was located on a 1.5-kb Bam HI and a 7.3-kb Eco RI fragment in *P. diminuta* and *Flavobacterium* sp., respectively (76, 89). Hybridization studies demonstrated that the *opd* genes from both sources was homologous (76, 90) and showed homology with total DNA from a *Pseudomonas* sp. that hydrolyzed MP (55). Mulbry *et al.* reported the size of the plasmids pCMS1 and pPDL2 to be 70 and 39 kb, respectively.

Besides, the *opd* genes of both plasmids were located within a highly conserved region (78).

Mulbry and Karns sequenced the *opd* gene of the plasmid pPDL2 and found that the amino acid composition of the purified protein corresponded well with that predicted from the nucleotide sequence. The data suggested that the parathion hydrolase protein is processed at its amino terminus in *Flavobacterium* sp. The *opd* coding region failed to express in *P. putida*. However, the cloned gene fused with lacZ expressed at higher levels in *E. coli* compared with the parent strain (79).

Similarly, the cloned *opd* gene from *P. diminuta* expressed poorly in *E. coli* and in a *Pseudomonas* sp. High level of hydrolase activity was obtained when the *opd* gene was placed under the control of the lambda P_L promoter in *E. coli* (80).

The expression of *opd* gene in Gram-positive organisms has also been reported. Steiert *et al.* have introduced the *opd* gene into *Streptomyces lividans* and shown that the parathion hydrolase enzyme is secreted into the culture medium (91). Rowland *et al.* purified parathion hydrolase from the recombinant *S. lividans* to homogeneity. The recombinant and the native hydrolases had similar characteristic, including molecular weight and temperature optima. Analysis of the native membrane-bound parathion hydrolase from *Flavobacterium* sp. (ATCC 27551), *P. diminuta* strain MG, and the recombinant hydrolase from *E. coli* and *S. lividans* system can be used effectively to produce high levels of the enzyme (92).

In addition, Dave *et al.* studied the expression of *opd* gene in the filamentous fungus *Gliocladium virens*. The *opd* gene was cloned into the *Gliocladium virens* and shown that integration occurred non-specifically at multiple sites. Parathion hydrolase

enzyme was produced in a processed form but the enzyme activity could not be found in culture media (93).

The aryldialkyl phosphatase (ADPase)-encoding gene, *adp* B gene, from *Norcadia* sp. strain B-1, which degrades organophosphate insecticide coumaphos, was cloned and sequenced. A comparison of *adp* B gene with the *Flavobacterium opd* gene, revealed no significant homology at both the nucleotide and amino acid levels (81, 94).

8. Analytical Method of Organophosphate Insecticides by High Performance Liquid Chromatography (HPLC)

The main advantage of HPLC is its ability to analyze compounds that are both labile, such as phenylurea and carbamates. In 1978, Lawrence and Turton reported the HPLC conditions for 166 pesticides including MP (95). HPLC analysis has been achieved using reverse phase chromatography, with acetone/water (60:40) (96) or methanol/acetic acid/water (32: 0.6: 47.4) as solvents, and UV-detection (97). Sharma *et al.* (1990) developed a method for the rapid quantitative analysis of organophosphate (including MP) and carbamate pesticides using HPLC and refractive index detection (98).

Shelton and Karns investigated the degradation of coumaphos in cattle-dipping vats to obtained information on the fate of coumaphos. Coumaphos was extracted with methanol and then the supernatant was stored at 4°C until analysis. The mobile phase was 40% methanol-60% 0.75 M phosphoric acid (pH 2) and the flow rate was 4.5ml/min. The result of this experiment showed that under aerobic

conditions, coumaphos was degraded at all dip vat solutions. In anaerobic conditions, coumaphos were reductively dechlorinated to potasan only in high-use vat (99).

9. The Methyl Parathion-Degrading Bacteria in Thailand

In Thailand, Keprasertsup reported two mixed cultures, which isolated from agricultural soil, capable of degrading commercial grade MP in basal mineral medium (BMM). *Pseudomonas* sp. isolated from the mixed bacterial culture could use MP, both commercial grade and analytical grade, for growth in BMM without glucose or other carbon sources.

The result of this study indicated that the *Pseudomonas* sp. used MP as a sole source of carbon, degrading it to PNP and also degrading PNP further. The quantification of MP and PNP in the culture medium at various times over the time course of the bacterial growth was determined by high performance liquid chromatography (HPLC). Moreover, this microorganism was studied for its ability to degrade MP at various conditions such as pH, salinity, presence or absence of glucose, and inoculum size.

From this study, the *Pseudomonas* sp. hydrolyzed MP and grew in BMM, which contained 50 μg/ml of MP at various pH, from pH 4 to pH 8. The microorganisms hydrolyzed MP at the concentration of 0 to 3.5% of sodium chloride (NaCl). The addition, glucose in the culture medium had no effect on growth of the microorganisms. On the other hand, glucose has effect on the degradation of MP. *Pseudomonas* sp. at the inoculum size of $6x10^7$ cells/ml, was found to hydrolyzed MP rapidly and the half life values of MP were to be 0.55, 0.58, and 0.87 h at the concentration of 50, 100, and 150 μg MP/ml, respectively. Moreover, the

Pseudomonas sp. also hydrolyzed PNP, which is the hydrolytic product of MP. At present, *Pseudomonas* sp. was tentatively identified as a *Pseudomonas stutzeri* (7). So far, the molecular study of this bacterium has not been performed at all, even though it is very interesting. This molecular study will be very useful for cloning and improving of the MP degradative gene and for the efficient application of the bacteria in the future.

CHAPTER III

MATERIALS AND METHODS

1. Studies on Some Basic Properties of *P. stutzeri*

1.1 Growth Curve and the Appearance of *p*-Nitrophenol (PNP)

1.1.1 Culture Media and Reagents

The culture media which were used for growing and for measuring the concentration of this MP-degrading *P. stutzeri* were basal mineral medium (BMM) and Luria broth (LB) agar plate, respectively, as shown in Appendix 1.1. BMM contained methyl parathion (MP), which was used as a carbon source, at the concentration of 50 μg/ml. In this study, a commercial grade MP, Folidol-E 605 M 50, that was filter-sterilized (0.45 μm-pore size membrane, Acrodisc) was used.

The solution used for diluting the bacterial cells was 0.85% sodium chloride (NaCl). The concentration of bacterial cells is determined as colony-forming unit/ml (CFU/ml).

1.1.2 Growth Conditions

The MP-degrading *P. stutzeri* was obtained from Keprasertsup C. (7). They were grown in BMM containing 50 µg/ml of MP (BMM-MP) for 12 h at 26°C, shaking at 200 rpm. The 12h old culture was subcultured into a fresh BMM-MP medium. Aliquots of the culture were collected at 0, 3, 6, 9, 12, 24, and 48 h, and used to determine the bacterial concentration and the appearance of PNP.

1.1.3 Measurement of the Concentration of Cells

Growth measurement for this study was done by Plate Count technique (100, 101). The culture of bacterial cells was diluted with 0.85% NaCl resulting in the initial concentration of approximately 10^5 viable cells/ml. Then the dilutions were spread evenly onto the surface of LB agar plates and incubated for 24 h at room temperature. This was done in duplicate for each dilution and the numbers of colonies were counted from plates, which contained only 30-300 colonies. The colony-forming unit/ml (CFU/ml) and the generation time or doubling time (102) were calculated as mentioned in Appendix 2.1.

1.1.4 Determination of *p*-Nitrophenol (PNP)

The appearance of the yellow-colored PNP, which is the metabolite of methyl parathion hydrolysis by the bacterial cells, was determined by monitoring the increase in the absorbance at 410 nm (Jasco, model 7800 UV/visible) (7). BMM-MP and BMM medium were used for the control and the blank, respectively. The ability of the bacterial cells to degrade MP to PNP was studied. The sample was collected and studied the growth of the bacterial cells and the appearance of PNP.

1.2 Analysis of Methyl Parathion (MP) and *p*-Nitrophenol (PNP) by High Performance Liquid Chromatography (HPLC)

1.2.1 Apparatus and Reagents

The decrease of MP and increase of PNP in the medium during the bacterial growth were quantified by HPLC. The HPLC system used (Waters Associates, Inc., Milford, Mass.) consisted of 510 pump, 721 Waters Automated Gradient Controller,

and Waters ™ 486 Tunable Absorbance Detector set at 220 nm for the determination of MP and PNP (7).

Standard chemical substances used were analytical grade methyl parathion (99% purity), and 4-nitrophenol (>99.5% purity). The stock standard solutions were prepared to the concentration of 1 mg/ml in methanol and diluted to 0, 2, 4, 6, 8, and 10 μg/ml as final concentrations. The standard solutions were filtered through a 0.45 μm-pore size filter membrane (Millipore). HPLC grade methanol and water, which were used as solvents, were also filtered through filter membrane (0.45 μm-pore size, Millipore) and degassed by sonicator before use.

1.2.2 Extraction and HPLC Analytical Procedure

For determination of MP and PNP concentrations, 0.2ml aliquots of the culture taken at various time points as mentioned above were extracted vigorously with 0.8 ml of methanol by using vortex for 3 min, and centrifuged at 12,000 rpm for 3 min. The supernatants were filtered through 0.45 μm-pore size filter membrane (Millipore) and stored at -20°C until analyzed. MP and PNP concentrations were quantified on a Water HPLC System. The separations were achieved on a C-18 μ Bondapak column. The mobile phase was 70% methanol-30% water (v/v) at the flow rate of 1.0 ml/min and the injection volume was 20 μl. The detection was carried out at 220 nm and peak areas were used for determining the concentration of MP and PNP (7).

2. Certain Basic Properties of the Methyl Parathion Hydrolase Activity of the MP-Degrading *P. stutzeri*

2.1 Expression of the Methyl Parathion Hydrolase Enzyme

In this study, we would like to know whether the expression of the hydrolase enzyme is an inducible or constitutive one. The disappearance of MP and the appearance of PNP were compared together, when the bacterial cells were grown in different media such as BMM-MP medium, LB-MP medium, and LB medium.

2.1.1 Culture Conditions

The experimental set was designed as (i) treatment 1; the bacteria were grown in BMM-MP medium, (ii) treatment 2; the bacteria were grown in LB medium supplemented with 50 μg/ml of MP, and (iii) treatment 3; the bacteria were grown in LB medium. These treatments were grown at 26°C. The 12h culture of treatment 1 and the 24h culture of the other treatments were subcultured to fresh BMM-MP that had MP as the only carbon source. The MP and PNP concentrations of the experimental set were compared with the control set, which was composed of uninoculated media in duplicate. The duplicate flasks of the control set were uninoculated. The experimental set and the control set were incubated at 26°C, 200 rpm. Aliquots of both sets were collected at 0, 3, 6, 9, 12, 24, and 48h. HPLC and a spectrophotometer were used to determine the concentrations of MP and PNP.

2.1.2 Expression of the Hydrolase Enzyme

The time course of the degradation of MP to PNP in the experimental set and the control set were analyzed by HPLC, detected at 220 nm, and compared with that

determined by using a spectrophotometer at 410 nm. The procedures of both methods were followed as described in Section 1.1 and 1.2.

2.2 Localization of Methyl Parathion Hydrolase Enzyme in Bacterial Cells

2.2.1 Growth Condition and Lysis of the Cells

The 8h culture of *P. stutzeri* was subcultured to fresh BMM medium containing 50 μg/ml of MP and grown continuously on a shaker incubator at 200 rpm for 15 h at 26°C. The bacterial cells were harvested by centrifugation (Sorvall type GSA rotor) at 5,000 rpm for 10 min at 4°C. Then the cell pellet was resuspended and washed with 20 mM 2-(*N*-Morpholino)ethane sulphonic acid (MES, pH 6.0) and it was disrupted by passage through a cold French pressure cell (SLM Aminco) at 16,000 lb/in^2.

2.2.2 Cellular Preparation and Analytical Methods

The crude lysate was centrifuged (Haraeus type HFA 15.16 rotor) at 15,000 x g for 5 min at 4°C. The debris/nuclei fraction was resuspended with 20 mM MES, pH 6.0 buffer while the supernatant was subjected to ultracentrifugation (Beckman type Ti-70.1 rotor) at 100,000 x g for 1 h at 4°C. After that, the membrane fraction was resuspended with the same buffer whereas the supernatant was transferred to ultracentrifugation at 300,000 x g for 2 h at 4°C. The supernatant is a cytosolic fraction and the pellet is a ribosomal fraction.

These fractions were collected and stored at -20°C until analyzed. The localization of the enzyme at various fractions was found by using the reaction mixture for testing the enzyme activity. Moreover, the PNP concentration was

calculated by using HPLC. These data obtained from this study were useful for the purification of the enzyme from the bacteria.

3. To Determine the Homology between Methyl Parathion Hydrolase Gene of Methyl Parathion-Degrading *P. stutzeri* and other Relevant Genes

In this study, I would like to test whether the methyl parathion gene of *P. stutzeri* has significant homology with the *opd* gene of the parathion-degrading *Flavobacterium* sp. ATCC 27551 and *adp* B gene of coumaphos-degrading *Nocardia* sp. strain B-1. This was achieved by Southern blot hybridization of the genomic DNA of *P. stutzeri*, using *opd* and *adp* B gene as probe.

3.1.1 Bacterial Strains and Medium

P. stutzeri were grown in BMM-MP for 24 h at 26°C. *Pseudomonas aeruginosa*, *Bacillus* sp., and *Escherichia coli*, which used as a negative controls, were grown in LB medium for 24 h at 37°C. *E. coli* JM 105 harboring plasmid pWWM513, which contains the *opd* gene of *Flavobacterium sp.* were grown in LB medium containing 100 μg ampicillin/ml for 24 h at 37°C. *E. coli* DH5α were used as $CaCl_2$-treated competent cells for transformation of *adp* B gene (103, 104) (*E. coli* JM 105 harboring plasmid pWWM513 which contains the *opd* gene and the *adp* B gene were kindly received by Karns, JS. and Mulbry WW.).

3.1.2 Probe Labeling

The plasmid DNAs which contained *opd* gene and *adp* B gene were isolated by Miniprep method and digested with restriction enzyme *Pst* I (103, 104). Then, these genes were labeled with non-radioactive digoxigenin (DIG). The method were

performed as indicated in The DIG Non-radioactive Nucleic Acid Labeling and Detection System instruction manual (Boehringer, Mannheim) (105). Plasmids DNA were used as a template DNA and hexanucleotide, dNTP mixture, and Klenow enzyme were added. The labeling reaction was incubated for 16 h at 37°C. Finally, the reaction was stopped with adding 0.2 M EDTA.

3.1.3 Southern (DNA) Transfer

The genomic DNA of *P. stutzeri* and another ones were isolated by using CTAB (104) and the method as described by Sambrook *et al.* (103). The genomic DNAs were digested with the restriction enzyme *BamH* I and the lambda DNA (λ DNA), which used as the size marker was cut with *Hind* III and *EcoR* I. These DNAs were incubated for 2 h at 37°C and loaded onto a 0.7% agarose gels. After that, there were immersed in 0.25 N HCl for 30 min at room temperature. The DNAs were denatured by soaking gel in denaturation solution for 30 min and neutralized by neutralization solution for 20 min, 2 times. Finally, the gels were soaking in transfer buffer (10x SSC) for 30 min. The membranes were soaked with distilled water and transfer buffer.

After DNA transfer, the membranes were soaked in 5x SSC for 5 min and the DNAs were fixed to the membrane by baking for 2 h at 80°C. Then, the membranes were hybridized to non-radioactive labeled DNA containing the 1.3-kb *opd* gene and 3.5-kb *adp* B gene. The hybridized membrane was used for determining the location of the labeled restriction fragment on the gel.

3.1.4 Hybridization for Southern Blots

The blotting membranes were incubated in hybridization buffer for 30 min at the hybridization temperature 50°C. The denatured DNA probes were added, mixed well and incubated at 50°C overnight. After that, the hybridized membranes washed 2 times for 5 min in 2x SSC, 0.1% SDS at room temperature and in 0.1x SSC, 0.1% SDS for 15 min, 2 times at 50°C.

The membranes were rinsed in washing buffer for 5 min and incubated in 1x blocking solution for 30 min. Added 1: 5000 anti-DIG-AP conjugate in blocking solution and incubated for 30 min at room temperature. Then, the membranes were washed with washing buffer for 15 min twice and these membranes were equilibrated in detection buffer for 5 min. Finally, the membranes were incubated in freshly prepared color-substrate solution in the dark and the reaction was stopped by washing with distilled water.

4. To Isolate and purify the Methyl Parathion Hydrolase Enzyme

4.1 Purification of Methyl Parathion Hydrolase Enzyme

4.1.1 Culture Conditions

This experiment was performed as described by Mulbry and Karns (38). The MP-degrading *P. stutzeri* were grown in BMM-MP for 9 h and subcultured 9 liters of BMM-MP for 15 h at 26°C. The bacterial cells were harvested by centrifugation (Sorvall type GSA rotor) at 5,000 rpm for 10 min at 4°C and the cells pellet was washed with 20 mM MES, pH 6.0. Subsequently, a French pressure cell disrupted the cells at 16,000 lb/in^2. Then the sample was subjected to ultracentrifugation at 100,000 x g for 1 h at 4°C. Thereafter, the pellet was resuspended in 20 mM MES, pH 6.0-

0.4% Triton X–100 and incubated for 16 h at 4°C before subjected to ultracentrifugation as described above. Finally, the supernatant (crude Triton X extract) was used as the source of methyl parathion hydrolase for further purification.

4.1.2 Purification of Methyl Parathion Hydrolase

The purification of MP-degrading in this study were composed of 3 steps: (i) ion exchange chromatography, (ii) size-exclusion chromatography or gel filtration chromatography , and (iii) hydrophobic interaction chromatography.

(i) Ion Exchange Chromatography (IEC). Seven ml of crude Triton X extract was concentrated by using centricon filtration YM-10 membrane (BIO-RAD) to 1 ml. The Resource S prepacked column (Pharmacia LKB Inc., 6.4 x 30 mm, 1 ml) was equilibrated with 20 mM MES, pH 6.0 - 0.1% Triton X-100 (buffer A). After that, the sample was loaded at the rate of 1 ml/min onto Resource S via fast protein liquid chromatography (FPLC). The FPLC system (Pharmacia LKB Inc.) consisted of P-500 pump, Controller LCC-501 Plus, and Pharmacia LKB-control Unit UV-1.

The sample was washed to get rid of the unbound protein and eluted with a linear NaCl gradient, from 0.05 to 1 M, using NaCl in 20 mM MES, pH 6.0, 0.1% Triton X-100, and 1 M NaCl (buffer B) and the sample was collected 1 ml/fraction. The fractions containing methyl parathion hydrolase activity were pooled (fraction no. 3 to 8; 6 ml) and concentrated with centricon filtration YM-10 membrane to 0.55 ml. 0.3 ml of the concentrated sample was further purified while the rest of the sample was used for measuring the protein concentration by Lowry method (107) and determining the enzyme activity by enzyme spectrophotometric assay at 410 nm which contained MP as a substrate in the reaction mixture.

(ii) Gel Filtration Chromatography (GFC). The Sephadex G-100 powder was swollen in 50 mM Tris-HCl, pH 7.8 and let stand for 72 h at room temperature. The gel was degassed until no air bubble was found, then cooled at 4°C in order to packe the column at the same condition as when the sample was run. Afterwards, Sephadex G-100 (Pharmacia LKB Inc., 1.5 x 100 cm) was washed and equilibrated with 50 mM Tris-HCl, pH 7.8-0.1% Triton X-100. The gel was calibrated with the molecular weight marker and their elution volumes (V_e) were established. This protein molecular weight marker contained Blue Dextran 2000, Orange Dextran, and Vitamin B 12, with the molecular weights of 2,000, 20, and 2 kilodalton (kDa), respectively. The void volume (V_o) was determined from the elution volume of Blue Dextran 2000 (Appendix 3).

The column was equilibrated at 4°C before 0.3 ml concentrated pooled Resource S fractions was loaded onto the column at the flow rate of 0.5 ml/min. The sample was collected as 3 ml/fraction. Methyl parathion hydrolase positive fractions were pooled (fraction no. 29 to 33; 15 ml) and used for further purification. Each fraction was estimated for the amount of protein by measuring the optical density at 280 nm and tested for the enzyme activity by enzyme spectrophotometric assay at 410 nm.

(iii) Hydrophobic Interaction Chromatography (HIC). Seven ml pooled Sephadex G-100 fractions was added with solid ammonium sulfate ($(NH_4)_2SO_4$), stirred slowly on-ice, then incubated for 30 min at -20°C. The sample was adjusted to 1 M $(NH_4)_2SO_4$ for preparing the sample with 50 mM sodium phosphate buffer (NaP_i, pH 7.0) buffer. Octyl Sepharose 4 Fast Flow prepacked column (Pharmacia LKB

Inc., 0.7 x 2.5 cm, 1 ml) was washed with 50 mM NaP_i, pH 7.0 (buffer A) and equilibrated with 50 mM NaP_i, pH 7.0 - 1 M $(NH_4)_2SO_4$ (buffer B).

