Characterization of Enzyme and Encoding Gene of Thermostable Peroxidase from *Bacillus* sp. PHS 155

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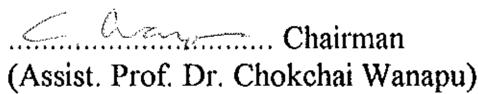
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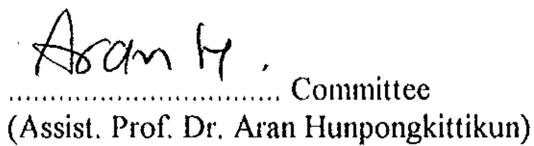
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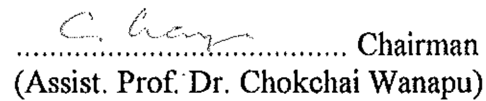
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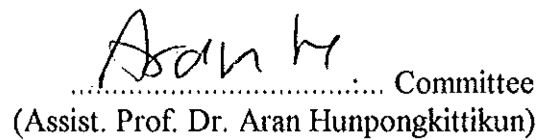
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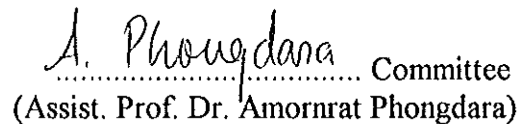
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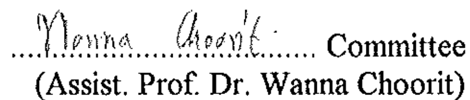
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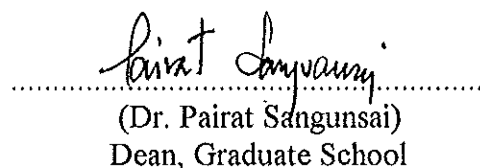
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ชื่อวิทยานิพนธ์	คุณสมบัติของเอนไซม์และยีนเปอร์ออกซิเดสทนร้อนจากเชื้อ *Bacillus* sp. PHS 155
ผู้เขียน	นางสาว ศศิธร ทองมา
สาขาวิชา	เทคโนโลยีชีวภาพ
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บทคัดย่อ

เอนไซม์เปอร์ออกซิเดส (EC 1.11.1.7) มีบทบาทสำคัญในงานตรวจวิเคราะห์ต่างๆ โดยใช้เทคนิค ELISA แต่จุลินทรีย์ส่วนใหญ่ผลิตเอนไซม์เปอร์ออกซิเดสโดยมีแอคติวิตี้ของเอนไซม์คาทาเลสรบกวนอยู่ อย่างไรก็ตามพบว่าเชื้อ *Bacillus* sp. PHS 155 ซึ่งแยกได้จากบริเวณบ่อน้ำร้อนในภาคใต้ สามารถผลิตเอนไซม์เปอร์ออกซิเดสที่ปราศจากการรบกวนของคาทาเลส และทนอุณหภูมิสูงได้ดี เอนไซม์นี้ถูกทำให้บริสุทธิ์โดยใช้เทคนิคโครมาโตกราฟฟีแบบแลกเปลี่ยนอิออนชนิด DEAE-Sephacel และเจลฟิวเตรชั่นชนิด Sephadex G-200, G 50-150 และ Sephacryl S-300 เอนไซม์บริสุทธิ์ที่แยกได้มีน้ำหนักโมเลกุล 67 กิโลดาลตัน และ ทำงานได้ดีในช่วง พีเอช 5-7 อุณหภูมิ 60°ซ โดยมีค่า K_m และ V_{max} สำหรับ H_2O_2 เป็น 50 mM และ 0.5 U/ml ตามลำดับ ส่วน K_m และ V_{max} สำหรับ *o*-dianisidine เป็น 50 mM และ 1.11 U/ml ตามลำดับ โดยที่อุณหภูมิ 60°ซ และ 70°ซ เอนไซม์จะมีแอคติวิตี้ลดลงครึ่งหนึ่งภายในเวลา 2 ชั่วโมงครึ่ง และ 30 นาที ตามลำดับ สับสเตรทจำเพาะของเอนไซม์ได้แก่ *o*-dianisidine และ *o*-aminophenol นอกจากนี้เอนไซม์ยังถูกกระตุ้นได้ด้วย Fe^{2+} แต่ถูกยับยั้งได้ด้วย Zn^{2+} เมื่อศึกษาลักษณะของยีนเปอร์ออกซิเดสพบว่ามีขนาด 5.5 กิโลเบสที่ถูกโคลนลงไปในพลาสมิด pBluescript SK+ และ *E. coli* ลูกผสมสามารถผลิตเอนไซม์ที่ 37°ซ สูงกว่า *E. coli* ที่ไม่มียีนประมาณ 1.79 เท่า แต่ผลิตเอนไซม์ได้น้อยกว่า *Bacillus* sp. PHS 155

Thesis Title Characterization of Enzyme and Encoding Gene of Thermostable Peroxidase from *Bacillus* sp. PHS 155

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Abstract

Peroxidase (EC 1.11.1.7) is an enzyme which plays an important role in medical analysis such as ELISA technique. Most of the microorganisms produce peroxidase which have also catalase activity. However, *Bacillus* sp. PHS 155 was isolated from hot pond in the south of Thailand. This strain produced peroxidase without catalase activity and was tolerated high temperature. The crude enzyme from this strain was purified for peroxidase using ion exchange chromatography on DEAE-Sephacel, gel filtration on Sephadex G-200, G 50-150 and Sephacryl S-300. It was shown that the partially purified enzyme had a molecular weight of 67 kDa. Its optimum pH was 5-7 at 60°C. For enzyme kinetic, the value of K_m and V_{max} for H_2O_2 was 50 mM and 0.5 U/ml, respectively while the K_m and V_{max} for *o*-dianisidine was 50 mM and 1.11 U/ml, respectively. A half life of this purified enzyme at 60°C and 70°C was 2 hrs 30 minute and 30 minute, respectively. The specific substrates for this enzyme were *o*-dianisidine and *o*-aminophenol. The activity of enzyme was activated by Fe^{2+}, but was inhibited by Zn^{2+}. The encoding gene considered to have a size of 5.5 kb has been transformed into *E. coli* using the pBluescript SK+ plasmid as vector. Although the recombinant *E. coli*

produced 1.79 times higher peroxidase activity than its wild type, the activity was still lower than in *Bacillus* sp. PHS 155.

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List of Abbreviations

μM = Micromolar

BSA = Bovine serum albumin

EDTA = Ethylenediaminetetraacetic acid

kb = Kilo base pairs

kDa = Kilodaltons

K_m = Michaelis-Menten constant

l = Litre

mg = Milligram

ml = Millilitre

mM = Millimolar

MW = Molecular weight

OD = Optical density

°C = Degree celsius

PAGE = Polyacrylamide gel electrophoresis

rpm = Revolutions per minute

SDS = Sodiumdodecyl sulfate

TEMED = N,N,N',N'-tetramethyl ethylenediamine

V_{max} = Maximum velocity

w/v = Weight by volume

v/v = Volume by volume

HRP = Horseradish peroxidase

Chapter 1

Introduction

The deliberate use of enzymes by man is central to the application of biotechnology since enzymes are involved in all aspects of biochemical conversion from the simple enzyme or fermentation conversion to the complex techniques of genetic engineering. Therefore, a thorough understanding of the structure, source and mode of action of an enzyme is essential for the development of any biotechnological process. This thesis concentrates on peroxidase, because it has been widely used as an important component for clinical diagnosis and various laboratory experiments. This enzyme can be applied with a wide range of chemical substrates. In addition, several novel applications for peroxidase have been suggested including the treatment of wastewater containing phenolic compounds, the synthesis of various aromatic chemical and the removal of peroxidase from materials such as foodstuffs and industrial wastes (Kim and Yoo, 1996).

Currently a major limitation for the widespread use of peroxidase is the high cost of production. A reduction in enzyme cost is dependent on the development of an efficient method of enzyme production. Peroxidases are widely distributed throughout plants, animals and microorganisms.

Nowadays, the commercial source of peroxidases in enzyme immuno assays and diagnostic assays is plant peroxidase, Horseradish (*Armoracia rusticana*). However, the horseradish peroxidase group consists of more than 40 components (Intapruk, *et al.*, 1994). Although peroxidases are

highly catalytic enzymes, they have very low specificity and exist in a multitude of isoenzyme forms, making it difficult to ascertain their actual functions (Kawaoka, *et al.*, 1994). In animal peroxidase, an expensive technique must be used to extract the enzyme, which tends to be limiting its supply and hence it is difficult to match the supply to the demand.

It was early recognized that microbial cells have a great potential as sources of enzymes. Peroxidases from microorganisms would be less expensive than plant or animal peroxidases and could be made available in unlimited quantities. However, peroxidase from microorganisms usually act as hydroperoxidase or catalase-peroxidase enzyme, that can catalyze both catalase and peroxidase reactions at significant rates. For this reason, the microbial peroxidase has attracted comparatively little attention for research. In contrast, thermostable peroxidase are of interest because of their potential industrial applications (Loprasert, *et al*, 1988).

Thermostable hydroperoxidase (catalase I) from a thermophilic bacteria, *Bacillus stearothermophilus* IAM 1101, has been purified to homogeneity as a peroxidase by Loprasert and colleagues (1988). The apparent optimum temperature of the enzyme is 70°C. The gene encoding of this enzyme (the *perA* gene) was cloned into *Escherichia coli* (*E. coli)* UM228 (a catalase HPI-deficient mutant) and sequenced. Because of its remarkable heat stability, it is potentially useful for industrial application. However, it also showed catalase activity.

In addition, the molecular Biology and Biotechnology Laboratory of the Department of Biochemistry, Faculty of Science, Prince of Songkla University could isolate *Bacillus* sp. PHS 155 in the south of Thailand. The strain was a thermophilic bacterium which had only peroxidase

activity. This unique property will provide us with important information about thermal resistance in peroxidase and could be an alternative source for peroxidase preparation. In a recent study, Na Pathalung (1996) has constructed the *Bam*HI fragment (9 kb), encoding the peroxidase gene from a genomic library of *Bacillus* sp. PHS 155 and inserted into a Lambda GEM^{-11} vector. It was transfected into *E. coli* LE 392. The positive clone, which was screened using the peroxidase gene from pOD68 (obtained from Dr. Savitr Trakunaleamsai, Osaka University, Japan) (Trakunaleamsaias, *et al.*, 1990) as a probe has been isolated. The aim of this work was to purify and study various characteristics of the thermostable peroxidase from *Bacillus* sp. PHS 155, the size of the peroxidase gene, including the expression in *E. coil* UM228.