The pooled Sephadex G-100 sample was loaded onto the column at the rate of 1 ml/min and the sample was collected as 2 ml/fraction. A linear gradient of 1 to 0 M $(NH_4)_2SO_4$ in buffer B was run to elute the bound material from the column. The amount of protein and the enzyme activity of each fraction were estimated as described above. The fractions containing methyl parathion hydrolase activity (fraction no. 9 to 12; 8 ml) were pooled.

The fractions from each column were measured for the concentration of protein by Lowry method. The enzyme activity was determined by the enzyme spectrophotometric assay, which contained MP as a substrate in the reaction mixture. These data were used for calculating the total protein, total activity, specific activity, % yield recovery, and purification fold.

The purity of protein, the activity staining, the molecular weight, and the effect of some reagents on the methyl parathion hydrolase activity were studied in the next section.

4.1.3. Protein Determination

Generally, absorbance at 280 nm is used for generating a protein elution profile after column chromatography. Total protein concentrations of the samples in all purification steps were measured by Lowry method with bovine serum albumin (BSA) as a protein standard.

4.1.4 Enzyme Assay

The enzyme assay was employed to estimate the hydrolysis of MP. This assay was performed in a cuvette that contained the reaction mixture, MP act as the substrate, and the protein of interest. Adding the enzyme followed by mixing started the reaction. The activity of methyl parathion hydrolase was measured the increase in terms of PNP as measured spectrophotometrically at 410 nm. The optical density measurements of the samples were compared with that of PNP standard concentrations shown in Appendix 3. All assays were run for 1 min at room temperature.

4.1.5 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein was performed by the method of Laemmli (108). The 0.75 mm-thick slab gels contained 12.5% acrylamide resolving gel and 5% acrylamide stacking gel. The partially purified methyl parathion hydrolase were concentrated with trichloroacetic acid (TCA). The stock concentration of TCA was 70% and final concentration was 10%. The samples were added with 70% TCA, which were diluted to 10% and mixed by vortex. The samples were allowed to precipitate for 30 min at -20°C and then centrifuged at 12,000 x g for 5 min at 26°C. The supernatants were removed and the pellets were washed with approximately 100 μl of distilled water and centrifuged again (109). The samples were dried at room temperature.

The crude Triton extract and the concentrated partially purified protein were boiled in 5X loading buffer for 2-3 min and loaded onto the gel. The SDS gel was run at a constant voltage of 120 voltage (BIO-RAD, Mini PROTEAN 3 Cell) for 1 h. The

proteins in the gel were fixed and stained in a solution of 0.1% Coomassie Blue R-250 in 45% methanol-10% glacial acetic acid for 1 h and were destained in 10% methanol-10% glacial acetic acid for 1 h (109, 110, 111).

4.2 Activity Staining of Methyl Parathion Hydrolase Enzyme in SDS Gel

4.2.1 Renaturation Treatment

After separation of the protein by SDS–PAGE, the gels were incubated for 30 min in the renaturation buffer containing 0.1 M Tris-HCl, pH 7.0, and 2% Triton X-100. The gel was washed by washing buffer for 2 h (four times, 30 min each). The washing buffer contained 100 mM Maleic acid, 150 mM NaCl, and 0.3% Tween 20, pH 7.5 (112).

4.2.2 Localization of Methyl Parathion Hydrolase in SDS Gel

The proteins in SDS gel were renatured and washed before the gel was immersed in the activity-staining buffer for 2-5 min at room temperature. The activity-staining buffer contained 20 mM MES, pH 6.0, 0.1% Triton X-100, and 50 μg/ml of MP. The location of methyl parathion hydrolase in the gel was visible as yellow color of PNP, which slowly diffused with time (112, 113).

4.3 Effects of Reagents on the Activity of the Enzyme

4.3.1 Chemical Agents

Chemical agents used for testing the activity of the enzyme were cupric chloride ($CuCl_2$), dithiothreitol (DTT), and ethylenediamine tetraacetic acid (EDTA). Stock solutions were prepared to the concentration of 10 mM in water. The reaction mixture used for determining the activity of the enzyme contained 50 mM Tris-HCl,

pH 8.5, 20 mM Tris-HCl, pH 7.8, 0.1% Triton X-100, and 50 μg/ml MP, which served as substrate (82).

4.3.2 Enzyme Assay

The effects of chemical agents on the enzyme activity were studied by determining the change in the rate of appearance of PNP as determined by spectrophotometric assay at 410 nm. Methyl parathion hydrolase enzyme was added with $CuCl_2$, DTT, and EDTA to a final concentration of 1mM, 1mM, and 0.1 mM, respectively. The reaction mixtures were added with 0.15 ml of partially purified enzyme in a total volume of 1ml and the assays were run for 1 min at room temperature (82).

5. Statistical Analysis

Data in Section 1.1, 1.2, and 2.1 were presented as means and the standard deviation of mean (mean ± SD) (114, 115).

CHAPTER IV

RESULTS

1. Studies on Some Basic Properties of *P. stutzeri*

1.1 Growth Curve and an Appearance of PNP

The MP-degrading *P. stutzeri* were grown in BMM containing MP at the concentration of 50 μg/ml, which served as carbon source. Aliquots of the culture were collected at 0, 3, 6, 9, 12, 24, and 48h. The concentration of bacterial cells were determined by plate count technique and shown in terms of colony-forming unit/ml (CFU/ml). The concentration of cells was calculated as shown in equation I in Appendix 2.1. The yellow-colored PNP, which is the hydrolytic metabolite of MP, appeared in the culture when MP was hydrolyzed by the bacterial cells. The appearance of PNP was determined quantitatively by using a spectrophotometer at 410 nm. Moreover, a more simple method in the reaction mixture within the microtiter plate was used to test the ability of the bacterial cells to degrade MP to PNP (Appendix 1) but this method could only determine PNP qualitatively, not quantitatively (Figure 7).

The initial concentration of bacterial cells was 6.35×10^5 cells/ml and increased to 7.15×10^7 cells/ml at 9h of incubation, which was within the log phase. Then, the concentration of the bacterial cells decreased from 7.15×10^7 cells/ml to 6.95×10^7 cells/ml at 24h of incubation, which was the stationary phase. After that, the concentration of the bacterial cells decreased to 5.1×10^7 cells/ml at 48h of incubation

(Figure 8). The generation time of the bacteria was 53 min, which was calculated according to equation II in Appendix 2.1.

As illustrated in Table 1, the optical density of PNP determined by a spectrophotometer and the concentrations of the bacterial cells determined by plate count technique were shown. The optical density at 410 nm of *P. stutzeri* increased from 0.007 to 0.549 within 9h of incubation and decreased to 0.106 at 48h of incubation, while the initial absorbance of the control increased from 0.002 to 0.133 at 48h of incubation.

Figure 9 shows the correlation of the growth curve of this bacterium and the appearance of PNP. It was found that the highest concentration of the bacterial cells and the concentration of PNP appeared at 9h of incubation. These concentrations were decreased rapidly at various time intervals. Meanwhile, the absorbance of control increased slowly.

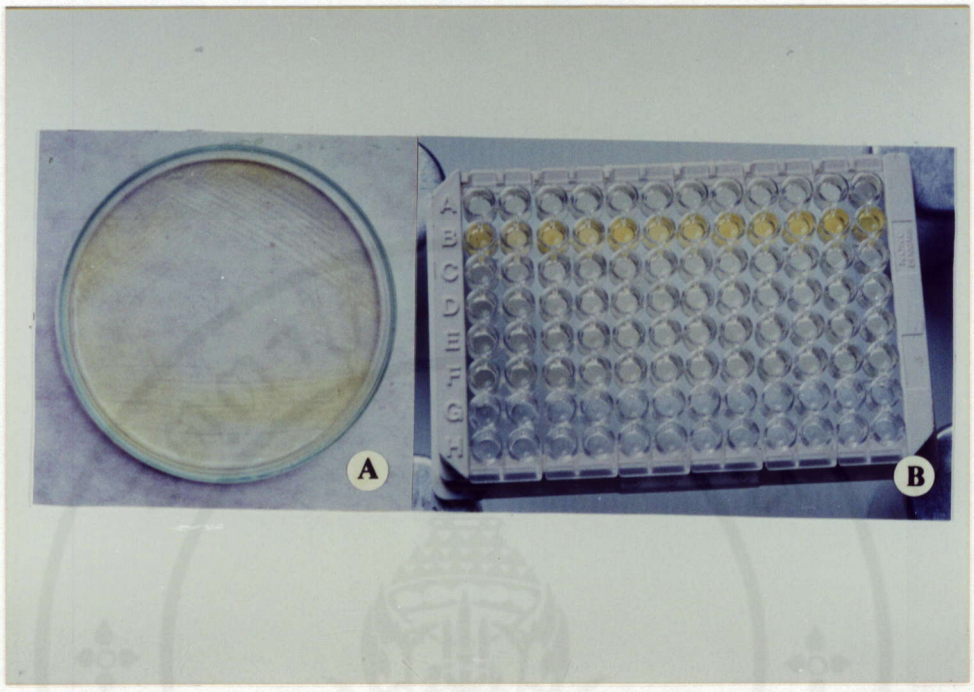

Figure 7. Observation of the yellow-colored PNP on BMM-MP plate and in microtiter plate. (A) A yellow color zone of PNP on BMM-MP plate. (B) Simple method was used to test the appearance of PNP with the reaction mixture[a].

[a]: The reaction mixture formula is shown in Appendix 1.

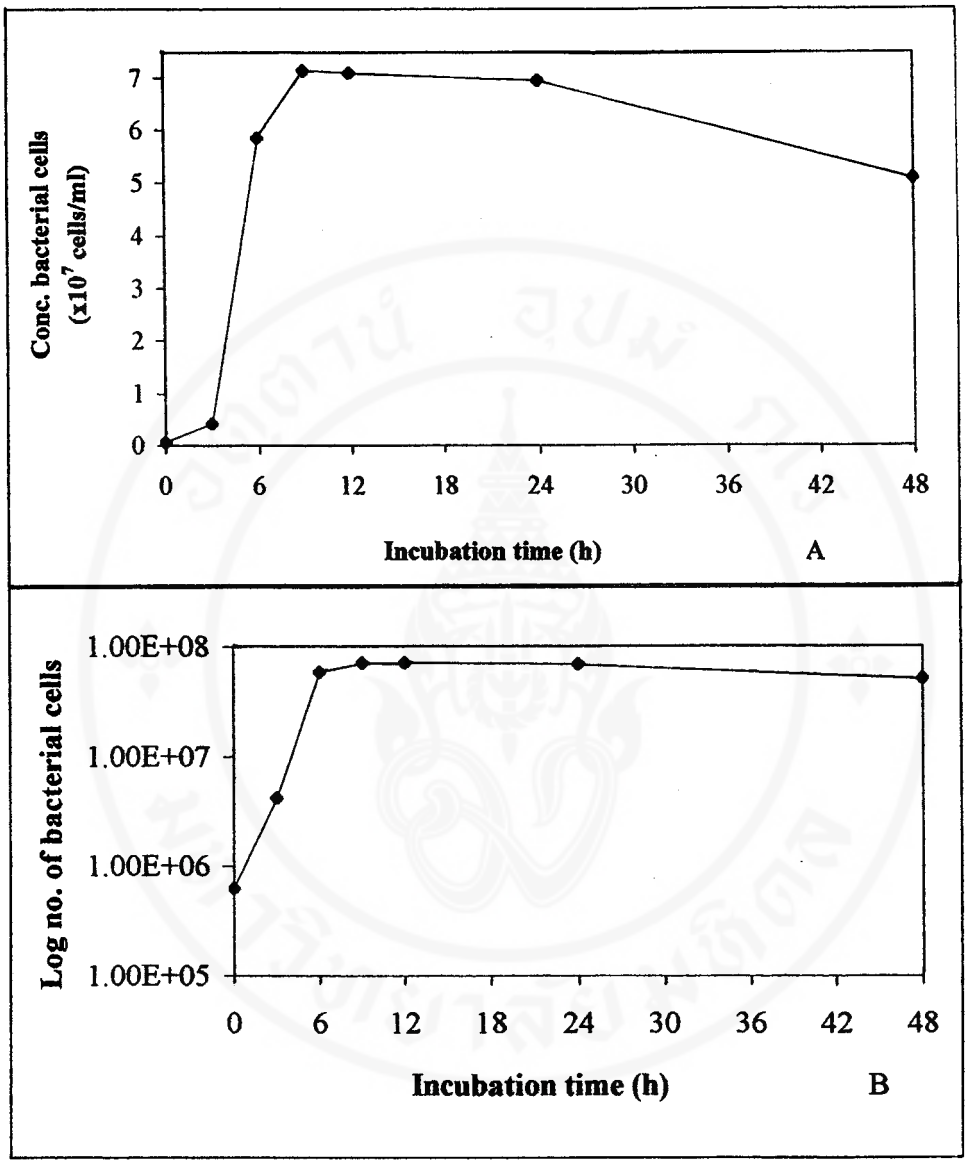

Figure 8. Growth curve of *P. stutzeri*, which aliquots of the culture was collected at various time intervals. (A) The concentration of bacterial cells (CFU/ml; $x10^7$cells/ ml). (B) Log number of bacterial cells.

Table 1. The concentration of bacterial cells and the appearance of PNP determined by a spectrophotometer.

Incubation time (h)	Concentration of bacterial cells (x 10^7cells/ml)	A_{410} nm
	Control ($\bar{X}$ ± SD)[a]	
0		0.002 ± 0.001
3		0.031 ± 0.002
6		0.064 ± 0.005
9	ND[b]	0.093 ± 0.008
12		0.105 ± 0.012
24		0.121 ± 0.013
48		0.133 ± 0.026
	***P. stutzeri* ($\bar{X}$ ± SD)[c]**	
0	0.063 ± 0.707	0.007 ± 0.001
3	0.42 ± 1.414	0.202 ± 0.001
6	5.86 ± 2.828	0.353 ± 0.003

[a]: mean ± standard deviation without *P. stutzeri*

[b]: not determined

[c]: mean ± standard deviation with *P. stutzeri*

Table 1. The concentration of bacterial cells and the appearance of PNP determined by a spectrophotometer (Cont.).

Incubation time (h)	Concentration of bacterial cells ($\times 10^7$ cells/ml)	A_{410} nm
	P. stutzeri ($\bar{X} \pm SD$)[c]	
3	0.42 ± 1.414	0.202 ± 0.001
6	5.86 ± 2.828	0.353 ± 0.003
9	7.15 ± 3.535	0.549 ± 0.012
12	7.1 ± 1.414	0.461 ± 0.009
24	6.95 ± 0.707	0.12 ± 0.015
48	5.1 ± 2.828	0.106 ± 0.022

[a]: mean ± standard deviation without *P. stutzeri*

[b]: not determined

[c]: mean ± standard deviation with *P. stutzeri*

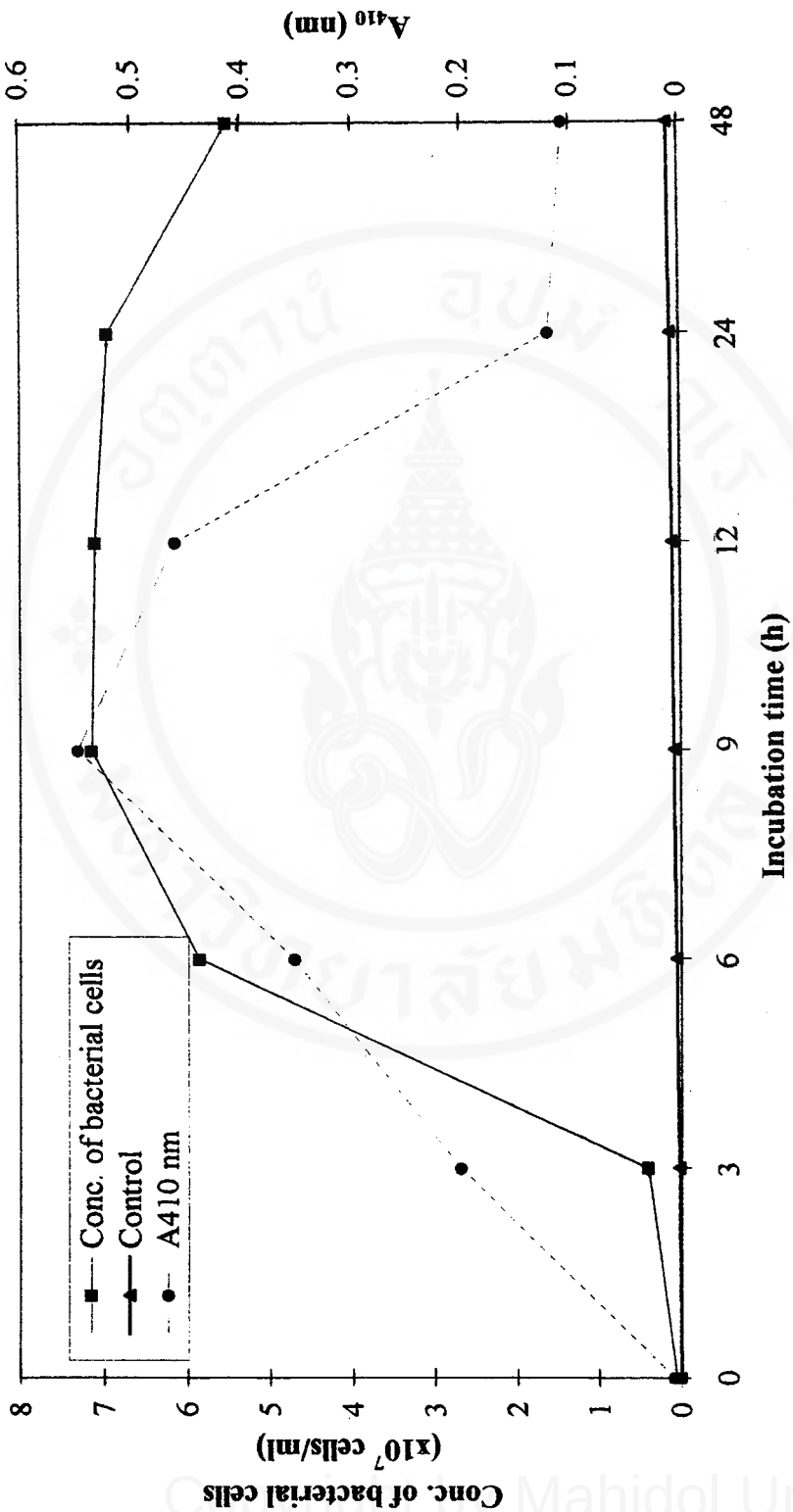

Figure 9. The growth curve of MP-degrading *P. stutzeri* and the appearance of PNP (A410 nm) during.

1.2 Analysis of MP and PNP by HPLC

The concentrations of MP and PNP in the medium during the growth of MP-degrading *P. stutzeri* were detected by HPLC. Using the external standard method by taken the peak area value (Appendix 2.1) carried out the quantitative determination of the sample. The retention time (RT) of MP and PNP were approximately 5.8 and 3.9 min, respectively. The MP and PNP concentrations of sample were compared with that of the standard of known concentration. These concentrations were calculated by following an equation III in Appendix 2.1. The MP and PNP concentrations of the control and the bacterial culture are shown in Table 2. The initial MP concentration of the bacterial culture was 56 μg/ml and decreased rapidly. It was degraded completely within 24h of incubation but the initial MP concentration of the control was 55 μg/ml and decreased slowly throughout 48h of incubation.

On the other hand, the PNP concentration of the sample increased from 0.31 to12.2 μg/ml at 9h of incubation, which was the highest concentration. It was degraded completely after 24h of incubation. Meanwhile, the PNP concentration of the control increased continually to 2.64 μg/ml within 48h of incubation (Figure 10).

The comparisons of MP and PNP concentrations between the bacterial culture and the control showed that the MP concentration decreased, while the concentration of PNP increased with time, as shown in Figure 11. Moreover, Table 3 shows the concentration of MP, PNP, and the growth of the bacterial culture. (Figure 12). The results showed that the MP concentration decreased rapidly, and degraded completely after 12h of incubation, while the PNP concentration increased and degraded completely after 24h of incubation when the initial concentration of the

bacterial cells were $x10^6$ cells/ml. Moreover, the concentration of bacterial cells and the concentration of PNP reached the highest at 9h of incubation.

As illustrated in Table 4, the percentage of MP-residue of the bacterial culture could not be found after 12h of incubation but the other one still remained to 85% at 48h of incubation. After that, the PNP concentration and the concentration of bacterial cells were decreased. The percentage of MP-residue was calculated by following equation IV and V in Appendix 2.3. In addition, the percentage of residual MP is presented as the percentage of MP-degradation, which can be calculated according to equation VI in Appendix 2.3. The percentage of MP-degradation of MP-degrading *P. stutzeri* was 100% when MP was degraded completely but the control was still remained 85% at the end of incubation period.

Table 2. The MP and PNP concentrations of the control and the bacterial culture.

Incubation time (h)	Control (μg/ml)	Bacterial culture (μg/ml)
	Concentration of MP ($\bar{X} \pm SD$)	
0	55.03 ± 1.96	56.55 ± 2.83
3	54.89 ± 0.59	35.69 ± 2.1
6	54.7 ± 3.58	26.85 ± 0.08
9	52.08 ± 2.25	21.26 ± 0.45
12	50.87 ± 3.20	5.37 ± 0.59
24	49.7 ± 2.21	0
48	46.77 ± 2.59	0
	Concentration of PNP ($\bar{X} \pm SD$)	
0	0	0.31 ± 0.17
3	0.4 ± 0.12	1.24 ± 0.15
6	0.42 ± 0.12	6.2 ± 0.84
9	1.77 ± 0.02	14.6 ± 0.63
12	1.82 ± 0.08	10.65 ± 1.02
24	2.75 ± 0.12	0.76 ± 0.42
48	2.64 ± 0.03	0

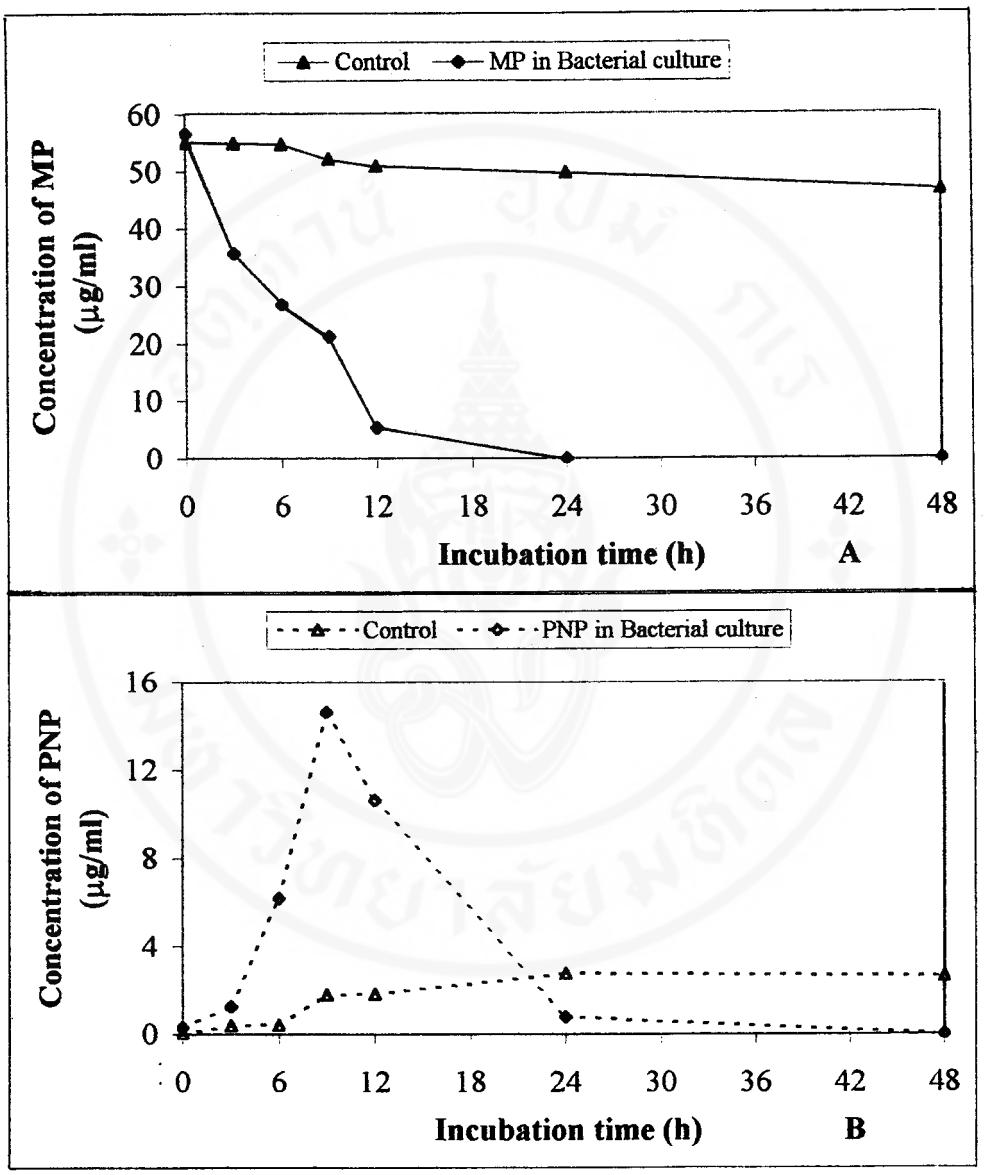

Figure 10. MP and PNP concentrations of the control set and the bacterial culture determined by HPLC. (A) MP concentrations of the control set and the bacterial culture. (B) PNP concentrations of the control set and the bacterial culture.

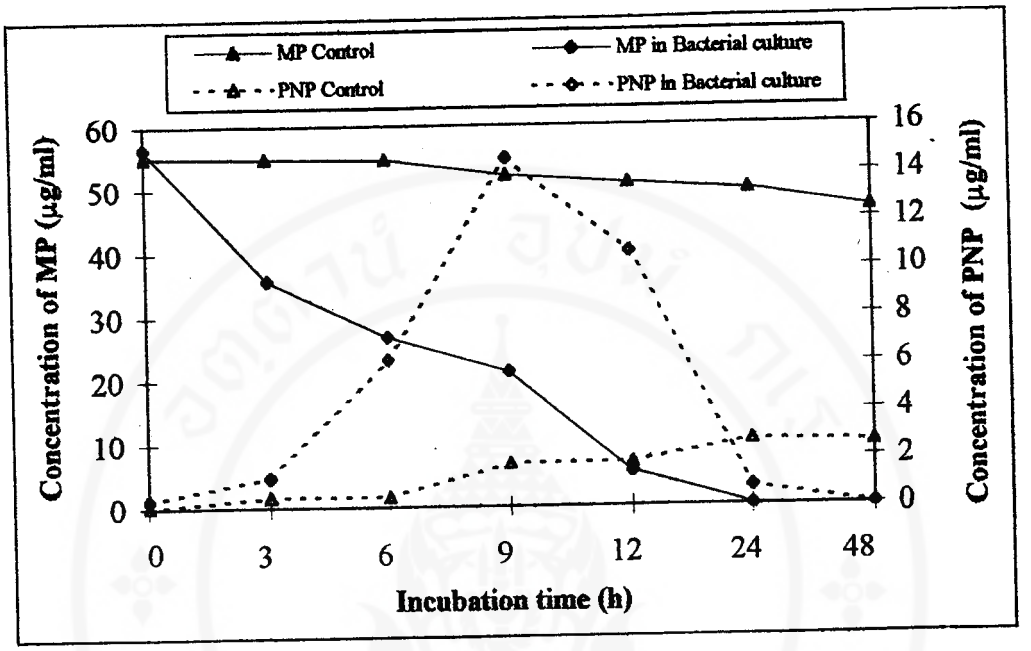

Figure 11. The fates of MP and PNP during the growth of MP-degrading *P. stutzeri*.

Table 3. MP concentration, PNP concentration, and the concentration of MP-degrading *P. stutzeri* (x10^7 cells/ml) during the bacterial growth.

Incubation time (h)	Concentration of MP (μg/ml)		Concentration of PNP (μg/ml)		Concentration of bacterial cells (x10^7 cells/ml)
	Control	*P. stutzeri*	Control	*P. stutzeri*	
0	55.03	56.55	0	0.31	0.321
3	54.89	35.69	0.4	1.24	1.365
6	54.7	26.85	0.42	6.2	7.95
9	52.08	21.26	1.77	14.6	9.3
12	50.87	5.37	1.82	10.65	8.55
24	49.7	0	2.75	0.76	7.975
48	46.77	0	2.64	0	7.675

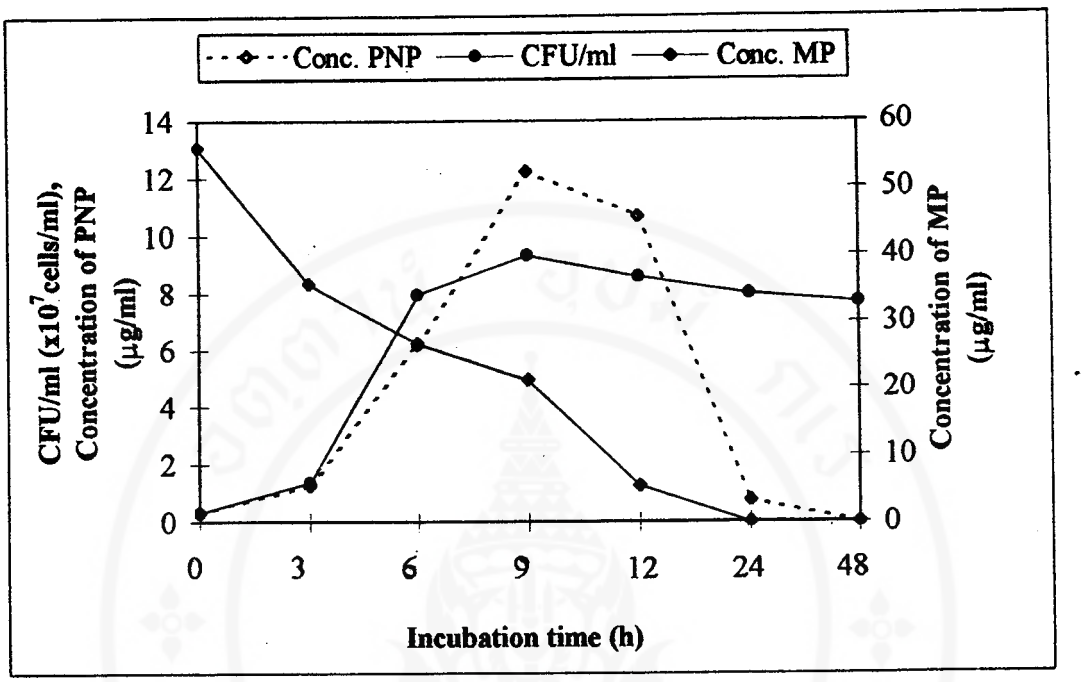

Figure 12. The fates of MP, PNP, and the growth curve of the bacteria.

Table 4. The percentages of MP-residue and MP-degradation in the control and MP-degrading *P. stutzeri* culture calculated at various time intervals.

Incubation time (h)	Percentage of MP-residue ($\bar{X} \pm SD$)		Percentage of MP-degradation
	Control	*P. stutzeri*	
0	100	100	0
3	99.78 ± 2.48	63.11 ± 0.56	36.68
6	99.42 ± 2.96	47.53 ± 2.23	51.83
9	94.63 ± 0.72	37.62 ± 1.08	57.01
12	92.44 ± 2.52	9.49 ± 0.56	82.91
24	90.31 ± 0.78	0	100
48	84.99 ± 1.67	0	100

2. Certain Basic Properties of the Methyl Parathion Hydrolase Enzyme

2.1 Expression of the Methyl Parathion Hydrolase Enzyme

The experiments were divided into control set, which was BMM-MP without bacterial cells, and experimental set, consisted of treatment 1, 2, and 3, which were bacterial cells grown in BMM-MP, LB-MP, and LB, respectively. The concentrations of MP of the control set and the experimental set (all treatments) were determined by HPLC, which can be calculated as equation III in Appendix 2.2. Table 5 shows that the initial MP concentration of the control was 55 μg/ml and slowly decreased to 46.77 μg/ml when the experiment finished at 48h of incubation. Meanwhile, the initial MP concentrations of treatment 1, 2, and 3 were 56.55, 55.3, and 54.55 μg/ml, respectively. The MP concentrations of treatment 1 and treatment 2 were degraded completely after 12h of incubation but that of the other treatment was degraded to 0.08 μg/ml at 48h of incubation (Figure 13 A).

Table 6 shows that the PNP concentration in the control set was increased continually from 0 to 2.64 μg/ml when the experiment finished at 48h of incubation. The PNP concentrations in treatment 1, 2, and 3 were 0.31, 0.1, and 0.72 μg/ml, respectively. After that the PNP concentration in treatment 1 increased and reached the highest concentration at 9h of incubation, while the highest PNP concentration in the other treatments ones occurred at 12h of incubation. The highest concentration of all treatments were 14.6, 12.2, and 3.36 μg/ml, respectively. PNP concentration in treatment 1 and 2 were degraded completely after 24h of incubation. The other one still remained in the medium at 48h of incubation (Figure 13 B).

Figure 14 shows the correlation of the MP and PNP concentrations in the control set and the experimental set. The highest PNP concentration of treatment 1

appeared at 9h of incubation but those of treatment 2 and 3 occurred at 12h of incubation. After that, the concentration of MP and PNP of both treatment 1 and 2, which contained MP in the media, could not be found after 12h and 24h of incubation, respectively. In these conditions, both MP and PNP were able to degrade completely. However, the concentrations of MP and PNP of treatment 3, which did not have MP in it, still remained little at 48h of incubation.

Beside, as shown in Table 7, the percentage of MP-residue of the control set remained approximately at 85% and that of treatment 3 also remained at 0.15%. However, in the other treatments (treatment 1 and 2) could not found MP-residue after 12h of incubation, even though the percentages of MP-residue treatment 2 were always higher than that of treatment 3 (Figure 15).

Table 8, showing the percentages of MP-degradation, indicated that the percentages of MP-degradation in treatment 1 and 2 were 100%, while that of treatment 3 was 84.81%.

Table 5. Concentrations of MP in the control, treatment 1, treatment 2, and treatment 3 determined by HPLC.

Incubation time (h)	concentration of MP (μg/ml; $\bar{X} \pm SD$)			
	Control[a]	Treatment 1[b]	Treatment 2[c]	Treatment 3[d]
0	55.03 ± 1.96	56.55 ± 2.83	55.3 ± 0.014	54.55 ± 0.735
3	54.89 ± 0.59	35.69 ± 2.1	53.89 ± 0.82	49.83 ± 4.384
6	54.7 ± 3.58	26.85 ± 0.08	38.14 ± 1.87	41.79 ± 1.591
9	52.08 ± 2.25	21.26 ± 0.45	34.63 ± 0.332	39.58 ± 2.503
12	50.87 ± 3.2	5.37 ± 0.59	33.85 ± 0.311	37.54 ± 1.251
24	49.7 ± 2.21	0	0	1.14 ± 0.289
48	46.77 ± 2.59	0	0	0.08 ± 0.056

[a]: BMM-MP without bacterial cells

[b]: The culture was grown in BMM-MP

[c]: The culture was grown in LB-MP before subcultured to BMM-MP

[d]: The culture was grown in LB before subcultured to BMM-MP

Table 6. Concentrations of PNP in the control, treatment 1, treatment 2, and treatment 3 determined by HPLC.

Incubation time (h)	concentration of PNP (μg/ml; $\bar{X} \pm SD$)			
	Control[a]	Treatment 1[b]	Treatment 2[c]	Treatment 3[d]
0	0	0.31 ± 0.17	0.1 ± 0.07	0.72 ± 0.01
3	0.4 ± 0.12	1.24 ± 0.15	1.18 ± 0.08	0.83 ± 0.15
6	0.42 ± 0.12	6.2 ± 0.84	5.45 ± 0.63	1.48 ± 0.19
9	1.77 ± 0.02	14.6 ± 0.63	8.98 ± 0.50	2.44 ± 0.48
12	1.82 ± 0.08	10.65 ± 1.02	12.2 ± 0.49	2.86 ± 0.83
24	2.75 ± 0.12	0.76 ± 0.42	1.29 ± 0.37	1.93 ± 0.24
48	2.64 ± 0.03	0	0	0.21 ± 0.29

[a]: BMM-MP without bacterial cells

[b]: The culture was grown in BMM-MP

[c]: The culture was grown in LB-MP before subcultured to BMM-MP

[d]: The culture was grown in LB before subcultured to BMM-MP

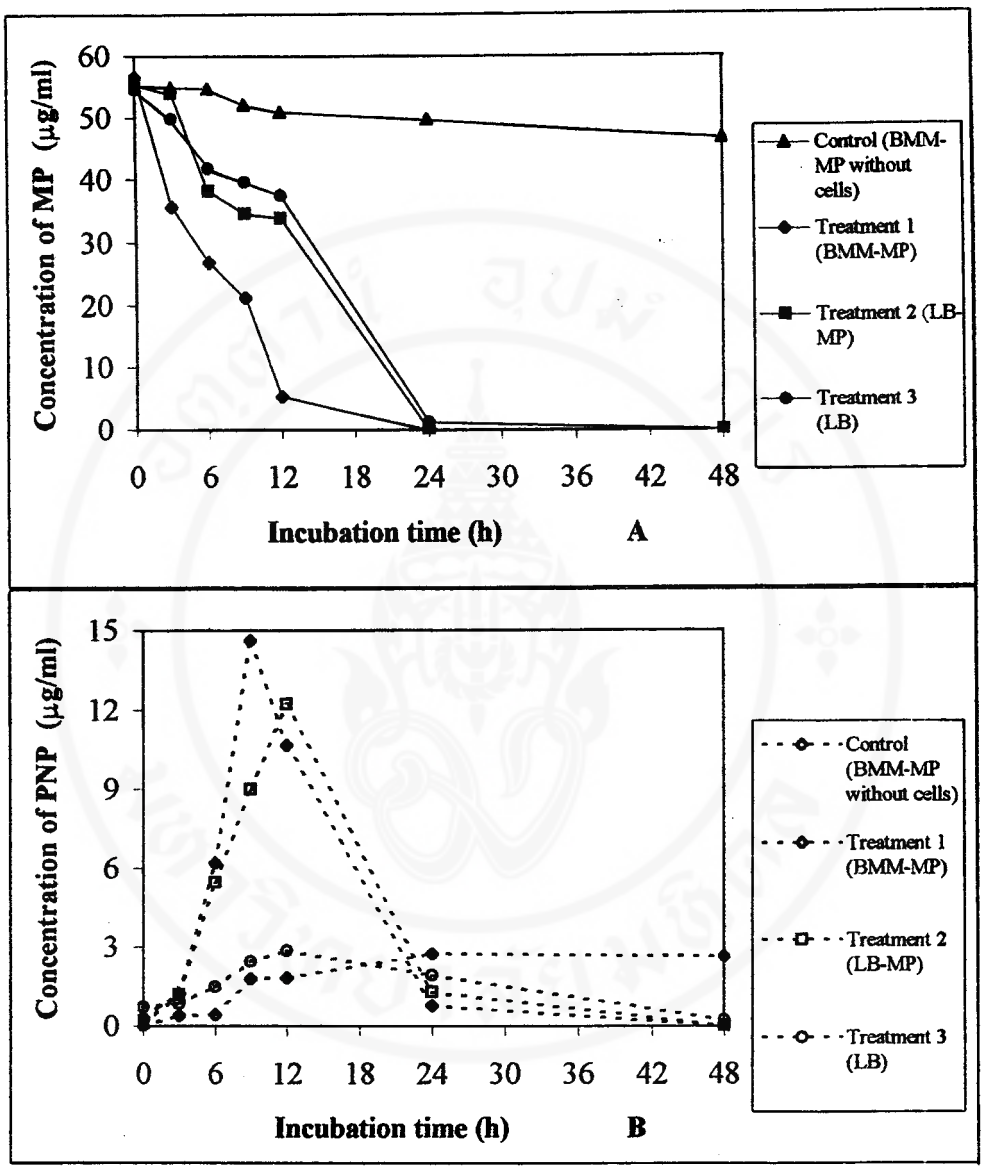

Figure 13. The concentrations of MP and PNP in various medium. (A) MP concentrations of the control set and the experimental set. (B) PNP concentrations of the control set and the experimental set.