Literature Review

1. Structure of peroxidase

Peroxidase (EC 1.11.1.7) is a member of the oxidoreductases. The feature of a common active site or prosthetic group of enzyme composes of an iron-porphyrin complex (Loew, 1983).

The biosynthesis of porphyrin is the condensation of glycine and succinyl CoA to form δ-aminolevulinate, which is catalyzed by δ-aminolevulinatase. Two molecules of δ-aminolevulinate then condense to form porphobilinogen. This dehydration reaction is catalyzed by δ-aminolevulinate dehydrase. Four porphybilinogens condense head-to-tail to form a linear tetrapyrrole, which remains bound to the enzyme (Fig. 1). An ammonium ion (NH_4^+) is released for each ethylene bridge formed. This linear tetrapyrrole cyclizes by losing NH_4^+. The cyclic product is uroporphyrinogen III, which has an asymmetric arrangement of side chains. These reactions require a synthetase and a cosynthetase. In the presence of synthetase alone, uroporphyrinogen I, the symmetric isomer, is produced. The cosynthetase is essential for isomerizing one of the pyrrole rings to yield asymmetric uroporphyrinogen III.

With the porphyrin skeleton formed, subsequent reactions alter the side chains and the degree of saturation of the porphyrin ring. Coproporphyrinogen III is formed by decarboxylation of the acetate side chains. Unsaturation of the porphyrin ring and conversion of two of the propionate side chains into vinyl groups yield protoporphyrin IX. Chelation of iron finally gives heme (Stryer, 1981).

Fig. 1 Pathway for the synthesis of heme from porphobilinogen.

(Abbreviations : A, acetate; M, methyl; P, propionate; V, vinyl.)

source : Stryer (1981)

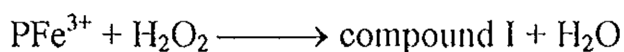
2. The reaction of peroxidase

The term peroxidase in its widest sense includes a group of specific enzymes such as NAD peroxidase, NADP peroxidase, fatty acid peroxidase, cytochrome peroxidase and glutathione peroxidase as well as a group of very non-specific enzymes from different sources, which are simply known as peroxidase. Peroxidase catalyses the dehydrogenation of a large number of organic compounds such as phenols and aromatic amines, especially benzidine derivatives: *o*-cresol, *o*-toluidine, guaiacol, pyrogallol, homovanillnic acid, *p*-hydroquione, *o*- or *p*-phenylenediamine, leucomalachite green, reduced 2,6-dichlorophenolidophenol, 4,4'-diaminodiphenylamine, benzidine, *o*-toluidine, *o*-dianisidine and some azo dyes derived from these. The typical peroxidase belonging to group 1.11.1.7 are haemoproteins. Although the real peroxidase reaction consists of the transfer of hydrogen from a donor to H_2O_2, there are examples of peroxidase acting like oxidase ($SH_2 + O_2 \rightarrow S + H_2O_2$) and mono-oxygenases (e.g. $S\text{-}H + O_2 + NADPH \rightarrow S\text{-}OH + NADP^+ + OH^-$). On the other hand, apart from the true peroxidase, peroxidative reactions can be catalysed by catalase with certain substrates, e.g. phenols, alcohols and several inorganic compounds, as well as by haemoglobin and some of its break-down products with typical peroxidase substrates. Finally, oxidases can in many cases react like peroxidase and use H_2O_2 as a source of oxygen. Although in the classical peroxidase reactions the specificity for the hydrogen donor is very low, that for the peroxide is much higher. It appears that apart from H_2O_2, only compounds having the group -O-OH, e.g. acetyl-, methyl-, and ethyl-hydroperoxide can act as substrates.

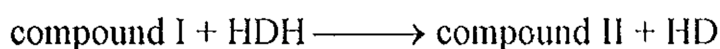
Peroxidase can be determined by the decrease of H_2O_2 or the hydrogen donor and the formation of the oxidized compound. Usually the second method is employed, and many different substrates have been used (Putter, 1974).

Peroxidase catalyzes the oxidation of a variety of organic substrates. The first step involves the two-electron oxidation of the ferric heme prosthetic group of the enzyme by H_2O_2 or an organic hydroperoxide. The interaction of the resting enzyme ferriperoxidase (PFe^{3+}) with H_2O_2 results in the formation of an unstable intermediate, two oxidizing equivalents above the ferric state, consisting of ferryl iron and a porphyrin cation radical. This intermediate known as compound I, reacts with an electron donor (HDH), with the loss of one oxidizing equivalent, to form compound II. The compound II removes an additional electron to return to the resting form.

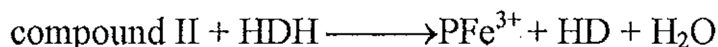
Schematically:



$PFe^{3+} + H_2O_2 \longrightarrow$ compound I + H_2O



compound I + HDH $\longrightarrow$ compound II + HD



compound II + HDH $\longrightarrow PFe^{3+} + HD + H_2O$

The cycle involving PFe^{3+} and compound I and II is common to most peroxidase reactions, electron donor substrates being oxidized to free radicals, whose individual chemical characteristics and reactivity determine the specific compounds formed in each case (Campa, 1991).