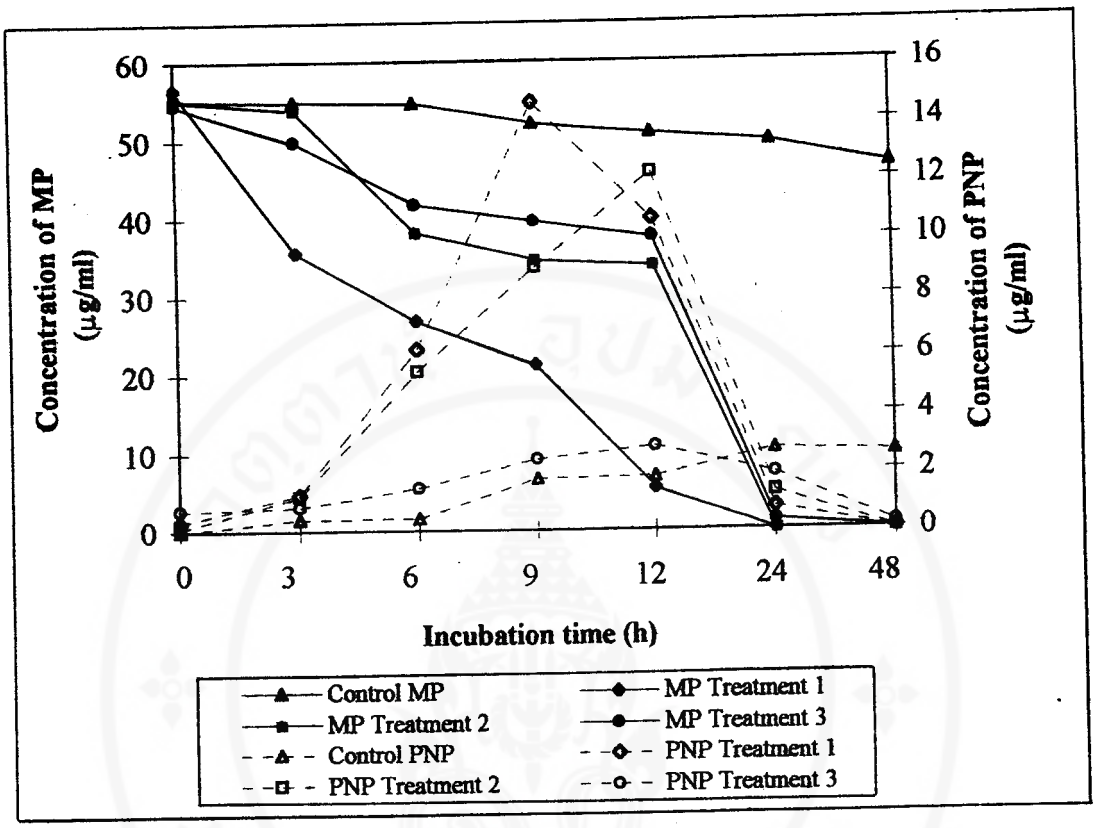

Figure 14. The MP and PNP concentrations of the control set were compared with that of the experimental set.

Table 7. The percentage of residual MP in the control set and the experimental set at various time intervals.

Incubation time (h)	Percentage of MP-residue ($\bar{X} \pm SD$)			
	Control[a]	Treatment 1[b]	Treatment 2[c]	Treatment 3[d]
0	100	100	100	100
3	99.78± 2.48	63.11± 0.56	91.35 ± 7.73	97.45 ± 1.46
6	99.42 ± 2.96	47.53 ± 2.23	68.98 ± 0.58	76.61 ± 2.66
9	94.63 ± 0.72	37.62 ± 1.08	64.44 ± 3.37	72.55 ± 4.34
12	92.44 ± 2.52	9.49 ± 0.56	62.21 ± 0.55	68.82 ± 2.06
24	90.31 ± 0.78	0	0	16.75 ± 0.54
48	84.99 ± 1.67	0	0	0.15 ± 0.1

[a]: BMM-MP without bacterial cells

[b]: The culture was grown in BMM-MP

[c]: The culture was grown in LB-MP before subcultured to BMM-MP

[d]: The culture was grown in LB before subcultured to BMM-MP

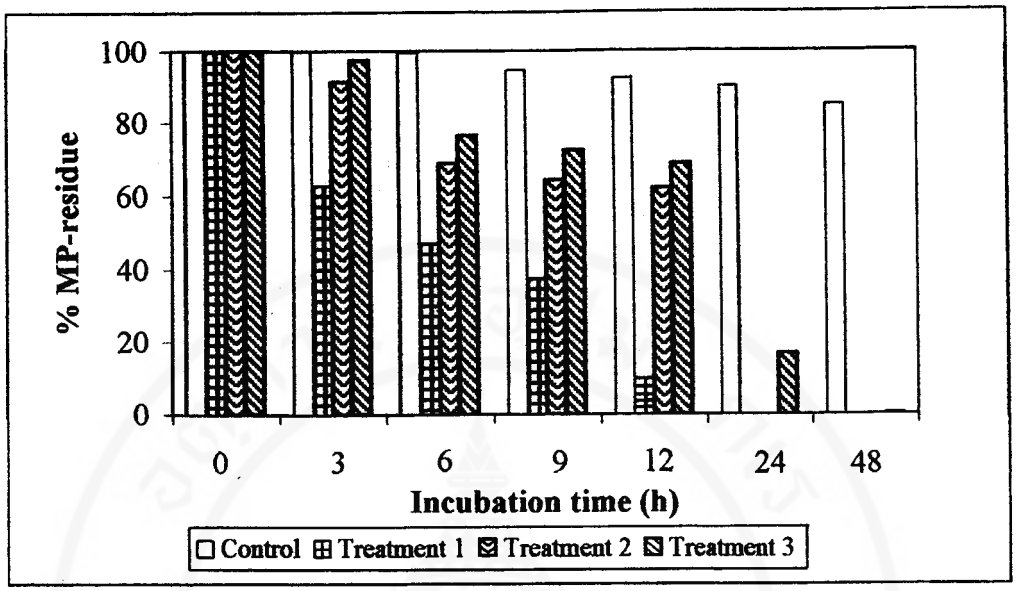

Figure 15. Degradation of MP by MP-degrading *P. stutzeri* at various time intervals.

Table 8. The percentage of MP-degradation in the control set and the experimental set at various time intervals.

Incubation time (h)	Percentage of MP-degradation		
	Treatment 1[b]	Treatment 2[c]	Treatment 3[d]
0	0	0	0
3	36.68	8.44	2.34
6	51.83	30.39	22.75
9	57.01	30.2	22.08
12	82.91	31.19	23.58
24	100	100	73.55
48	100	100	84.81

[b]: The culture was grown in BMM-MP

[c]: The culture was grown in LB-MP before subcultured to BMM-MP

[d]: The culture was grown in LB before subcultured to BMM-MP

In addition, the degradation of MP and PNP in the control set and the experimental set were analyzed by a spectrophotometer for the optical density at 410 nm. Table 9 shows that PNP in the control increased slowly, while that of treatment 1 increased rapidly. The highest optical density at 410 nm for PNP in treatment 1 was 0.65 at 9h of incubation but those of the other treatments were 0.553 and 0.393 at 12h of incubation, respectively. After that, the PNP of all treatments decreased to 0.185, 0.202, and 0.242 at 48h of incubation, respectively. We found that the amount of PNP of treatment 1 decreased faster than the other treatments (Figure 16).

Table 10 shows the comparisons between the PNP concentrations determined by HPLC and by a spectrophotometer. The results of the study showed that the highest PNP concentration from both methods of treatment 1 occurred at 9h of incubation, while those of the other treatments occurred at 12h of incubation (Figure 17).

Table 9. An appearance of PNP analyzed by a spectrophotometer for A_{410} nm.

Incubation time (h)	Control[a] ($\bar{X} \pm SD$)	Treatment 1[b] ($\bar{X} \pm SD$)	Treatment 2[c] ($\bar{X} \pm SD$)	Treatment 3[d] ($\bar{X} \pm SD$)
0	-0.011 ± 0.004	0.035 ± 0.00	0.028 ± 0.001	0.029 ± 0.001
3	0.024 ± 0.00	0.154 ± 0.00	0.098 ± 0.001	0.076 ± 0.005
6	0.049 ± 0.00	0.559 ± 0.024	0.326 ± 0.003	0.309 ± 0.007
9	0.052 ± 0.001	0.657 ± 0.029	0.414 ± 0.013	0.334 ± 0.001
12	0.059 ± 0.00	0.441 ± 0.114	0.553 ± 0.028	0.393 ± 0.018
24	0.067 ± 0.00	0.206 ± 0.023	0.225 ± 0.013	0.273 ± 0.025
48	0.064 ± 0.00	0.185 ± 0.017	0.202 ± 0.014	0.242 ± 0.007

[a]: BMM-MP without bacterial cells

[b]: The culture was grown in BMM-MP

[c]: The culture was grown in LB-MP before subcultured to BMM-MP

[d]: The culture was grown in LB before subcultured to BMM-MP

Table 10. The comparison between PNP concentration determined by HPLC and the amount of PNP from a spectrophotometer (A_{410}).

Incubation time (h)	Control[a]	Treatment 1[b]	Treatment 2[c]	Treatment 3[d]
	Concentration of PNP by HPLC ($\bar{X} \pm SD$)			
0	0	0.31 ± 0.17	0.1 ± 0.07	0.72 ± 0.01
3	0.4 ± 0.12	1.24 ± 0.15	1.18 ± 0.008	0.83 ± 0.15
6	0.42 ± 0.12	6.20 ± 0.84	5.45 ± 0.63	1.48 ± 0.19
9	1.77 ± 0.02	14.6 ± 0.63	8.98 ± 0.5	2.44 ± 0.48
12	1.82 ± 0.08	10.65 ± 1.02	12.2 ± 0.49	2.86 ± 0.83
24	2.75 ± 0.12	0.76 ± 0.42	1.29 ± 0.37	1.93 ± 0.24
48	2.64 ± 0.03	0	0	0.21 ± 0.29
	PNP from A_{410} ($\bar{X} \pm SD$)			
0	-0.011 ± 0.004	0.035 ± 0.00	0.028 ± 0.001	0.029 ± 0.001
3	0.024 ± 0.00	0.154 ± 0.00	0.098 ± 0.001	0.076 ± 0.005

[a]: BMM-MP without bacterial cells

[b]: The culture was grown in BMM-MP

[c]: The culture was grown in LB-MP before subcultured to BMM-MP

[d]: The culture was grown in LB before subcultured to BMM-MP

Table 10. The comparison between PNP concentration by HPLC and the amount of PNP from a spectrophotometer (A_{410}) (Cont.).

Incubation time (h)	Control[a]	Treatment 1[b]	Treatment 2[c]	Treatment 3[d]
	PNP from A_{410} ($\bar{X} \pm SD$)			
3	0.024 ± 0.00	0.154 ± 0.00	0.098 ± 0.001	0.076 ± 0.005
6	0.049 ± 0.00	0.559 ± 0.024	0.326 ± 0.003	0.309 ± 0.007
9	0.052 ± 0.001	0.657 ± 0.029	0.414 ± 0.013	0.334 ± 0.001
12	0.059 ± 0.00	0.441 ± 0.114	0.553 ± 0.028	0.393 ± 0.018
24	0.067 ± 0.00	0.206 ± 0.023	0.225 ± 0.013	0.273 ± 0.025
48	0.064 ± 0.00	0.185 ± 0.017	0.202 ± 0.014	0.242 ± 0.007

[a]: BMM-MP without bacterial cells

[b]: The culture was grown in BMM-MP

[c]: The culture was grown in LB-MP before subcultured to BMM-MP

[d]: The culture was grown in LB before subcultured to BMM-MP

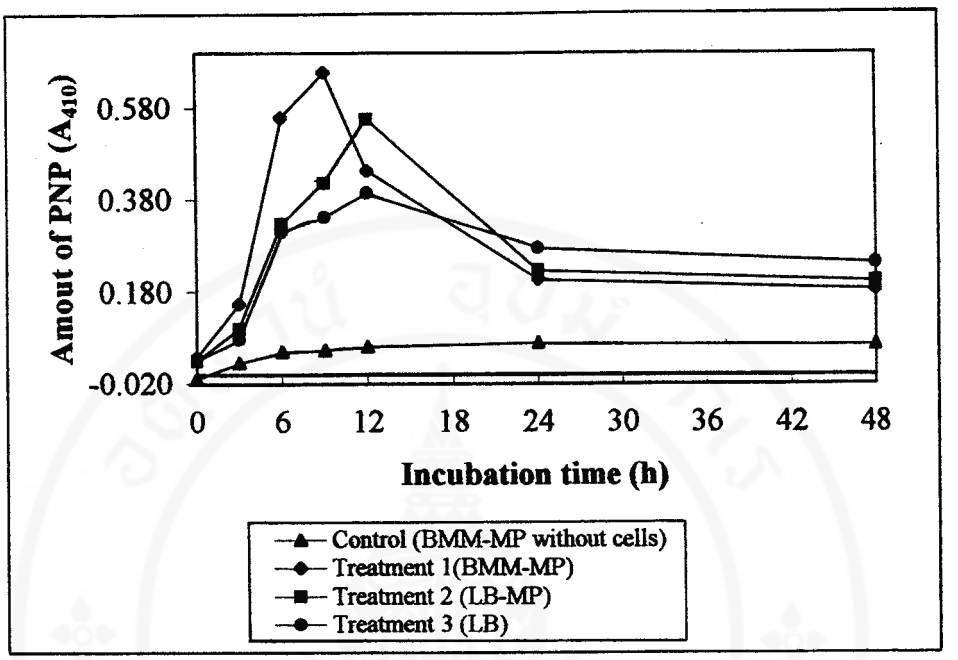

Figure 16. PNP of the control set and the experimental set determined by a spectrophotometer.

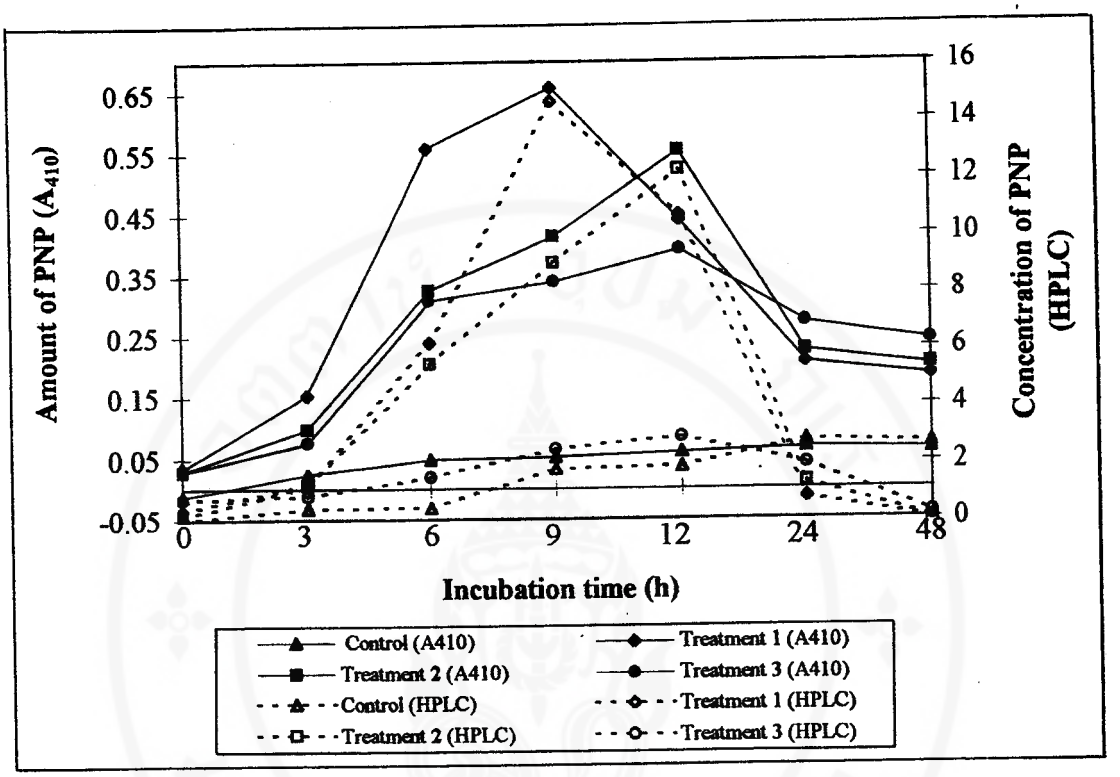

Figure 17. PNP concentrations from HPLC and A_{410} nm of the control set compared with those of the experimental set.

2.2 Localization of Methyl Parathion Hydrolase in Bacterial Cells

It is essential to know the location of the enzyme within the cells and whether the enzyme is present in cytoplasm or bound to the membrane. Crude cell lysate was subjected to differential centrifugation and divided into debris fraction, membrane fraction, ribosomal fraction, and cytosolic fraction. The cellular location of the enzyme was determined the concentration of PNP from fractions by using HPLC and the enzyme activity of each fraction were measured by enzyme assay method.

The experiment showed that the concentrations of PNP from debris fraction, membrane fraction, and cytosolic fraction, which were analyzed by HPLC, were 8.126, 8.451, and 0.016 μg/ml, respectively, as shown in Figure 18.

These results were also confirmed by the specific activity of the enzyme. The enzyme activity of cellular fraction: debris fraction, membrane fraction, ribosomal fraction, and cytosolic fraction were measured for the enzyme specific activity. The specific activity of these fractions was calculated as equation IX in Appendix 3. The enzyme specific activity of debris fraction, membrane fraction, ribosomal fraction, and cytosolic fraction were 3.53, 3.71, 0.78, and 0.82 units/mg of protein (Table 11).

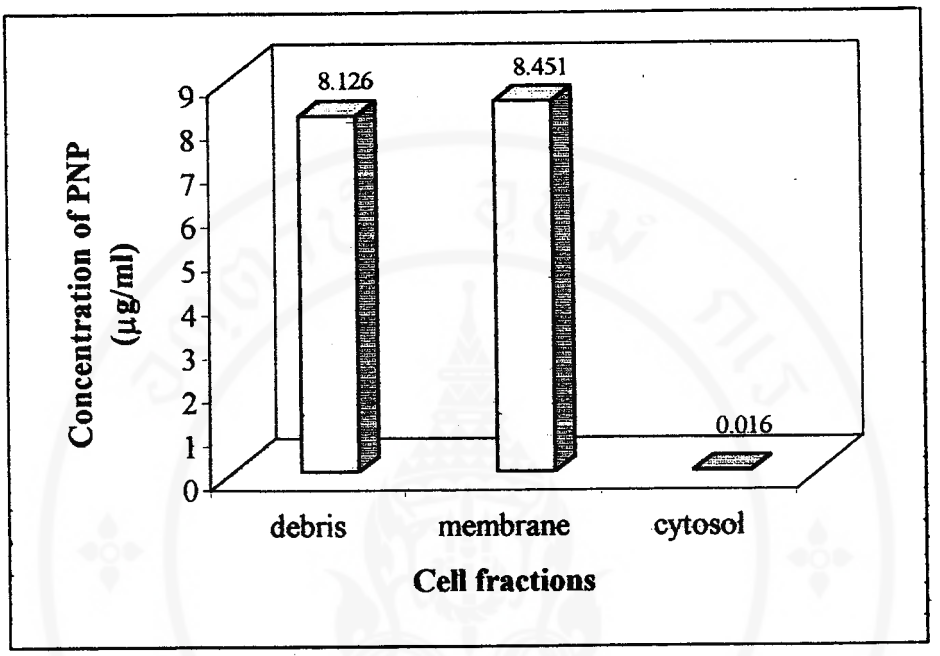

Figure 18. Comparisons of MP degradation between various cell fractions by HPLC.

Table 11. The specific activity of various cell fractions determined by enzyme assay.

Fractions	Total Protein (mg/ml)	Total Activity (Unit; μmole)	Specific Activity (Unit/mg protein)
Debris fraction	5.48	19.33	3.53
Membrane fraction	13.22	49.1	3.71
Ribosomal fraction	1.65	1.28	0.78
Cytosolic fraction	1.44	1.18	0.82

3. Determine the Homology between Methyl Parathion Hydrolase Gene of Methyl Parathion-Degrading *P. stutzeri* and Other Relevant Genes

The DNA homology of the methyl parathion hydrolase gene of MP-degrading *P. stutzeri*, the *opd* gene of parathion-degrading *Flavobacterium* sp. ATCC 27551, and the *adp B* gene of coumaphos-degrading *Nocardia* sp. strain B-1 was analyzed by Southern blot hybridization. The molecular size of the *opd* gene and the *adp B* gene, which is 1.3-kb and 3.55-kb, respectively is shown in Figure 19. The result of the transformation of *adp B* gene by calcium chloride procedure is shown in Figure 19 B. It was found that transformant colonies could degrade MP to PNP and the *Pst* I fragment of these plasmids were 3.55-kb which is the same size as the *adp* B gene. After that, the 1.3-kb *Pst* I fragment from plasmid pWWM513 and the 3.55-kb *Pst* I fragment of pWWM1327 were eluted from a 0.7% agarose gel. Both fragments were labeled non-radioactive with digoxigenin (DIG) and used as hubridization probes. The concentrations of the prepared DIG-probes were 20 ng/μl.

The results of the Southern blot hybridization of the *opd* gene probe and *adp* B gene probe are shown in Figure 20. The genome size of the MP-degrading *P. stutzeri* is not known but will be comparable to those of *P. aeruginosa*, *Bacillus* sp., and *E. coli*. The DNA of these bacterial species was used as a negative control. All bacterial DNAs were digested with *BamH* I. Five micrograms digested-DNA from each bacterial species was loaded on a 0.7% agarose gel and blotted onto a nylon membrane. The *Pst* I fragments of pWWM513 and pWWM1327 were loaded as controls in separate lanes (100 pg). Only the *Pst* I fragments were detected by the hybridization. The result shows that both the *opd* probe and the *adp* B probe do not hybridize to genomic DNA from the MP-degrading *P. stutzeri*.

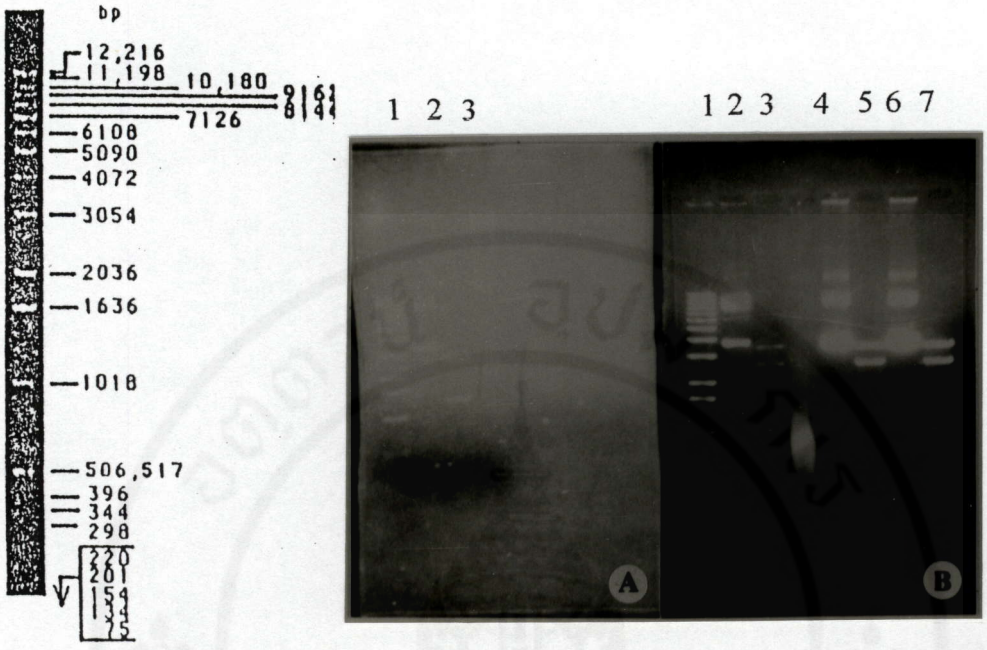

Figure 19. (A) 0.7% agarose gel electrophoresis of plasmid pWWM513 containing the 1.3-kb *opd* gene. Lanes: 1, 1 Kb DNA ladder, used as molecular standard marker; 2, undigested pWWM513; 3, *Pst* I-digested pWWM513. (B) 0.7% agarose gel electrophoresis of plasmid pWWM1327 containing the 3.55-kb *adp* B gene. Lanes: 1, 1 Kb DNA ladder; 2, undigested pWWM1327; 3, *Pst* I-digested pWWM1327; 4 and 6, undigested inserted plasmid; 5 and 7, *Pst* I-digested inserted plasmid.

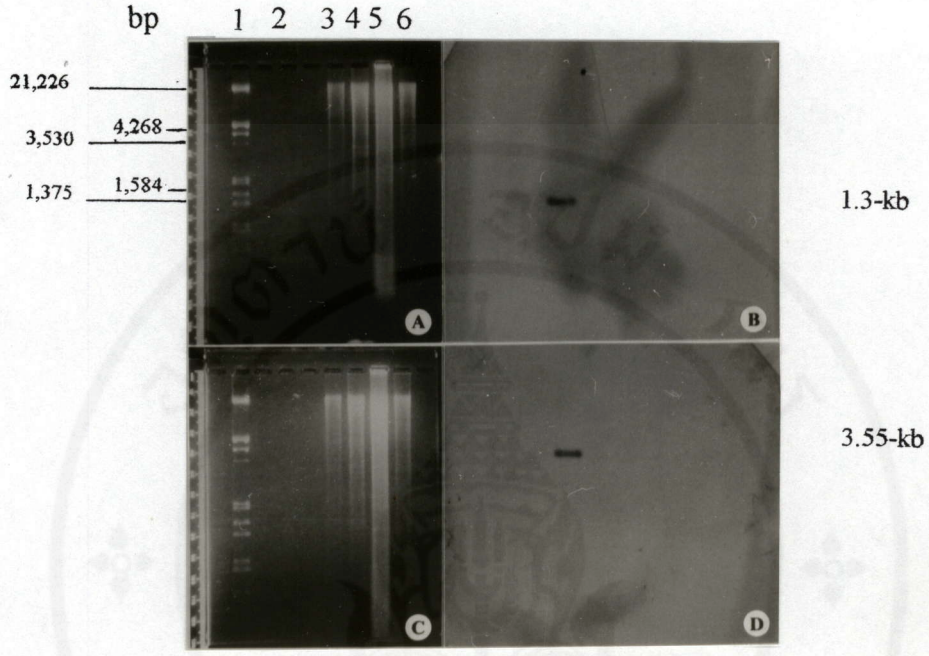

Figure 20. Southern blotting and hybridization of the 1.3-kb *opd* probe and 3.55-kb *adp* B probe. (A, C) 0.7% agarose gel electrophoresis of various DNAs. Lanes: 1, 0.9 μg *Eco*R I-*Hin*d III-digested λ DNA as a molecular size marker; 2, 100 pg *Pst* I-digested pWWM513 in panel A and *Pst* I-digested pWWM1327 in panel C; 3, 4, 5, and 6, 5 μg *Bam*H I-digested DNAs from *P. stutzeri*, *P. aeruginosa*, *Bacillus sp.*, and *E. coli*, respectively; (B, D) Southern hybridization of the membrane transformed DNA shown in A, C. Random primed DIG-labeled DNA probes of the opd and adpB *Pst* I fragments were used for the hybridization.

4. Purification of Methyl Parathion Hydrolase Enzyme and Determination of Its Molecular Weight

4.1 Purification of the Methyl Parathion Hydrolase Enzyme

In this study, the crude Triton X extract was purified in 3 steps, which were composed of ion exchange chromatography (IEC, Resource S column), gel filtration chromatography (GFC, Sephadex G-100 column), and hydrophobic interaction chromatography (HIC, Octyl sepharose 4 Fast Flow column).

Ion Exchange Chromatography. Crude Triton X extract was concentrated by using a Centricon YM-10 filter membrane. The total protein of the crude Triton X extract was 66 mg/ml, total activity was 133.74 units, and specific activity was 2.03 units/mg of protein/min (Table 12). The concentrated crude Triton X extract was fractionated on a 1 ml Resource S column at the flow rate of 1 ml/min as described under the Materials and Methods section. The fractions no. 3 to 8 containing methyl parathion hydrolase activity, at approximately 0.3-0.8 M NaCl, were pooled.

The concentration of protein was estimated by Lowry method and the enzyme activity was determined, using MP as a substrate. Specific activity of the enzyme was expressed as μmole of PNP formed per minute for each milligram of protein under specified conditions (μmole of PNP/mg of protein/min). In the present study, the methyl parathion hydrolase was purified by using a Resource S column, which is a cation exchange chromatography column. The total protein of pooled Resource S fractions was 3.86 mg/ml, total activity was 48.17 units, and the specific activity was 12.46 units/mg of protein/min (Table 12).

Gel Filtration Chromatography (Size-Exclusion Chromatography). The pooled Resource S, which contained methyl parathion hydrolase enzyme, was loaded

onto a Sephadex G-100 column at the flow rate of 0.5 ml/min. Fractions were assayed for enzyme activity and the protein concentration was measured at the optical density 410 nm and 280 nm, respectively, as shown in Figure 21. The results showed that the methyl parathion hydrolase enzyme appeared in fractions 29-53. However, only fractions 29-33 were pooled because these fractions had the higher enzyme activity.

Ten ml of pooled Sephadex G-100 fractions was added with $(NH_4)_2SO_4$, which was brought to 1 M $(NH_4)_2SO_4$. The total protein was declined to 0.09 mg/ml, total activity was 23.23, and enzyme specific activity of this step was expressed as 270.13 units/mg of protein/min and the purification was 133 fold (Table 12).

Hydrophobic Interaction Chromatography. The pooled gel filtration was loaded onto Octyl Sepharose 4 FF column at the flow rate of 1 ml/min. Protein concentration was estimated by measuring optical density at 280 nm and the enzyme activity was determined by enzyme assay, as shown in Figure 22. The methyl parathion hydrolase enzyme activity did not occurred until fraction no. 8, whereas the high enzyme activities occurred in fraction no. 9 to 12 and these fractions were pooled for further SDS polyacrylamide gel electrophoresis. The partially purified enzyme was found to have a total protein as 0.006 mg/ml, total activity as 2.58 units, specific activity as 430 units/mg of protein/min, and corresponding to a 212 fold purification. Total protein, total activity, specific activity, purification fold, and % yield (recovery) were calculated as followed an equation VII, VIII, IX, X, and XI, respectively, in Appendix 3.2. The purification is summarized in Table 12.

After that, pooled fractions of each column were analyzed for the purity and determined the molecular weight of protein of interest by SDS-PAGE. From Figure

21 the protein profile (chromatogram) of the final purification step showed that the protein of interest was not purified to homogeneity, however, the purification of protein increase to approximate 212 folds in specific activity, as shown in Table 12.

From Table 12, total activity decreased along the purification scheme, being 133.74, 48.17, 23.23, and 2.58 μmole, while specific activity increased along the purification step, being 2.03, 12.46, 270.13, and 430 units/mg of protein for crude Triton X extract, Resource S, Sephadex G-100, and Octyl sepharose 4 FF samples, respectively. The protein purification in the final step was as high as approximately 200 folds in specific activity, however, the final yield was very low (1.93%).

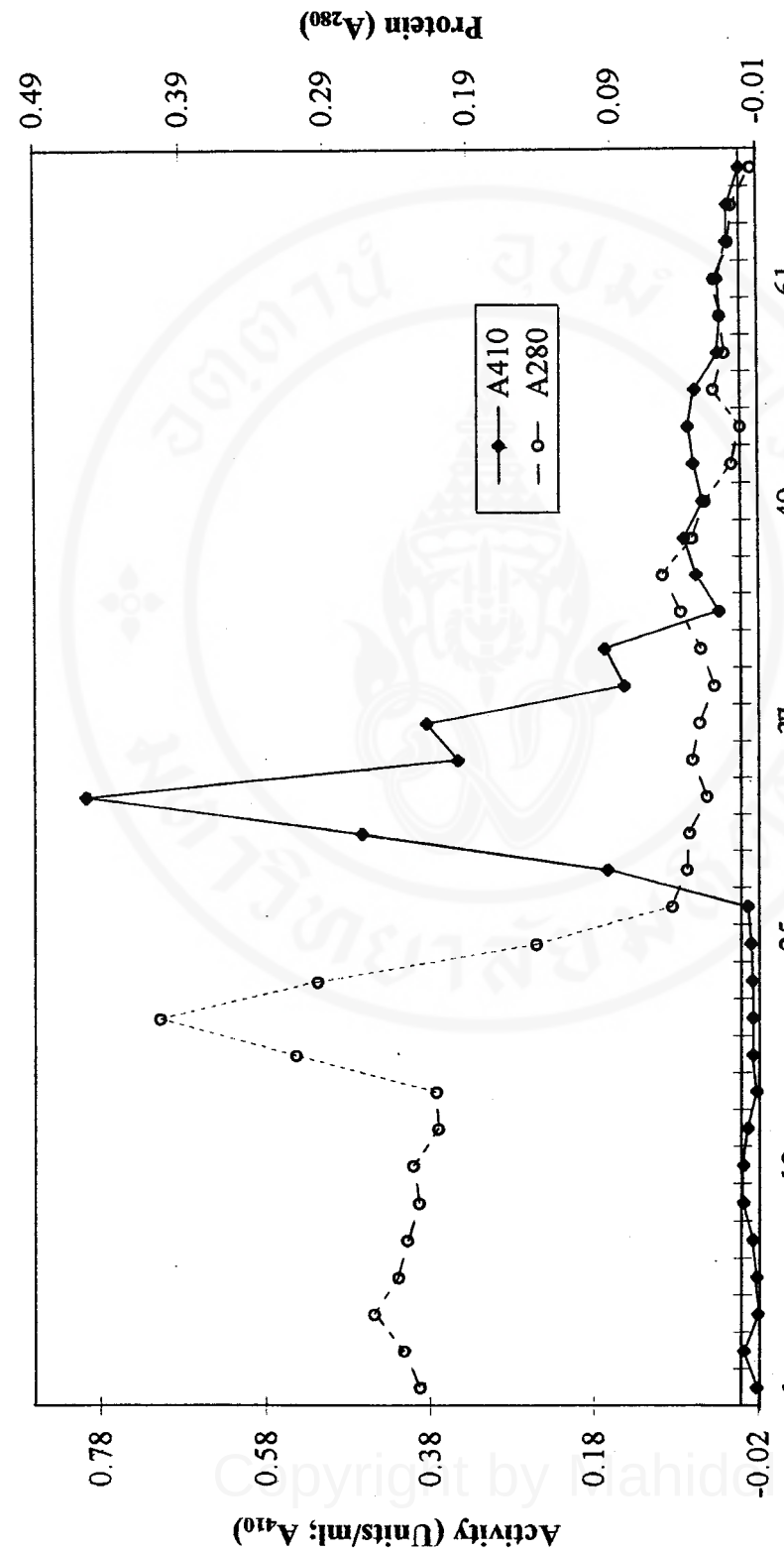

Figure 21. Pooled Resource S sample was chromtographed over Sephadex G-100 column.

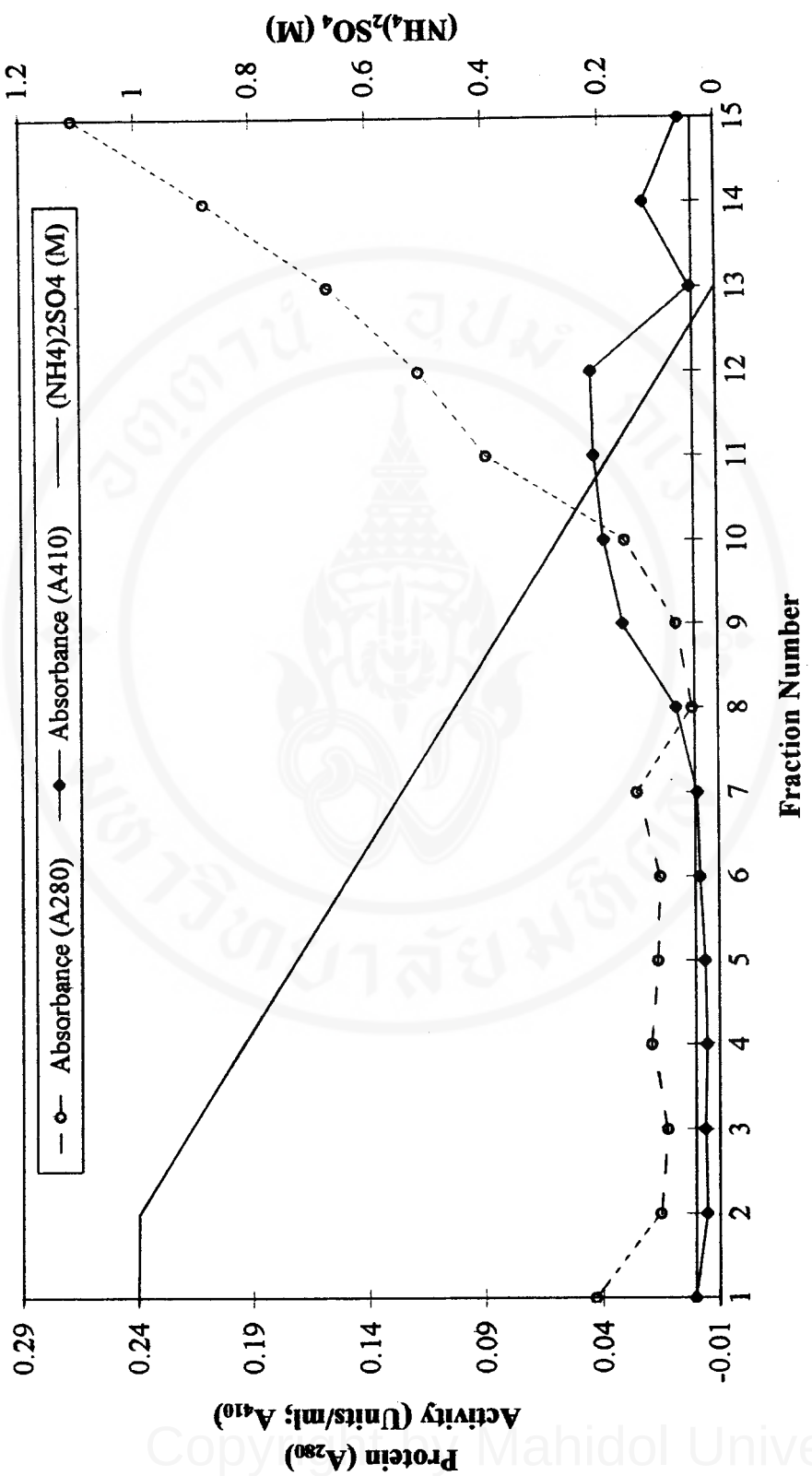

Figure 22. Pooled Sephadex G-100 sample was chromatographed over Octyl Sepharose 4 FF column.

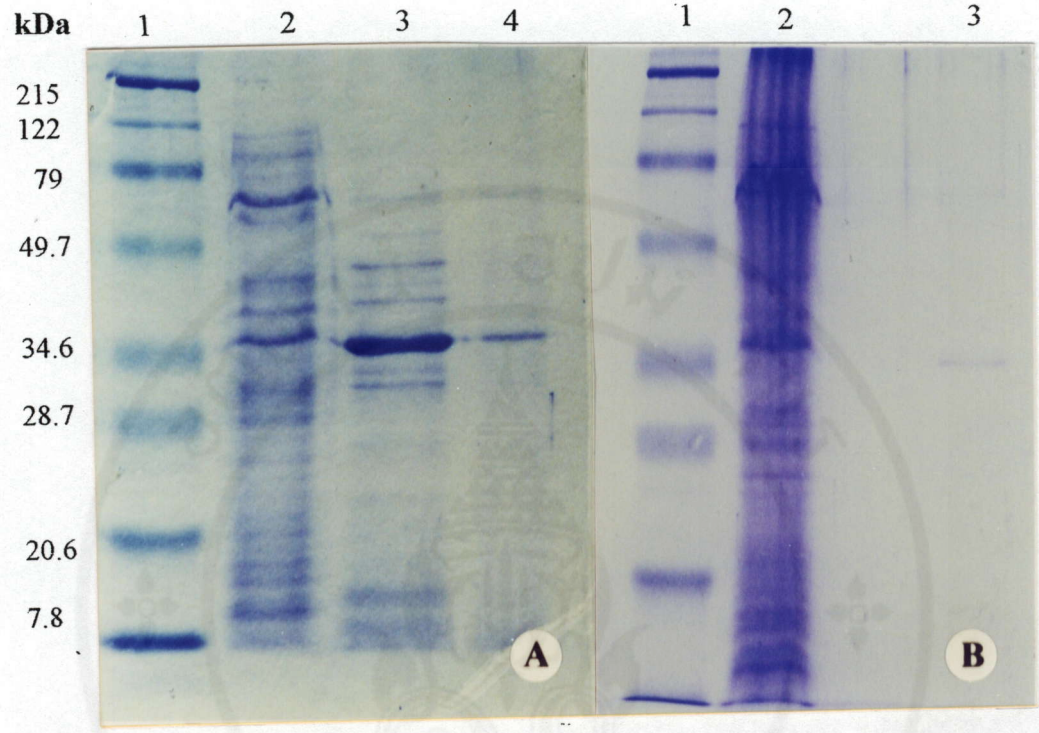

Figure 23. SDS-PAGE of fractions from methyl parathion hydrolase purification. (A) Lanes; 1, molecular weight standards; 2, crude Triton X extract; 3, pooled resource S samples; 4, pooled sephadex G-100 samples. (B) Lanes; 1, molecular weight standards; 2, crude Triton X extract; 3, pooled octyl sepharose 4 FF samples. The molecular weight markers included Myosin (215,000), β-galactosidase (122,000), BSA (79,000), Ovalbumin (49,700), Carbonic anhydrase (34,600), Soybean-trypsin inhibitor (28,700), Lysozyme (20,600), and Aprotinin (7,800).

Table 12. Purification of methyl parathion hydrolase from MP-degrading *P. stutzeri*.