However, catalase is usually defined as the enzyme catalyzing the dismutation of H_2O_2 to O_2 and H_2O whereas peroxidase use H_2O_2 to oxidize a variety of compounds. The study of the peroxidase reaction was frustrated by the ability of the enzyme to act as a catalase and decompose

H_2O_2. Therefore, the K_m value would be lower than estimated as the actual concentration of H_2O_2 was reduced by decomposition (Loprasert, *et al.*, 1988).

3. Sources of peroxidase

The peroxidase has been extensively studied in animals, plants, and microorganisms.

3.1 Plants

In plants, peroxidases have been implicated in a variety of secondary metabolic reactions including polysaccharide cross-linking (Fry, 1986), indole-3-acetic acid oxidation (Hinnman and Lang, 1965), lignification (Grisebach, 1981), wound-healing (Espelie, *et al.*, 1986), pathogen defense (Hammerschmidt, *et al.*, 1982) and the regulation of cell elongation (Goldberg, *et al.*, 1986). Morever, peroxidase activity has been investigated in relation to several development processes: fruit ripening (Rothan and Nicolas, 1989), seed germination (Lewak, 1986), senescence (Abeles, *et al.*, 1988), pollination-induced aging (Bredemeijer and Blaas, 1983), sex expression (Ghosh and Basu, 1984), organogenesis of vegetation and floral buds (Kay and Basile, 1987). Plant peroxidases are highly catalytic enzymes with broad substrate specificity and in higher plants, they are expressed as multiple isozyme (Gaspar, *et al.*, 1982). However, due to the many different isoenzymes whose pattern of expression is tissue specific and developmentally regulated by environmental stimuli, it has proven difficult to verify actual physiological roles for peroxidase.

Peroxidase is of interest to biology researches and has been widely used as an important component for clinical diagnosis, for laboratory experiments, and for industrial uses. Nevertheless, the only source of commercial peroxidase available is obtained from horseradish (*Armoracia rusticana*) roots cultivated and harvested in various countries having relatively cool climates (Lin, *et al.*, 1996). Horseradish peroxidase (HRP) is a monomeric glycoprotein of a molecular weight of 40 kDa and contains ferriprotoporphyrin IX as its heme prosthetic group (Shannon, *et al.*, 1966). Hoyle (1977) found 42 isozymes in commercial preparations of HRP, usually classified as acidic, neutral and basic isoenzymes by their isoelectric points or elution profiles during ion-exchange column chromatography. However, the distribution and function of each isoenzyme is still unclear. Yeung and Cavry (1990) detected the peroxidase from bean (*Phaseolus vulgaris*) during seed development, and Stich and Ebermann (1988) found peroxidase isoenzymes in bark, sap wood and hard wood of some trees. In addition, the peroxidase isozyme patterns of tobacco in the cell suspension culture, peroxidases found in the medium were different from those of inside the cell (Mader 1992). Until a general investigation of the distribution of peroxidase activity in 60 species of high plants (covering 31 families) was carried out, and the novel source for peroxidase preparation was selected instead of horseradish for industrial applications, Lin and colleagues (1996) showed that the purified *Ipomoea cairica* peroxidase assessed in assays for glucose in comparison with a commercially available horseradish peroxidase for glucose determination could be an alternative commercial source of high activity peroxidase enzyme.

3.2 Animal

The presence of a peroxidase in high concentration in the cytoplasmic granules of polymorphonuclear leukocytes has been known since the application of cytochemical stains for peroxidase to blood smears early in the century (Klebanoff, 1991). Mammalian blood cells contained specific peroxidases such as myeloperoxidase, eosinophil peroxidase, and platelet peroxidase. They were heme-containing glycoproteins and were present in lysozomes of neutrophilic granulocytes and eosinophilic granulocytes, respectively. In the presence of peroxidase and halide ion, human myeloperoxidase and eosinophil peroxidase work as potent anti-microbial agents and play important roles in human defense against microorganisms in neutrophils and eosinophils (Sakamaki, *et al.*, 1989).

A peroxidase activity was also found in exocrine secretions including milk, tears, saliva, and perhaps in other secreted fluids. The enzyme responsible for this activity was synthesized in the glands that produce the secretions (Thomas, *et al.*, 1991). For example thyroid peroxidase was involved in the biosynthesis of thyroid hormones, and was shown to be a major component of the thyroid microsomal antigen involved in autoimmune thyroid diseases (Kimura, *et al.*, 1989). All contain a heme group located at the active site except the glutathione peroxidase, which have a selenocysteine residue at the active site (Tyson, 1992). Glutathione peroxidase was shown to be chromatographically different from that of catalase. Because its enzymatic activity was not inhibited by either azide or cyanide, nor was a soret band found, it was concluded that glutathione peroxidase was not a heme containing

peroxidase. It was further determined that glutathione was the preferred thiol of glutathione peroxidase in the reduction of hydrogen peroxidase. The cytosolic selenoenzyme glutathione peroxidase was first identified in rat erythrocytes and has since been widely found in many mammalian and nonmammalian species (Spallholz and Boylan, 1991). The most important problems in animal enzyme production are related to the quantity, stability and purification costs.

3.3 Microorganisms

Many microorganisms have been reported to produce peroxidase enzyme as shown in Table 1. Several peroxidases of microbial origin which act as both peroxidases and catalase have been described. These enzymes share biochemical and physicochemical properties with both catalase and peroxidase.

Microorganisms have become increasingly important as producers of industrial enzymes and which in fact represent most enzymes used in industry today. Furthermore, advantages of using microorganisms for enzyme production are short fermentation times, inexpensive media, ease of developing simple screening procedures and the existence of distinct proteins from different strains which catalyze the same reaction. The last point allows flexibility in choice of fermentation conditions since these different enzymes may have different stabilities and different pH and temperature optima. In addition, microorganisms may be manipulated by genetic engineering. Strains constructed in this manner will be able to produce abnormal amounts of enzymes inherent in this organism as well as to synthesize foreign proteins derived from animal cells (Borriss, 1987).

Table 1 The strains of microorganisms producing the peroxidase enzyme.

microorganisms	references
Pseudomonas fluorescens	Lenhoff and Kaplan, 1956
Streptococcus faecalis	Dolin, 1957
Escherichia coli	Claiborne and Fridovich, 1979
Halobacterium halobium	Fukumori, *et al.*, 1985
Rhodopseudomanas capsulata	Hochman and Shemesh, 1987
Pellicularia filamentosa	Ichikawa, *et al.*, 1981
Saccharomyces cerevisiae	Finzel, *et al.*, 1984
Bacillus stearothermophilus	Loprasert, *et al.*, 1990
Inonotus weirii	Mustranta, 1987
Rhodobacter capsulatus	Hochmam, *et al.*, 1992
Klebsiella vinosum	Hochman and Goldberg, 1991
Chromatium vinosum	Nadler, *et al.*, 1986
Salmonella typhimurium	Loewen and Stauffer, 1990
Vitreoscilla sp.	Abrams and Webster, 1990
Alkalophilic bacillus sp.	Yumoto, *et al.*, 1990
Streptomyces cyaneus	Mliki and Zimmermann, 1992

4. Application of peroxidase

4.1 Enzymatic removal of toxic organic compounds from wastewater

Phenols and aromatic amines are present in the wastewater of a number of industries, including coal conversion, oil and the production of plastics and resins. In the future, this problem is likely to become even more severe, because the coal conversion industry is rapidly growing which uses more than 50% of the world's resources of coal. Phenol, cresols and xylenols are the major organic contaminants of aqueous effluents produced by the coal conversion industry.

Virtually all phenols and aromatic amines are particular toxic to marine life. Phenol is toxic to fish in concentrations as low as 5 ppm. Moreover, many phenols and aromatic amines are mutagenic and carcinogenic. Numbers of methods are used today to remove phenols and aromatic amines from industrial wastewater, including extraction with organic solvents, microbial degradation, absorption on activated carbon, incineration, irradiation with ultraviolet light, etc. These methods are useful, and practical in certain areas. However, they suffer from very serious drawbacks such as high cost, complete purification, formation of hazardous by products, etc.

Horseradish peroxidase is remarkable enzyme. In 1980, Klibanov and colleagues that reported peroxidase oxidizes different phenols and aromatic amines with hydrogen peroxide to give certain coupled products. Peroxidase-catalyzed oxidation of a phenol or an aromatic amine molecule produced two free radicals. These free radicals diffused from the active center of the enzyme to the aqueous solution, where they could react

either with each other or with unoxidized molecules of phenols or aromatic amines. This chain reaction could go on for a long period of time, until the two free radicals recombine. The result of this reaction was a number of coupled aromatic products. The major characteristics of these products were low solubility in water and a high molecular weight. This was extremely important for removal of toxic organic compounds. Enzyme peroxidase could be used to convert water-soluble phenols and aromatic amines for the removal of water-insoluble polyaromatic products. It was relatively easy to remove these water-insoluble compounds because they precipitate and could be filtered or allowed to settle out. Alberti and Klibanov (1981) showed the removal efficiencies for some of the phenols and aromatic amines from water (Table 2).

Tatsumi and colleagues (1996) reported the removal of chlorophenols from wastewater by immobilized horseradish peroxidase. In addition, it was discovered that horseradish peroxidase was selectively adsorbed on magnetite by the crosslinking method.

4.2 The degradation of lignin

Lignin is the most abundant renewable aromatic material on earth. It is found in higher plants, including ferns, liverworts, mosses or plants of lower taxonomic wood and other vascular tissues, which contain approximately 20-30% lignin. Most lignin is found within the cell walls, where it is interspersed with the hemicellulose, forming a matrix that surrounds the orderly cellulose microfibrils. In wood, lignin in high concentration acts like glue that binds cells, forming the middle lamella (Kirk and Farrell, 1987). Degradated products and chlorinated lignin must be recovered from pulp and paper-mill effluents in order to reduce both

Table 2 Removal of aromatic amines and phenols from water by horseradish peroxides and H_2O_2.