Purification Step	Total Protein (mg/ml)	Total Activity (Unit; μmole)	Specific Activity (Unit/mg protein)	% yield (Recovery)	Purification (Fold)
crude Triton X extract	66	133.74	2.03	100	
pooled Resource S	3.86	48.17	12.46	36.02	6.14
pooled Sephadex G-100	0.09	23.23	270.13	17.37	133.07
pooled Octyl Sepharose 4 FF	0.006	2.58	430	1.93	211.82

4.2 Activity Staining of the Methyl Parathion Hydrolase Enzyme

The ability of reactive and activity-stain methyl parathion hydrolase following SDS gel electrophoresis has been crucial to the purification efforts. The partially purified protein was separated by SDS-gel electrophoresis, which was not stained with Coomassie Blue R. SDS-PAGE was renatured and stained by activity-staining method. The gel was incubated in a renaturation buffer and washed with washing buffer. The formula of a renaturation buffer and washing buffer were shown in Appendix 1. SDS gel was incubated with substrate activity and allowed assessment of the relationship of particular protein band to activity for 3-5 min at room temperature and observed the yellow colored band in the gel. The yellow color of PNP diffused with time.

Values for protein molecular size, expressed in kDa were determined by comparing the protein of interest migration relative to that of known molecular weight protein marker. SDS-PAGE of the degraded product revealed that a yellow band with R_f value of 0.368 cm coincided in location and color with PNP. The molecular weight of the MP hydrolase enzyme was approximately 40 kDa (Appendix 3.3), as shown in Figure 24. Molecular mass values of protein standard marker are shown in Appendix 3.2.

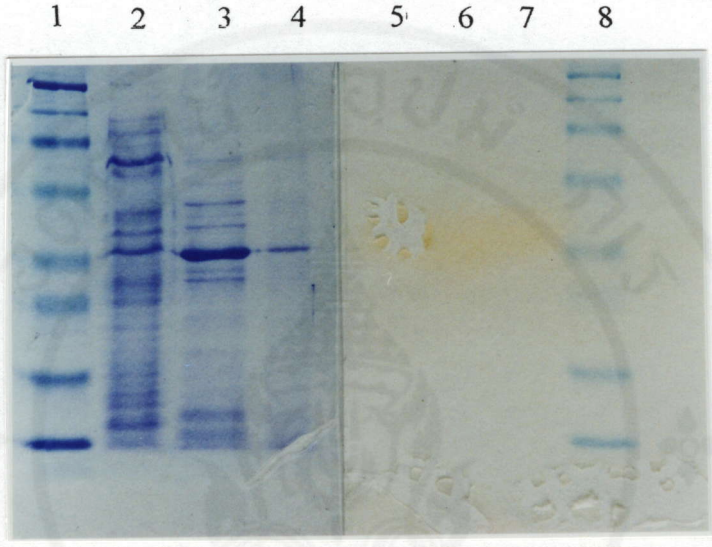

Figure 24. SDS-PAGE analysis of fractions from MP hydrolase purification steps: lane 1 and 8, molecular weight marker; lane 2 and 7, crude Triton X extract; lane 3 and 6, pooled Resource S samples; lane 4 and 5, pooled Sephadex G-100 samples. Lane 5-8 the samples were stained with MP substrate.

4.3 Effects of Reagents on the Activity of the Enzyme

The experiments were then conducted to study the effects of reagents on the activity of the enzyme. The purified enzyme appeared to be homogeneous as judged by its migration as a protein band in SDS-PAGE with an apparent molecular weight of approximately 40,000 Da (40 kDa). In this section, the partially purified enzyme eluted from hydrophobic interaction chromatography (Octyl sepharose 4 FF column) had a total protein 0.004 mg/ml. This partially purified enzyme was treated with sulfhydryl reducing agent (1 mM DTT), heavy metal ion (1 mM $CuCl_2$), and metal chelating agent (0.1 mM EDTA) in order to investigated the activity of the enzyme.

As illustrated in Table 13, the direct addition of various agents showed that the specific activity of the enzyme was decreased 50% (130 units/mg of protein) when the enzyme was treated with 1 mM DTT. In contrast, the percentage of the enzyme activity when treated with 1 mM $CuCl_2$ and 0.1 mM EDTA were 91% and 89%, respectively, compared with that of no addition control (Figure 25).

Table 13. Effects of various reagents on the methyl parathion hydrolase activity.

Reagents	Total Activity (Unit; μmole)	Specific activity (Unit/mg of protein)	% Relative Activity
no addition	1.048	262	100
1 mM DTT	0.52	130	49.62
1 mM $CuCl_2$	0.952	238	90.84
0.1 mM EDTA	0.936	234	89.31

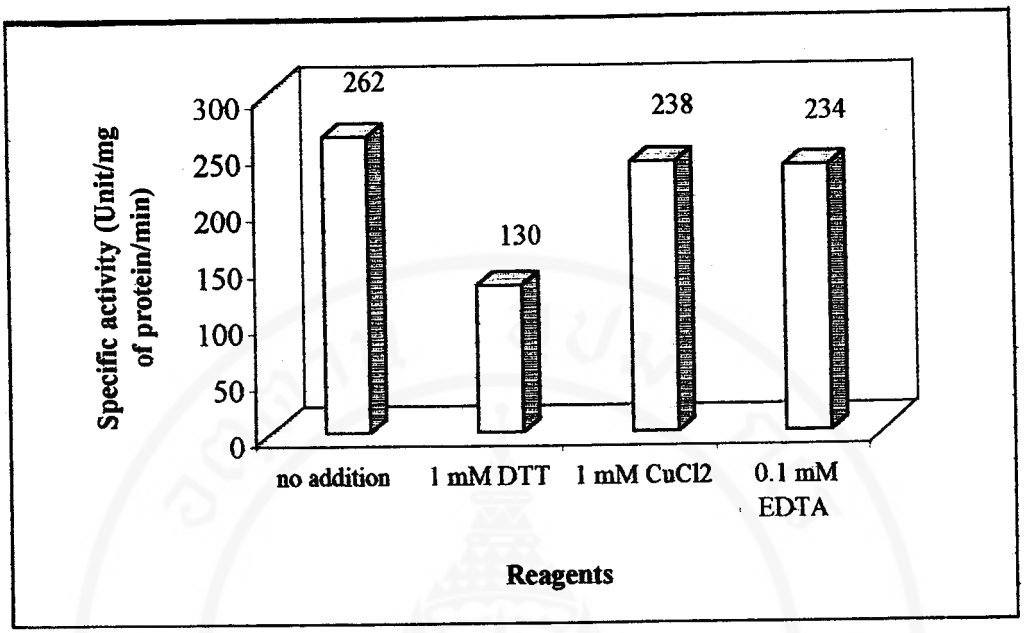

Figure 25. Comparisons of the enzyme activity between no addition control and treated sample with various reagents.

CHAPTER V

DISCUSSION

1. Studies on Some Basic Properties of *P. stutzeri*

1.1 Growth Curve and an Appearance of PNP

The study on growth curve and the optical density at 410 nm of *P. stutzeri* showed that the bacteria could not only use MP for growing and change it to PNP but could also degrade PNP further. The PNP concentration decreased and the bacterial growth also decreased. An increased in turbidity of the medium was reproducibly noticed with the depletion of MP. A yellow color of PNP could be observed directly from the medium.

The result of the present study is consistent with the report of Chaudhry *et al.* (55). The report described the isolation of mixed cultures from soil that was previously treated with parathion (PAR) and MP. The mixed bacterial cultures utilized both PAR and MP as a carbon source and hydrolyzed these compounds to PNP. One member of the mixed cultures was a *Pseudomonas* species, which required glucose or another carbon source to grow. Ou and Sharma reported that a *Bacillus* sp. was isolated from mixed culture and MP was utilized as a carbon source. This bacterium hydrolyzed MP to PNP rapidly (57).

In addition, the present study was supported by the report of Keprasertsup C. Her study showed that two mixed cultures, isolated from agricultural soil, capable of degrading both commercial grade and analytical grade for growth in BMM without glucose or other carbon sources. The result indicated that the *Pseudomonas* sp.

effectively utilized MP as a carbon source and hydrolyzed MP to PNP (7). The fact that MP and PNP concentrations decreased was interpreted as MP and PNP being degraded by this bacterium. At present, *Pseudomonas* sp. was tentatively identified as a *Pseudomonas stutzeri*. The results suggested that *P. stutzeri* effectively utilized MP as carbon source for growing and changed MP to PNP by its hydrolytic process.

1.2 Analysis of MP and PNP by HPLC

The concentrations of MP and PNP of MP-degrading *P. stutzeri* in the present study were detected by HPLC. The result of this study is distinct in retention time of MP and PNP with the result of Keprasertsup C (7). The retention times of MP and PNP of the previous study were 4.32 and 2.12 min, respectively. Meanwhile, the retention times of MP and PNP of the present study were 5.8 and 3.9 min, respectively. Because the present study used C-18 μBondapak column but the other study used Spherisorb ODS-2 column for separating MP and PNP.

Furthermore, the result of the present study is consistent with the result of Keprasertsup C. The previous study showed that if the initial concentration of the bacterial cells were $x10^7$ cells/ml, MP and PNP were degraded completely within 3h and 12h of incubation, respectively. Meanwhile, the concentrations of MP and PNP were degraded completely within 12h and 24h of incubation, respectively when the initial concentrations of the bacterial cells were $x10^6$ cells/ml. The result of the previous study is as same as the result of the present study. These results concluded that the MP-degrading *P. stutzeri* degraded MP to PNP and utilized MP as a carbon

source. In addition, the concentration of the bacterial cells affected the degradation rate of MP and PNP.

2. Certain Basic Properties of the Methyl Parathion Hydrolase Enzyme

2.1 Expression of the Methyl Parathion Hydrolase Enzyme

The degradation of MP and PNP were determined by HPLC. In addition, the PNP degradation was also analyzed by a spectrophotometer at 410 nm. The result of the present study showed that the disappearance of MP and the appearance of PNP in the cultures, which were grown in LB-MP and LB medium before subcultured to BMM-MP, were slower than the culture, which were grown only in BMM-MP medium. Because in LB-MP medium, the culture could use the other simple carbon source before used MP as a carbon source for their growth. In LB medium condition, MP-degrading *P. stutzeri* took longer time to degraded MP because of this bacteria used inducible substance, which is MP for producing the methyl parathion hydrolase enzyme. Meanwhile, the MP concentration of the control set remained rather stable due to its own degrading process and the PNP concentration increased continually. The result concluded that the highest degradation of MP occurred in the culture, which were grown in BMM-MP, while the culture in LB medium gave the lowest degradation rate.

It has noticed that the quantities of PNP, which were measured by using a spectrophotometer, were higher than that one when compared with that of by HPLC. Because a spectrophotometric method measures a yellow color in the solution that composed of MP, which is a yellow colored substance in commercial grade, and PNP.

On the other hand, PNP was extracted from the solution before analyzed by HPLC that analyses a substance from its chemical structure and retention time of each one.

The result of the present study is outstanding in the results of Serdar *et al.*, Munnecke, and Brown KA. These studies examined the isolation and identification of soil organisms, *Pseudomonas diminuta* strain MG and *Flavobacterium* sp. (ATCC 27551). The results showed that these bacteria could be grown in the medium without organophosphate insecticides and had possessed the ability to degrade extremely broad-spectrum organophosphorus compounds. It is concluded that these strains had a constitutive expression (66, 71, 77). In contrast, the result of the present study indicated that the expression of the MP-degrading *P. stutzeri* is an inducible one. Because when the bacteria grew in the medium without MP before subcultured to the MP-medium, it need the inducible substance and took longer time to produce the enzyme for degrading MP.

2.2 Localization of Methyl Parathion Hydrolase in Bacterial Cells

The cellular location of the enzyme was determined by using HPLC and confirmed the results by the enzyme assayed method. The result showed that the concentration of PNP from the membrane fraction was approximately 500 fold higher than that of cytosolic fraction. The enzyme specific activity of membrane fraction had the highest activity among various fractions, which implied that the MP-degrading enzyme is located in the membrane fraction.

It has noticed that both the PNP concentration and the enzyme specific activity in debris fraction seem to be high according to in the partial centrifugation step. Some pieces of membrane may be fallen in debris fraction, so the PNP

concentration and the enzyme specific activity of debris fraction were as high as the membrane fraction.

The result of the present study is accordance with the results of Adhya TK, Barik S, and Sethunathan N, and Brown KA. These researches reported that a *Flavobacterium* sp. was expressed as a membrane associated protein (54, 77). In addition, McDaniel CS, Harper LL, and Wild JR reported that the parathion hydrolase of *Pseudomonas diminuta* strain MG and *Flavobacterium* sp. were identified and found to be membrane-bound (83). The result of the present study suggested that the methyl parathion hydrolase enzyme is located in the membrane due to the specific activity of the membrane fraction was the highest.

3. Determine the Homology between Methyl Parathion Hydrolase Gene of Methyl Parathion-Degrading *P. stutzeri* and Other Relevant Genes

This experiment investigated whether the MP-degrading gene of *P. stutzeri* has significant homology with the *opd* gene of the parathion-degrading *Flavobacterium* sp. ATCC 27551 and the *adp* B gene of coumaphos-degrading *Nocardia* sp. strain B-1. The results were obtained by Southern blot hybridization of genomic DNA of the MP-degrading bacteria with *opd* gene and *adp* B gene fragments as probes.

The results of the present study are quite different when compared with other reports. *Pseudomonas diminuta* strain MG hybridized PAR to PNP and diethylthiophosphoric acid. The esterase responsible for this reaction is encoded by a gene located on a plasmid termed pCMS1, which was digested with restriction enzyme *Pst* I. The total number of base pairs in the *Pst* I fragment was observed to be

1322 (80, 89). Moreover, a hybridization study of *P. diminuta* and *Flavobacterium* sp., which used the *Pst* I fragment from the *P. diminuta* plasmid pCMS1 as a probe found that the *opd* gene of *Flavobacterium* sp. ATCC 27551 has a sequence identical to that of the plasmid-borne gene of *P. diminuta*. The results indicated that these bacteria have a homologous gene (76, 90) and also showed homology with total DNA from a *Pseudomonas* sp., which hydrolyzed MP (55).

Furthermore, the previous report of Mulbry WW *et al.* (78) showed both plasmids; pPDL2 is a 39-kb plasmid harboured by *Flavobacterium* sp. ATCC 27551 and pCMS1 is a 70-kb plasmid found in *P. diminuta* strain MG. These plasmids have been shown to share homologous parathion hydrolase gene (termed *opd* for organophosphate degradation) as judged by DNA-DNA hybridization and restriction mapping. The results were determined by DNA hybridization experiments using each of the restriction fragments from pCMS1 as probes against *Flavobacterium* plasmid DNA. Meanwhile, the result of the present study found that a genomic DNA of MP-degrading *P. stutzeri* could be not hybridized with a *opd* gene probe.

On the other hand, the results of the present study are accordant with the result of Shelton DR, Somich CJ and Mulbry WW (81, 94). A 3.55-kb *Pst* I fragment containing *adp* B gene was cloned into *E. coli* and the nucleotide sequence of a 1600-bp containing *adp* B was determined. Comparison of the *adp* B gene with the *Flavobacterium* ADPase-encoding gene, *opd*, revealed no significant homology at both the nucleotide and amino acid levels. *Escherichia coli* cells containing the plasmid pWWM1327, in which the 3.55-kb *Pst* I *adp* B fragment is inserted to pUC19, demonstrated ADPase activity. A search of DNA and protein databases for homologies to *adp* B demonstrated no significant homologies to known sequences.

The present study suggested that the MP-degrading *P. stutzeri* does not contain a gene with significant homology to the *opd* gene and the *adp* B gene. This indicated that this bacteria is not correlated with the previous organophosphate degrading microorganisms. So, the MP-degrading *P. stutzeri* is an interesting microorganism for studying the MP hydrolase enzyme further.

4. Purification of Methyl Parathion Hydrolase Enzyme and Determination of Its Molecular Weight

4.1 Purification of the Methyl Parathion Hydrolase Enzyme

The results of the present study are quite distinct with the result of the report of Mulbry WW and Karns JS (82). Resource S column, which is a cation exchange chromatography column, was used the first step for purifying the methyl parathion hydrolase in the present study because when the enzyme was purified by using DEAE column that is anion exchange chromatography. It was found that the enzyme did not bind to the column, even though the concentration and pH of the buffer was changed. From these reasons, the result of the present study indicated that the methyl parathion hydrolase is a positively charged protein.

On the other hand, the parathion hydrolase from a *Flavobacterium* sp. in the report of Mulbry WW and Karns JS (82) was purified by using a TSK-DEAE column, which is an anion exchange chromatography column. The result of the previous study suggested that the parathion hydrolase of a *Flavobacterium* sp. is a negatively charged protein.

The second purification step, the present study used a Sephadex G-100 column, while the study of Mulbry WW and Karns JS (82) used a Protein-Pak 300

SW column for purifying parathion hydrolase and determined the molecular weight of these enzymes. It was found that the molecular weights of the methyl parathion hydrolase and parathion hydrolase were approximately 40 and 36 kDa, respectively.

The last step, the methyl parathion hydrolase of the present study was purified by using a Octyl sepharose 4 FF column and the enzyme had a specific activity of 430 units/mg of protein. Meanwhile, the Mulbry WW and Karns JS (82) reported that the parathion hydrolase was purified by using a TSK-phenyl column and the enzyme had a specific activity of 3,018 units/mg of protein. It has noticed that the specific activity of the previous study had higher than the other one. Because high specificity columns were used for purifying the enzyme, so the purified enzyme was high purity.

Even though the enzyme was only partially purified and the final yield was low, the protein band of methyl parathion hydrolase could be determined, along with its molecular weight, if "protein regeneration" and "activity staining" of the SDS-polyacrylamide gel could be performed successfully.

4.2 Activity Staining of the Methyl Parathion Hydrolase Enzyme

The molecular weight of methyl parathion hydrolase from the MP-degrading *P. stutzeri* in the present study is higher than the parathion-degrading *Flavobacterium* sp. (ATCC 27551). The molecular weight of the methyl parathion hydrolase was approximately 40 kDa. The report of Mulbry WW and Karns JS concluded that the molecular weight of parathion hydrolase from a *Flavobacterium* sp. (ATCC 27551) was 36 kDa (82). The results of the present study suggested that the activity staining

method could determined the location of protein in SDS gel, which can be observed by a yellow colored band and determined the molecular weight of the enzyme when compared with that of the protein marker standard.

4.3 Effects of Reagents on the Activity of the Enzyme

The effects of reagents on the enzyme activity depend on chemical characteristic of the enzyme. The result of the present study showed that the methyl parathion hydrolase was inhibited by 1 mM DTT. Dithiothreitol may be interfered a functional group or changed a conformation of the enzyme at the active site. Meanwhile, 1 mM $CuCl_2$ and 0.1 mM EDTA had very little effect on the methyl parathion hydrolase activity from the present study.

The observation of the present study is compared with the report of Mulbry WW and Karns JS. The result of the previous study showed the effect of reagents on the activity of three parathion hydrolases, which were produced from *Flavobacterium* sp. (ATCC 27551), strain SC, and strain B-1. It was found that 1 mM DTT and 1 mM $CuCl_2$ inhibited the parathion hydrolase of *Flavobacterium* sp. (ATCC 27551). The hydrolase of strain SC was inhibited by 1 mM DTT but was stimulated by 1 mM $CuCl_2$. In addition, the organophosphate hydrolase enzyme from strain B-1 was stimulated by 1 mM DTT and 0.1 mM EDTA (82).

CHAPTER VI

CONCLUSION

1. *P. stutzeri* utilized MP as a carbon source for growth and MP was changed to PNP by hydrolytic processes of this bacterium.

2. The expression of the MP hydrolase is an inducible one.

3. The cellular location of the enzyme is at the membrane.

4. The MP-degrading *P. stutzeri* does not contain a gene with significant homology to the *opd* gene and the *adp* B gene.

5. The partially purified enzyme had the total protein 0.006 mg/ml, total activity of 2.58 units, and specific activity of 430 units/mg protein/min. The total purification was 212 fold.

6. The molecular weight of the enzyme was approximately 40 kDa.

7. The enzyme was inhibited by 1 mM DTT, while 1 mM $CuCl_2$ and 0.1 mM EDTA had very little effect.

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APPENDIX 1

1. Medium and Buffers

1. Basal Mineral Medium (BMM)-MP

BMM-MP solution (g/l)

Solution I:	K_2HPO_4	4.8
	KH_2PO_4	1.2
	NH_4NO_3	1.0
Solution II:	$MgSO_4.7H_2O$	0.25
	$CaCl_2$	0.04
	$FeSO_4.7H_2O$	0.005
MP		50 μg/ml

2. Luria Broth (LB) (g/l)

Tryptone	10
Yeast-extract	5
NaCl	10
distilled water up to	1000 ml

3. Reaction Mixture

25 mM Tris-HCl, pH 8.5

0.1% Triton X-100

MP 50 μg/ml

4. Enzyme Assay, pH 7.0

4.1 PNP standard solution (1000 μg/ml)

PNP	0.001 g
Methanol	1000 μl

Diluted stock PNP standard solution 20x to final concentration 50 μg/ml

4.2 Blank standard solution (50 mM Tris-HCl, pH 8.5, 50 ml)

Tris-HCl	0.303 g

Adjust pH with HCl and adjust volume up to 50 ml.

4.3 Blank sample solution

50 mM Tris-HCl, pH 8.5

20 mM Tris-HCl, pH 7.8

0.1% Triton X-100

Distilled water

5. Solutions for Isolating Genomic DNA and Plasmid DNA

5.1 CTAB/NaCl solution

5% CTAB

0.7 M NaCl

5.2 TE buffer, pH 8.0

10 mM Tris-HCl

1 mM EDTA

5.3 Glucose/Tris/EDTA (GTE) solution

50 mM glucose

25 mM Tris-HCl, pH 8.0

10 mM EDTA

Autoclave and store at 4°C.

5.4 NaOH/SDS solution

0.2 N NaOH

1% (w/v) sodium dodecyl sulfate (SDS)

Prepare immediately before use.

5.5 5 M Potassium acetate solution, pH 4.8

29.5 ml glacial acetic acid

KOH pellets to pH 4.8

H_2O to 100 ml

This solution can be store at room temperature (do not autoclave).

5.6 STET solution

8% (w/v) Sucrose

5% (w/v) Triton X-100

50 mM EDTA

50 mM Tris-HCl, pH 8.0

Filter sterilizes and store at 4°C

6. Solutions for Southern Transfer

6.1 Tris-borate (TBE) running buffer (5x)

225 mM Tris-base

225 mM Boric acid

10 mM EDTA, pH 8.0

6.2 Electrophoresis sample buffer

0.3% bromophenolblue

50% glycerol

6.3 0.5 μg/ml Ethidium bromide (EtBr)

10 mg/ml ethidium bromide

distilled water

6.4 0.25 N HCl

5 N HCl

distilled water

6.5 Denaturation solution

0.5 N NaOH

3 M NaCl

6.6 Neutralization solution

0.5 M Tris-HCl, pH 7.0

1.5 M NaCl

6.7 20 X SSC buffer

3.0 M NaCl

0.3 M Na-citrate, pH 7.0

6.8 Transfer buffer: 10X SSC

0.15 M Na-citrate, pH 7.0

1.5 M NaCl

7. Solutions for Hybridization

7.1 Blocking reagent

10X SSC

10% SDS

7.2 Standard hybridization buffer with formamide

5X SSC

50% formamide

0.1% (w/v) N-lauroylsarcosine

0.02% (w/v) SDS

2% Blocking reagent

7.3 Washing solution 1

2X SSC

0.1% SDS

7.4 Washing solution 2

0.1X SSC

0.1% SDS

8. Solutions for Immunological Detection

8.1 Maleic acid buffer

0.1 M maleic acid

0.15 M NaCl

Adjust to pH 7.5 at 20°C with solid NaOH

8.2 Blocking stock solution (10 x conc.)

10% (w/v) Blocking reagent

Maleic acid buffer

Dissolve blocking reagent by constantly stirring on a heating block at 65°C and store at 4°C. The solution remains opaque.

8.3 Blocking solution (1 x conc.)

1% (w/v) Blocking reagent

This reagent is dissolve in maleic acid buffer.

8.4 Washing buffer

Maleic acid buffer

0.3% (v/v) Tween® 20

8.5 Detection buffer

0.1 M Tris-HCl

0.1 M NaCl

50 mM $MgCl_2$

Adjust to pH 9.5 at 20°C.

8.6 Color-substrate solution

200 μl NBT/BCIP stock solution

10 ml Detection buffer

9. 20 mM MES-NaOH Buffer, pH 6.0, 250 ml

MES 1.015 g

Adjust pH with 0.1 N NaOH to 6.0 and adjust volume with distilled water up to 250 ml

10. 50 mM NaP_i Buffer, pH 7.0

Solution I: 1 M $Na_2HPO_4.2H_2O$, 1,000 ml

$Na_2HO_4.2H_2O$ 17.799 g

distilled water up to 100 ml

Dilute the combined 1 M stock solution to 1,000 ml with distilled water.

11. Solutions for Preparing SDS-PAGE

11.1 Solution A: Acrylamide stock solution, 100 ml 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide

acrylamide 29.2 g

bis-acrylamide 0.8 g

Add distilled water to make 100 ml and stir until completely dissolved. This can be stored for months in the refrigerator.

11.2 Solution B (4x Separating gel buffer), 100 ml

75 ml 2 M Tris-HCl, pH 8.8 1.5 M

4 ml 10% SDS 0.4%

21 ml H_2O

This solution is stable for months in the refrigerator.

11.3 Solution C (4x Stacking gel buffer), 100 ml

50 ml 1 M Tris-HCl, pH 6.8 0.5 M

4 ml 10% SDS 0.4%

46 ml H_2O

This solution is stable for months in a capped tube in the refrigerator.

11.4 10% Ammonium persulfate, 5 ml

0.5 g Ammonium persulfate

5 ml H_2O

11.5 Electrophoresis buffer, 1 liter

3 g Tris 25 mM

14.4 g glycine 192 mM

1 g SDS 0.1%

H_2O to make 1 liter

pH should be approximately 8.3. It can also be made as a 10x stock solution. Stable indefinitely at room temperature.

11.6 5x Sample buffer, 10 ml

0.6 ml 1 M Tris-HCl, pH 6.8	60 mM
5 ml 50% glycerol	25%
2 ml 10% SDS	2%
0.5 ml 2-mercaptoethanol	14.4 mM
1 ml 1% bromophenol blue	0.1%
0.9 ml H_2O	

Stable for weeks in the refrigerator or for months at –20°C.

12. Solutions for Preparing Lowry Method

12.1 Solution A (1% w/v $CuSO_4.5H_2O$), 50 ml

$CuSO_4.5H_2O$	0.5 g
distilled water	50 ml

12.2 Solution B (2% Sodium potassium tartrate), 50 ml

sodium potassium tartate	0.4 g
distilled water	50 ml

12.3 Solution C (0.2 M Sodium hydroxide), 50 ml

sodium hydroxide	0.4 g
distilled water	50 ml

12.4 Solution D (4% w/v Sodium carbonate), 50 ml

sodium carbonate	2 g
distilled water	50 ml

12.5 Solution E (Copper-alkali solution)

Solution A	1 ml
Solution B	1 ml

Solution C	49 ml
Solution D	49 ml

12.6 Solution F (Folin-ciocalteau reagent), 20 ml

Folin-ciocalteau reagent	10 ml
distilled water	10 ml

Solution A-D stable at room temperature. Solution E freshly prepares.

13. Renaturation Buffer

0.1 M Tris-HCl, pH 7.0

2% Triton X-100

14. Washing Buffer, pH 7.5

0.1 M Maleic acid

0.15 M NaCl

0.3% Tween® 20

Adjust pH with 0.1 N NaOH to pH 7.5

APPENDIX 2

1. Bacterial Growth Curve

1.1 The Calculation of Number of Bacterial Cells

The number of bacterial cells in the sample/ml could be calculated as follows:

$$\text{Number of bacteria} = \frac{\text{number of colonies}}{\text{dilution factor}} \quad \ldots\ldots\ldots\ldots \text{(I)}$$

1.2 The Calculation of Generation Time of Bacterial Cells

The generation time of bacterial culture can be calculated according to the following formula:

$$\text{Generation time (GT)} = \frac{t}{3.3 \log (b/B)} \quad \ldots\ldots\ldots\ldots \text{(II)}$$

where t = time interval between the number of cells at point in the log phase (B) and at a later point in time (b)

B = initial population

b = population after time t

log = $\log_{10}$

3.3 = $\log_{10}$ to $\log_2$ conversion factor

2. HPLC Analysis

2.1 The Concentration of MP and PNP

Methyl parathion (MP) and *p*-nitrophenol (PNP) were separated and detected with reverse-phase column as C-18 μBondapak column. Mobile phase was methanol-

water (70:30) at a flow rate was 1.0 ml/min. Standard solution of pure methanol, MP, and PNP were analyzed by HPLC (Figure 26).

The quantitative determination of each sample was carried out by using the external standard method by determining the peak area values. Calibration graph for MP and PNP standard solution were constructed by plotting concentration vs. peak area in Figure 27.

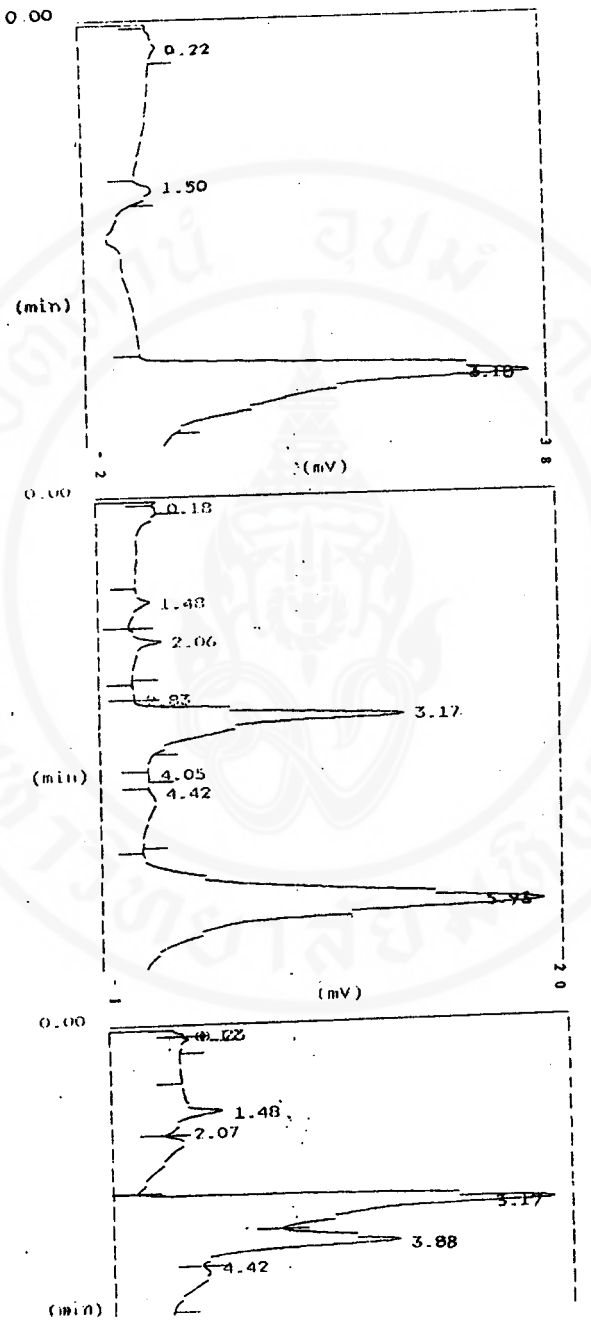


Figure 26. HPLC Chromatogram of pure methanol, MP, and PNP in standard solution, on a C-18 μBondapak column. Mobile phase, 70% methanol-30% water; flow rate, 1.0 ml/min; detection, UV at 220 nm.

Table 14. Standard Curve of MP.

Concentration (μg/ml)	Peak Area (uV.sec)	Retention time (min)
0	0	0
10	642,918	5.93
20	1,001,863	5.93
30	1,567,643	5.94
40	2,015,466	5.89
50	2,986,007	6.01

Table 15. Standard Curve of PNP.

Concentration (μg/ml)	Peak Area (uV.sec)	Retention time (min)
0	0	0
10	331,498	3.83
20	691,737	3.9
30	1,336,719	3.94
40	1,968,230	3.94
50	2,615,191	3.93

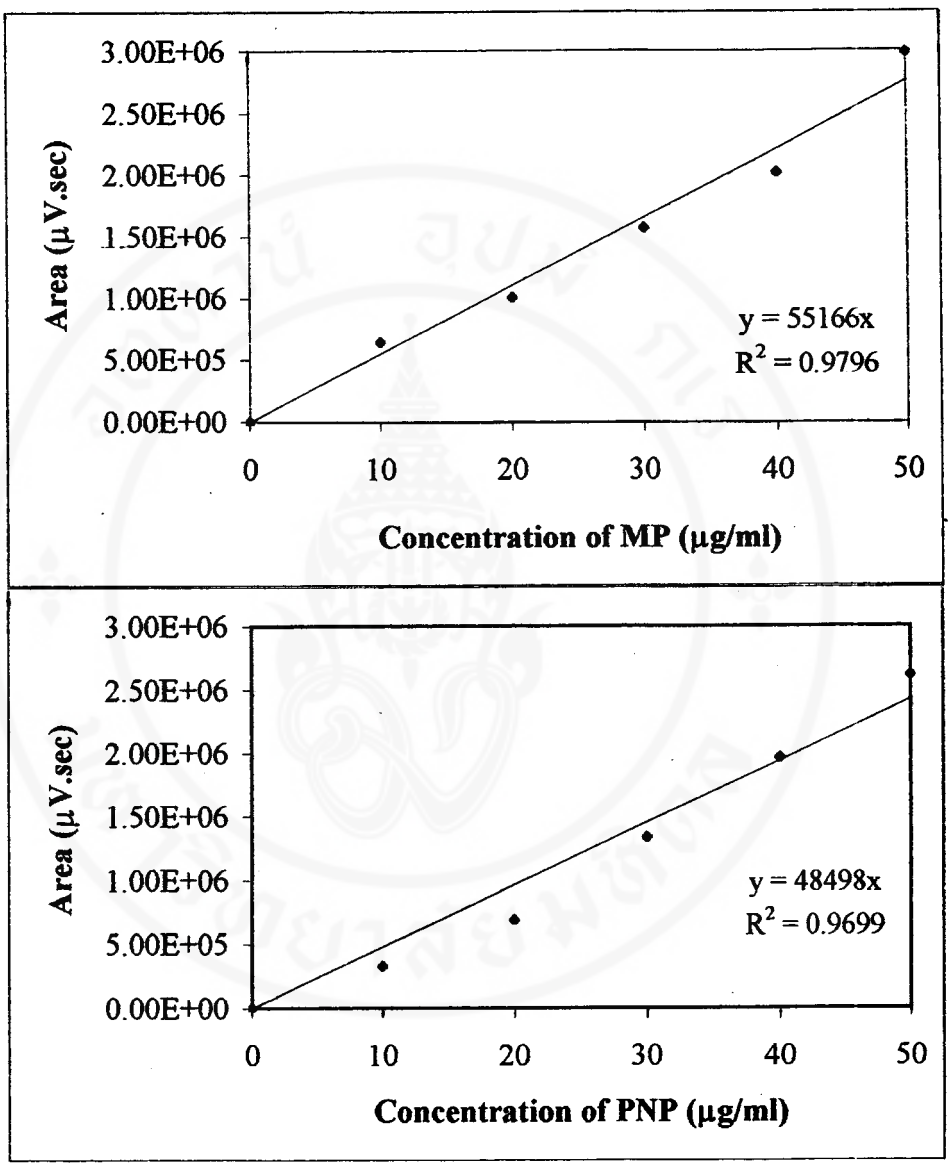
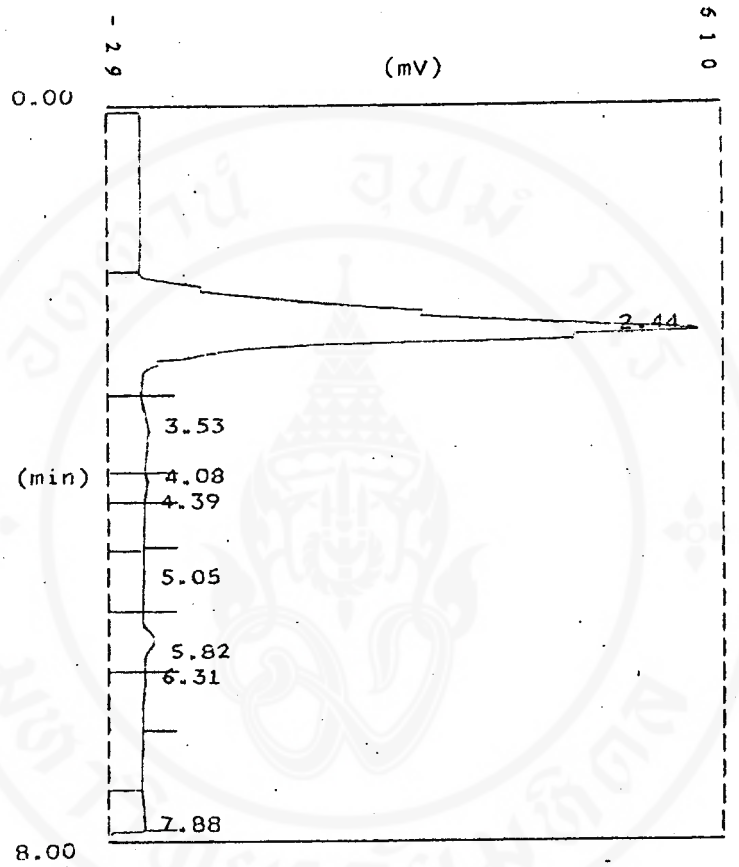


Figure 27. Calibration curve of MP and PNP. (A) Calibration curve of MP at concentration 0, 10, 20, 30, 40, and 50 μg/ml. (B) Calibration curve of PNP at concentration 0, 10, 20, 30, 40, and 50 μg /ml.

The chromatography was performed for each sample and compared with the retention time of standard solutions. The retention times of MP and PNP standard solutions were approximately 5.8 min and 3.9 min, respectively. An example of sample HPLC chromatogram at 6h of incubation is shown in Figure 28.



Quantitation Report for Chromatographic Analysis
Date : 2000-03-16 Acquisition Date : 1999-11-24
Time : 10:38:27 Acquisition Time : 15:52:26
File Name : pst206

No.	R.Time (min.)	Comp.	Height (uV)	Height%	Area (uV.sec)	Area%
1	2.44	Unknown	581817	96.11	12838187	97.38
2	3.53	Unknown	5987	0.99	135054	1.02
3	4.08	Unknown	3499	0.58	27225	0.21
4	4.39	Unknown	393	0.06	3756	0.03
5	5.05	Unknown	1027	0.17	16095	0.12
6	5.82	Unknown	10915	1.80	147828	1.12
7	6.31	Unknown	1140	0.19	7854	0.06
8	7.88	Unknown	578	0.10	7296	0.06
Total			605356	100.00	13183294	100.00

Figure 28. HPLC chromatogram of MP and PNP of MP-degrading *P. stutzeri*, which were extracted by methanol at 6h of incubation.

The concentration of MP and PNP of each sample were calculated according to equation III:

$$\text{Concentration of MP and PNP} = \frac{\text{peak area} \times \text{dilution factor}}{\text{slope}} \quad \ldots\ldots\ldots\ldots \text{(III)}$$

For example, the calculation of MP concentration of MP-degrading *P. stutzeri* at 6h of incubation is as follow:

$$\frac{147789.71 \times 10}{55166} = 26.79\ \mu g/ml$$

$\therefore$ The concentration of MP = 26.79

2.2 Percentage of MP-Residue and MP-Degradation Rate

The real concentration of residue (RC) was calculated from the detected concentrations of sample by using equation III. The concentration of MP-residue was transferred to the percentage of MP residues (% MP-Res) as equation V.

$$RC = \frac{A \times DC}{B} \quad \ldots\ldots\ldots\ldots \text{(IV)}$$

where A = the quantity of solvent used to extract the sample (0.8 ml)

RC = real concentration of MP residue

DC = detected concentration of MP sample

B = sample size (0.2 ml)

$$\%\ \text{MP-Res}_{(t)} = \frac{RC_{(t)} \times 100}{RC_{(0)}} \quad \ldots\ldots\ldots\ldots \text{(V)}$$

where $\%\ \text{MP-Res}_{(t)}$ = percentage of MP-residue at time t

$RC_{(t)}$ = real concentration of MP-residue at time t

$RC_{(0)}$ = real concentration of MP-residue at time 0

For example, the calculations of the real concentration of MP-residue (RC) and the percentage of MP-residue (% MP-Res) of MP-degrading *P. stutzeri* at 6h of incubation are as follows:

$$RC_{(6)} = \frac{0.8 \times 26.85 \pm 0.085}{0.2}$$

$$= 7.4 \pm 0.339$$

$$\%\ MP\text{-}Res_{(6)} = \frac{107.4 \pm 0.339 \times 100}{226.2 \pm 11.314}$$

$$= 47.53 \pm 2.227$$

The percentage of MP-degradation (% MP-Deg) were calculated by using the equation VI:

$$\%\ MP\text{-}Deg\ (t) = \%\ MP\text{-}Res\ of\ control\ (t) - \%\ MP\text{-}Res\ (t) \quad \ldots\ldots\ldots\ldots\ (VI)$$

where % MP-Deg (t) = percentage of MP-degradation at time t

APPENDIX 3

1. Calculation of Enzyme Assay

1.1 Quantitation of PNP by Spectrophotometric Assay

This technique was used for determining the enzyme activity by following the increase of PNP, which is the metabolite of MP degradation, by measuring A410 nm. PNP and protein standard curves in the study on localization of the MP hydrolase are shown in Figure 29.

The total protein concentration was calculated using the following equation.