Pollutant	Removal Efficiency
Benzidine	99.94
3,3-Dimethoxybenzidine	99.9
3,3-Diaminobenzidine	99.6
3,3-Dichlorobenzidine	99.9
3,3-dimethylbenzidine	99.6
1-Naphthylamine	99.7
2-Naphthylamine	98.9
5-Nitro-1-naphthylamine	99.6
N, N-Dimethylnapthylamine	93.2
Phenol	85.3
2-Methoxyphenol	98.0
3-Methoxyphenol	98.6
4-Methoxyphenol	89.1
2-Methylphenol	86.2
3-Methylphenol	95.3
4-Methylphenol	85.0
2-Chlorophenol	99.9
3--Chlorophenol	66.9
4-Chlorophenol	98.7
2,3-Dimethylphenol	99.7
2,3-Dimethylphenol	82.3
Aniline	72.9

Table 2 (continue)

Pollutant	Removal Efficiency
	62.5
4-Chloroaniline	84.5
4-Bromoaniline	86.4
4-Fluoroaniline	98.6
1,3-Diaminophenol	80.5
Diphenylamine	99.6
1-Naphthol	98.6
2-Nitroso-1-naphthol	99.9
4-Phenylphenol	99.8
8-Hydroxyquinoline	

source : Alberti and Klibanov (1981)

color and toxicity. This is presently done using a complex treatment, ultrafiltration and activated carbon adsorption. The successful biological treatment of these wastewaters would solve waste-disposal problems and allow the recovery of process waters, which could then be recycled within a closed-circuit paper-mill. Hammel and colleagues (1986) have proven the one-electron mechanism of ligninase action by showing that the key reaction of ligninase with lignin compounds, such as vertryl alcohol, was an one-electron oxidation. The degradation of lignin is limited by the presence of biologically stable carbon-carbon and other linkages in the molecule which protect the organic material from the degradative enzyme of microorganisms (Alfani and Cantarella, 1987). The recent discovery of several enzymes that are thought to play similar roles has stimulated lignin biodegradation research by many biochemists. These enzymes include lignin peroxidase (ligninase), manganase (Mn peroxidase), phenol-oxidizing enzymes, and H_2O_2-producing enzymes. Ligninase activity has been detected in white-rot fungi, including *Phlebia radiata, Panus tigrinus, Coriolus versicolor, Pleurotus ostreatus, Bjerkandera adusta* (Kirk and Farrell, 1987) and *Lentinus edodes* (Crestinic, *et al.*, 1996).

4.3 Regulation of plant peroxidase: defense mechanism

In contrast to many animals that can change location, plants are required to adapt to the change and stresses of the environment to survive and they do so in altering their growth pattern. Gaspar and colleagues (1991) have proposed a two-step mechanism in peroxidase induction, where growth control may occur through the basic and acidic peroxidase. A common feature of these physiological processes is lignification, which

is necessary for xylem formation. The perturbation of the normal lignification process mediated by auxin-peroxidase-ethylene is involved in the formation of local necrotic lesions, growth and morphogenetic changes in various stresses. The induction of the peroxidase formation can be regulated at the level of transcription probably to modify the composition of the cell wall and matrix, by an increased synthesis of lignin (Beardmore, *et al.*, 1983) or by enhancing the rate of intermolecular cross-linking between cell wall polymers. The consequence of these reaction make the cell wall more resistant to pathogen attack. They also constitute a wound healing mechanism.

4.4 Enzyme immunoassays

Following the widespread use of radioimmunoassays during the past two decades, many markers have been introduced for the quantitative measurement of primary antigen-antibody interactions. One of these methods utilizes an enzyme as a label which is referred to as enzyme immunoassay (EIA) or enzymes-linked immunosorbent assay (ELISA). These assays have gained increasing popularity in recent years not only for their simplicity but also for a variety of factors both technical and regulatory (e.g. availability, elimination of the problem of disposal of radioactive wastes). The method uses an enzyme-linked antibody, alkaline phosphatase and peroxidase, and the end point of measurement is the enzymatically generated product from its substrate that can be measured colorimetrically or visually. This method has wide clinical application in the serodiagnosis of many diseases, including infectious diseases and particularly viral infections, for example in hepatitis B antigen or antibody, and rubella detection. (Zmijeewski and Bellanti, 1985). In 1991 Conyers

and Kidwell reported on the development of two new detection systems for horseradish peroxidase for the strain of membranes used in immunoassays. These systems use dimethyl or diethyl analogues of *p*-phenylenediamine with 4-chloro-1-naphthol to generate a blue product, or 3-methyl-2-benzothialinone hydrazone with 4-chloro-1-naphthol to generate a red product. These reagents are have more sensitivity and lower background straining than currently available chromogenic detection substrates. In addition, the incorporation of these substrates increases the sensitivity of HRP labels to be comparable to that of alkaline phosphatase with the 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium substrate. Gonzalez and colleagues (1996) developed an indirect ELISA test for the detection of *Pseudomonas fluorescens* and related bacteria in refrigerated meat.

5. Purification of enzyme

Peroxidases play a variety of important roles in animal, microbial, and plant physiology, and continue to arise interest in the molecular and structural characteristics that generate such versatility. To study the characteristics of peroxidases in microorganisms, it is important to remove contaminant proteins. In 1956, Lenhoff and Kaplan purified a cytochrome peroxidase from *Pseudomonas fluorescences.* The extract precipitated between 40%-80% saturation with solid $(NH_4)_2SO_4$. Since the cytochrome peroxidase activity was associated with the cytochrome pigment, which was darker in the reduced state, the pigment was reduced with a few crystals of Na_2SO_4 before adding to the column. Small aliquots of the protein fraction were added to a chromatographic column consisting of