Total Protein Concentration (mg/ml) VII

$$= \left[\frac{\text{A750 of sample}}{\text{Slope}} \times \frac{1000\ (\mu l)}{\text{Amount of sample}} \right] \times 1000 \times \text{Total volume of sample}$$

Total Activity (μmole) VIII

$$= \left[\frac{\text{A410 of sample}}{\text{Slope}} \times \frac{10^{-3} \times 1000\ (\mu l)}{\text{Amount of sample}} \right] \times \text{Total volume of sample}$$

Specific Activity (Unit/mg of protein) IX

$$= \frac{\text{Total Activity}}{\text{Total Protein Concentration}}$$

Purification (fold) X

$$= \frac{\text{Specific Activity}}{\text{Initial Specific Activity}}$$

% Yield (Recovery) XI

$$= \frac{\text{Total Activity}}{\text{Initial Total Activity}}$$

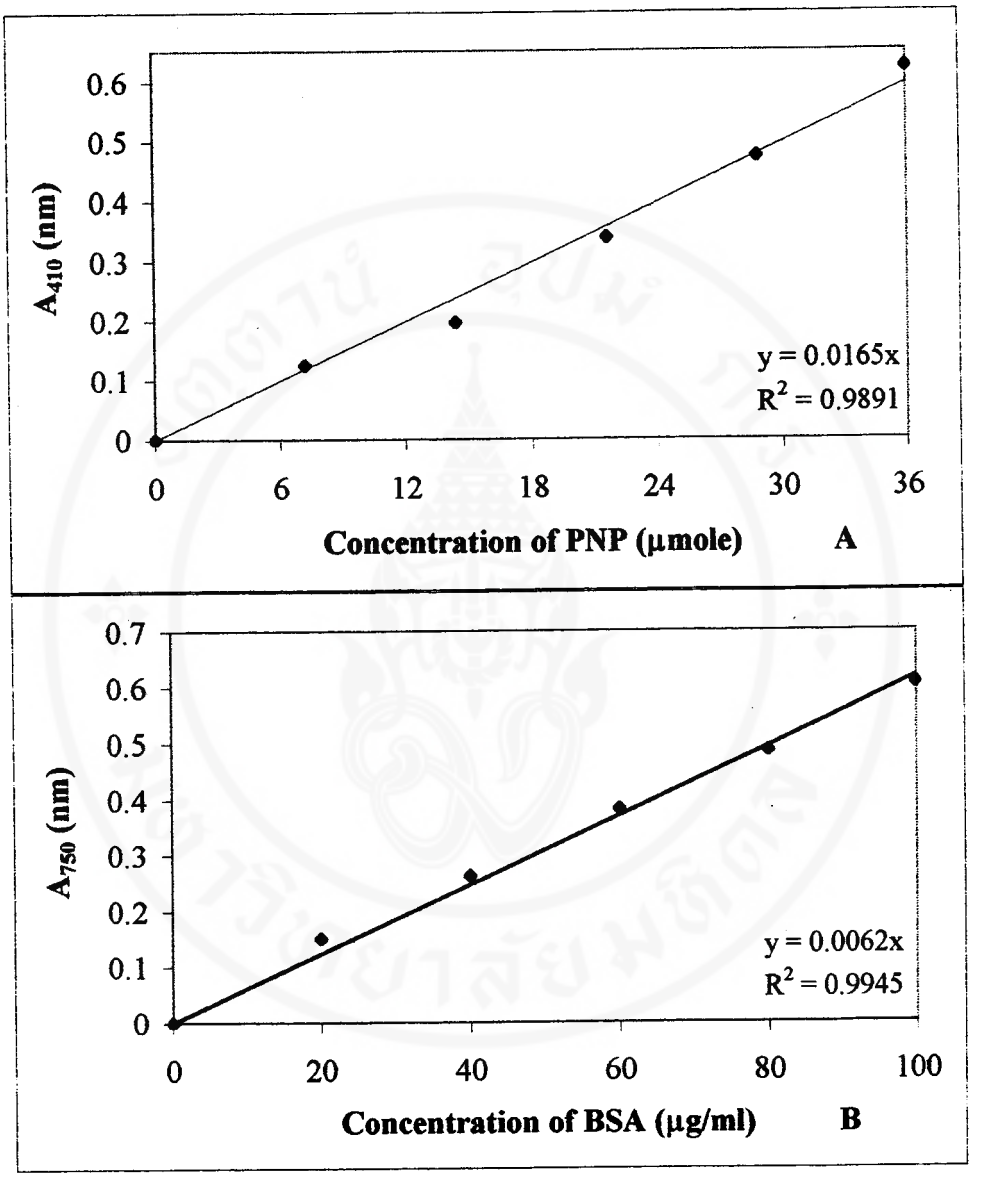


Figure 29. Standard curve of PNP and protein. (A) Standard curve of PNP (A_{410}). (B) Standard curve of Protein (A_{750}).

1.2 Purification of Protein

Proteins can generally be fractionated on the basis of size, hydrophobicity, and specificity. This study aims at obtaining MP hydrolyse enzyme at a sufficient purity for biochemical characterization, N-terminal amino acid sequence analysis and oligonucleotide probe designed for cloning. So the protein concentration, the enzyme activity, the enzyme purity, and the molecular weight of enzyme must be known. The protein concentration was measured by Lowry method and the appearance of PNP was estimated by determining the optical density at 410 nm. The enzyme activity was determined by following the increase of PNP via A_{410}. In addition, the purity and the molecular weight of the enzyme were demonstrated by SDS-PAGE, comparing with their standards.

PNP standard curve of gel filtration chromatography and hydrophobic interaction chromatography are shown in Figure 30. PNP standard curve and protein standard curves of purification steps are also shown in Figure 31. PNP and protein standard curves in the study on effect of reagents on the activity are shown in Figure 32.

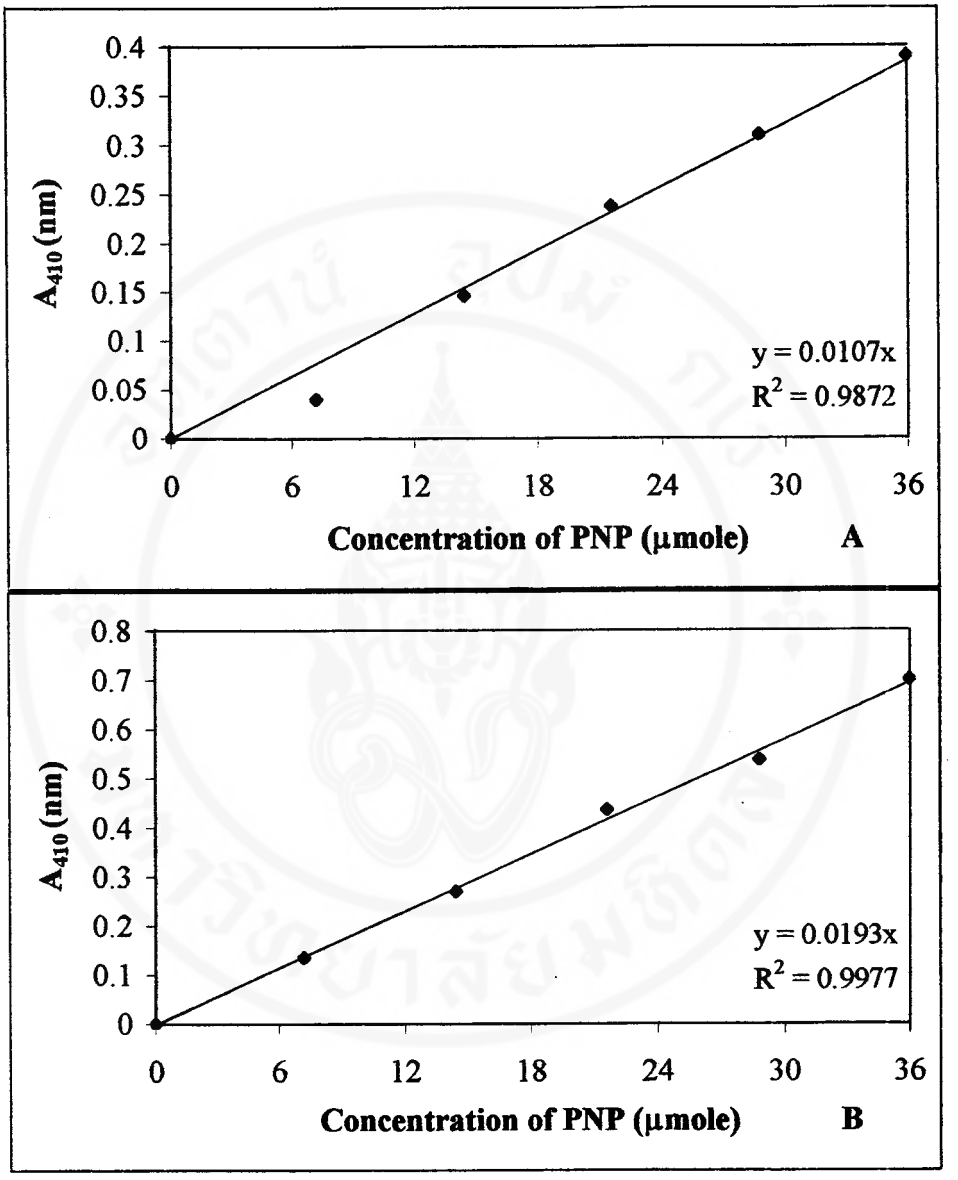


Figure 30. The PNP standard curve of the samples eluted from gel filtration chromatography and hydrophobic interaction chromatography at various PNP concentrations (7.2, 14.4, 21.6, 28.8, and 36 μmole). (A) The PNP standard curve of gel filtration chromatography (Sephadex G-100 column). Reaction mixture is shown in Appendix 1. (B) The PNP standard curve of hydrophobic interaction chromatography (Octyl Sepharase 4FF column).

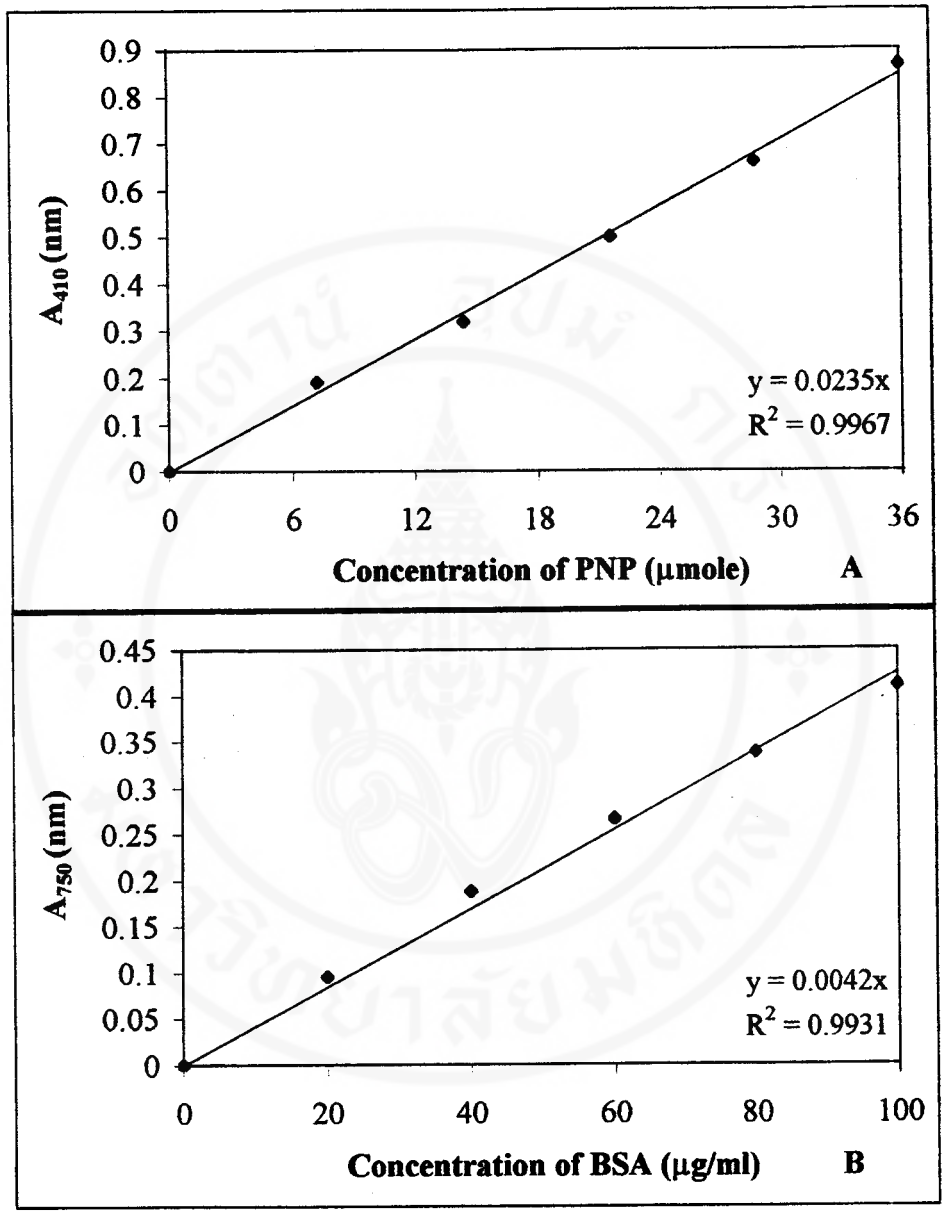


Figure 31. PNP and protein standard curves of protein purification steps. (A) PNP standard curve of protein purification step. (B) Protein standard curve of protein purification step.

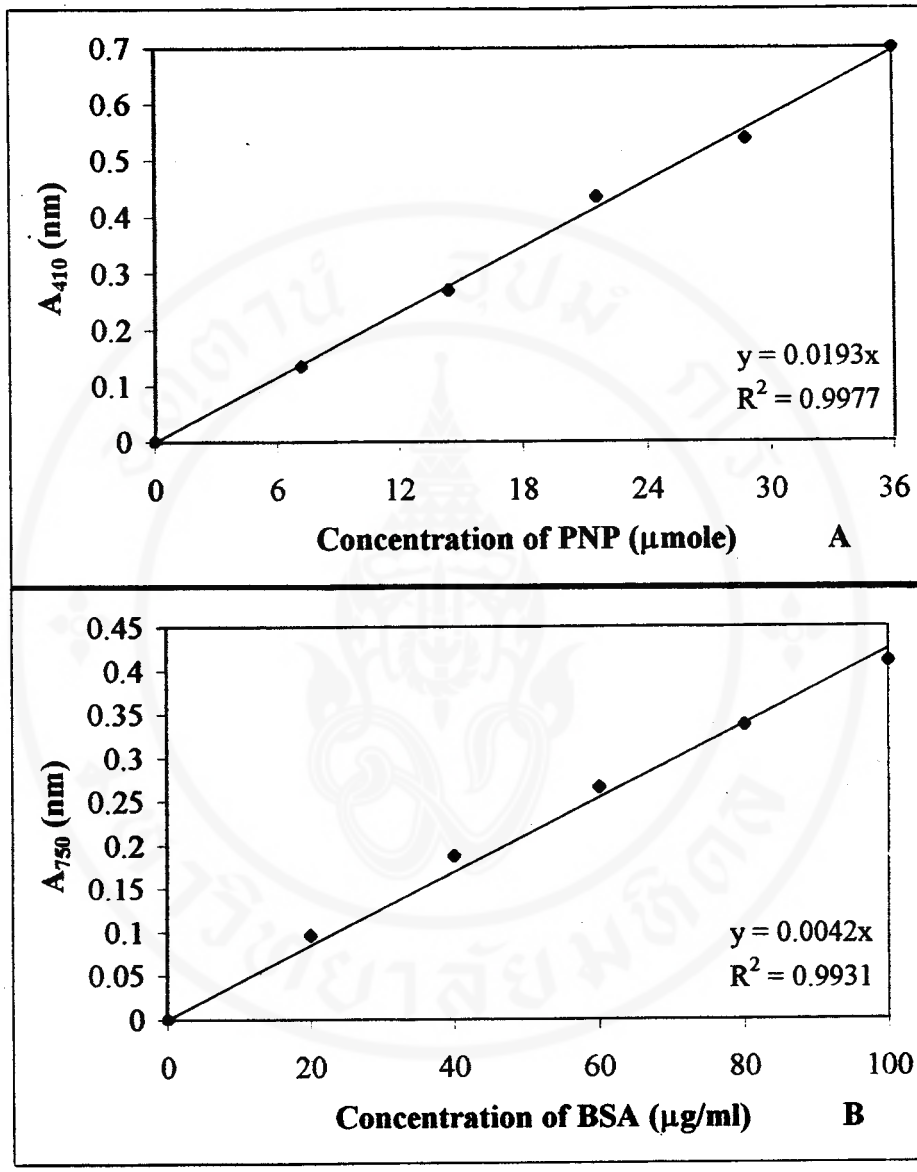


Figure 32. PNP standard curve and protein standard curve in the study on the localization of the enzyme.

The purity and the molecular weight of the enzyme were demonstrated by SDS-polyacrylamide gel electrophoresis. SDS-PAGE is used to determine the molecular weight of a protein since protein migration distance in the gel is proportional to the mass of the protein. A standard curve generated with protein of interest can be extrapolated from this curve. Proteins of known molecular weights (protein marker) are composed of Myosin, Mr 215 kDA; β-galactosidase, 122 kDa; BSA, 79 kDa; Ovalbumin, Mr 49.7 kDa; Carbonic anhydrase, 34.6 kDa; Soybean trypsin inhibitor, Mr 28.7 kDa; Lysozyme, 20.6 kDa; and Aprotinin, Mr 7.8 kDa (Figure 33). After SDS-polyacrylamide gel was stained, it was measured for the distance of migration of the protein as well as that of the tracking dye (bromophenol blue). Distance of migration is measured from the beginning of the separating gel to the leading edge of the protein band.

R_f values were calculated by the following equation:

$$R_f \text{ (cm)} = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration*}} \quad \text{..... XII}$$

*Distance of tracking dye migration was measured to be 5.7 cm.

Then the plot of $\log_{10}$ of the known protein molecular weights as a function of their R_f was constructed. Finally, the molecular weight of the unknown protein is read from the graph based on its R_f value.

Distance of unknown protein (sample; purified sample) was 2.1 cm, So R_f value can calculated by using the equation:

$$R_f = \frac{2.1}{5.7}$$

$$= 0.368 \text{ cm}$$

Molecular weight of the purified enzyme read from the graph was approximately 40 kDa.

Table 16. R_f values of standard protein marker.

Series of band	Standard protein	Molecular weight (kDa)	Distance (cm)	R_f (cm)
1	Myosin	215	0.2	0.035
2	β-galactosidase	122	0.5	0.088
3	BSA	79	0.9	0.158
4	Ovalbumin	49.7	1.6	0.281
5	Carbonic anhydrase	34.6	2.6	0.456
6	Trypsin inhibitor (soybean)	28.7	3.3	0.579
7	Lysozyme	20.6	4.4	0.772
8	Aprotinin	7.8	5.1	0.895

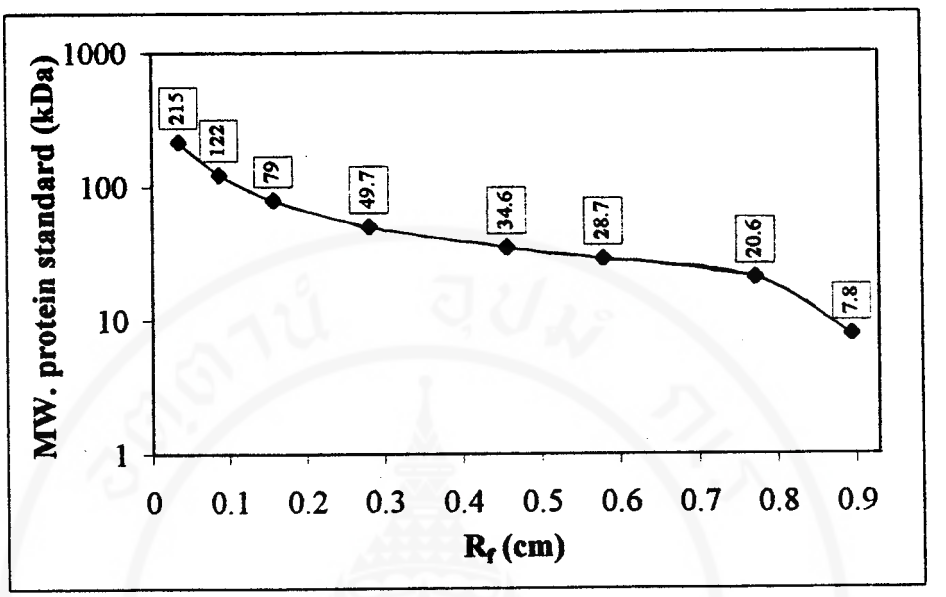


Figure 33. Graph of protein molecular weight versus their relative mobility.

BIOGRAPHY

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RESEARCH GRANT	-