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acid-treated kaolin. A cytochrome peroxidase obtained from this strain has been purified 13 fold. Claiborne and Fridovich (1979) reported that the extracts of aerobically grown *E. coli* exhibited both catalase and dianisidine peroxidase activities. Polyacrylamide gel electrophoresis (PAGE) exhibited two distinct catalases which have been designated hydroperoxidase I and II in order of increasing anodic mobility. Hydroperoxidase I has been purified by precipitation in 25%-60% saturation $(NH_4)_2SO_4$ at pH 7.0. After stirring the precipitate was collected by centrifugation and was dissolved in H_2O. The residual $(NH_4)_2SO_4$ was partially removed by passage over a column of coarse Sephadex G-20 and DE52. Catalase and peroxidase activities eluted together, and these fractions were pooled, clarified by centrifugation and concentrated over an Amicon PM-30 membrane. This sample was applied to a column of Bio-Gel A-1.5m and Bio-Gel HTP hydroxylapatile. The fractions collected were pooled and brought to 90% saturation with $(NH_4)_2SO_4$, and could then be stored for months at 4°C without loss of activity. The result showed catalase as the final product. The total purification was 20.4 fold for peroxidase and 39.5 fold for catalase. Fukumori and colleagues (1985) purified peroxidase from *Halobactrium halobium*. The extract was subjected to chromatography on a Sepharose CL-4B column, gel filtration on Sephacryl S-200, and following by Phenyl-Sepharose. Purification was 131 fold. Van P'ee and Lingens (1985) isolated and purified a bromoperoxidase from the chloramphenicol-producing actinomycete *Streptomyces phaeochromogense*. This purification was achieved by precipitating proteins in 40-70% saturation $(NH_4)_2SO_4$ The enzyme solution was adsorbed to a DEAE-cellulose DE52 column and a Bio-gel

HTP hydroxylapatite column. Those fractions having more than 25% of the maximal peroxidase activity were pool and concentrated using an Amicon concentrator with a PM-30 membrane. After dialysis, the enzyme was further purified by preparative polyacrylamide gel electrophoresis on 7.5% (w/v) gel under non-dissociating conditions at pH 7.5. The results of the purification procedure showed a 23.3-fold purification and a 16.2% yield. This report suggested that bacterial haloperoxidase could not be detected in crude extract or after $(NH_4)_2SO_4$ fractionation, because the crude extract contained a very efficient catalase. This catalase competed with bromoperoxidase from H_2O_2. Therefore bromoperoxidase activity could only be detected after $(NH_4)_2SO_4$ and ion-exchange chromatography had been carried out to remove of the catalase. However, bromoperoxidase from *S. phaeochromogenes* also had catalase and peroxidase activity. Loprasert and colleagues (1988) purified a peroxidase from *Bacillus stearothermophilus*. The crude enzyme was precipitated by 35-55% $(NH_4)_2SO_4$ and repeated with DEAE-Sephadex A50 column. Fractions containing peroxidase activity were pooled and concentrated by ultrafiltration, and put onto a second DEAE-Sephadex A50 column. The enzyme solution was adjusted to 4% $(NH_4)_2SO_4$ and adsorbed on a column of Phenyl-Sepharose CL-4B containing 4% $(NH_4)_2SO_4$. The peroxidase was eluted with a decreasing linear gradient of $(NH_4)_2SO_4$ (3.75%-0.9% in phosphate buffer) and put onto Sephadex G-200, DEAE-5PW HPLC column. The enzyme was purified 120-fold from the crude extract to a specific activity of 18 Units per mg proteins with a 10% recovery of its activity. Zeiner and colleagues (1988) detected the multiple bromoperoxidase in extracts of *Streptomyces griseus*. The extracts were

added $(NH_4)_2SO_4$ to a 40-90% saturation and adsorbed onto a DEAE-cellulose DE52 column. As several bromoperoxidase activities were detectable after cultivation in Erlenmeyer flasks, these were labeled according to their elution volume: BPO1a, BPO1b and BPO3 were further purified by heat treatment at 60°C for 5 min. The samples were then applied to a Sepharose Q Fast Flow column and a Bio-Gel HTP hydroxylapatite column for BPO 1, while BPO3 was passed through a Superose 12 column. The result showed an 1107-fold purification for BPO3, 538-fold for BPO1a and 933-fold for BPO1b. Loprasert and colleagues (1990) reported an overproduction and purification of *Bacillus stearothermophilus* peroxidase in *E. coli,* which was cloned with the peroxidase gene. The enzyme could easily be purified from *E. coli* by heat treatment and single-column Sephadex G-200 chromatography. It gave an activity of 12.2 Unit/mg and a 70% yield. The peroxidase produced by *E. coli* showed that thermostability, heme type and content identical with the peroxidase produced by *B. stearothermophilus.* Mliki and Zimmermann (1992) purified an intracellulose peroxidase and catalase from *Streptomyces cyaneus*. The peroxidase-catalase bound to the anion-exchange Q-Sepharose gel was eluted as a single peak, with 0.35 to 0.4 M NaCl. Gel filtration chromatography on Superose 12 also resulted in one peak containing peroxidase and catalase activities. Active fraction obtained by gel filtration chromatography were concentrated by ultrafiltration with a PM-10 membrane (Amicon) and then separated in a preparative isoelectric focusing (IEF)-agarose gel. The purification steps resulted in an enzyme preparation with a specific activity of 247 U/mg, a purification factor of 33.5, and a yield of 21.7% for the peroxidase

activity. After these steps of purification, the enzyme preparation was still not homogeneous, as indicated by minor protein bands detectable on native PAGE gel.

6. Enzyme properties

Hydrogen peroxide is metabolized by three different types of hydroperoxidases: catalase, peroxidase, and catalase-peroxidase. The typical catalase catalyzes the dismutation of H_2O_2 to O_2 and H_2O. It is generally accepted that the major physiological role of the typical catalase is the protection of the cells against the damaging effect of hydrogen peroxide (Chance, *et al.*, 1979). These enzymes are composed of four subunits of equal size, each containing one hemin (ferric heme IX) prosthetic group with a combined molecular weight in the range of 225 kDa-270 kDa. The catalytic activity of typical catalases is pH independent in the pH range of 5-10.5, is specifically inhibited by 3-amino-1,2,4-triazole, and has hydrophobic properties which are revealed by extractability in ethanol-chloroform and binds to phenyl-Sepharose (Hochman and Shemesh, 1987).

The typical peroxidase catalyzes the oxidation of H_2O_2 by a large variety of substrates. It also may function in detoxifying hydrogen peroxide as well as in various cellular activities of biosynthesis and degradation (Elstner, 1982). The peroxidases constitute a heterogeneous group of enzyme that share with catalases a number of structural and physicochemical properties. However, most peroxidases are monomeric with a wide range of molecular weights, and their prosthetic groups can be reversibly dissociated from the protein and even substituted with other

hemes; also, their iron can be reduced with dithionite and bound to CO in the ferrous state (Hochman and Shemesh, 1987). The catalase-peroxidase, which were only recently classified as a distinct group of enzymes, have been identified in various genera of microorganisms. The catalase-peroxidase are unique in that they catalyze both catalase and peroxidase activities at significant rates; most significantly, the enzyme can oxidize the pyridine nucleotides NADH and NADPH (Hochman and Goldberg, 1991) and cytochrome c. These enzymes share biochemical and physicochemical properties with catalases and peroxichlorophenoldases. Table 3. summarized The reports available on the characteristics of peroxidase.

7. Molecular genetics of peroxidase

Peroxidase was studied at the biochemical, physiological, genetical and molecular level to delineate the biological function of the enzyme. During the last nine years some efforts have been made in the isolation and cloning of peroxidase genes into different organisms. Loprasert and colleagues (1988) purified a peroxidase from *Bacillus stearothermophilus*, and the enzyme was found to have the catalase activity. The peroxidase gene (*perA*) was cloned and sequenced, and the deduced amino acid (aa) sequence showed 48% homology to that of *E. coli* catalase HPI (Loprasert, *et al.*, 1989). These results suggested that this peroxidase was a member of the bacterial catalases with broad-spectrum peroxidative activity. Fujiyama and his colleagues (1990) isolated two genes of horseradish peroxidase and compared these with the peroxidase genes from tobacco, potato, *E. coli, B. stearothermophilus* and cytochrome c

Table 3 Characteristics of peroxidase from different microorganisms

strains	MW (kDa)	feature	optimum		stability		pI	K_m for H_2O_2 (mM)	ref.
			pH	temp. (°C)	pH	temp. (°C)			
Bacillus stearothermophilus	175	di	6	70	5.5-6.5	30	UD	1.3	Loprasert, *et al.*, 1988
Caldariomyces fumago	42	mono	2.75	UD	UD	UD	UD	UD	Morris and Hager, 1966
Corallina pilulifera	790	twenlve	6.0	UD	UD	UD	3.0	0.09	Itoh, *et al.*, 1985
Escherichia coli	337	tetra	6.5	UD	UD	UD	UD	3.9	Claiborne and Fridovich, 1979
Halobacterium halobium	110	mono	UD	UD	UD	UD	UD	7.7 μM	Fukumori, *et al.*, 1985
Halobacterium halobium	240	tetra	6.5-8.0	UD	UD	40	3.8	0.14	Brown-Peterson and Salin, 1993
Penicillus capitatus	97	di	4.0	UD	UD	UD	UD	UD	Manthey and Hager, 1981
Penicillium simplicissimum	170	di	5.3	UD	UD	UD	UD	UD	Fraaije, *et al.*, 1996
Pseudomonas aureofaciens	155-158	di	5.3	UD	UD	UD	UD	UD	Van P'ee and Lingens, 1985
Rhodopseudomonas capsulata	236	tetra	5-5.3	UD	UD	UD	UD	UD	Hochman and Shemesh, 1987

Table 3 (continue)

strains	MW (kDa)	feature	optimum		stability		pI	K_m for H_2O_2 (mM)	ref.
			pH	temp. (°C)	pH	temp. (°C)			
Streptomyces aureofaciens	95-90	tri	4.5	UD	UD	UD	UD	3.1	Van P'ee, *et al.*, 1987
Streptomyces cyaneus	185	di	5.0	UD	UD	UD	6.1	2.07	Mliki and Zimmermann, 1992
Streptomyces grise BPO 1a	80-60	di	4.5	40	UD	60	4.6	0.43	Zeiner, *et al.*, 1988
BPO 1b	95-85	di	UD	UD	UD	60	4.7	UD	
BPO 3	95-85	tri	4.5	60	UD	60	3.6	6.4	
Streptomyces phaeochromogenes	150-144	di	5.0	UD	UD	UD	4.0	UD	Van P'ee and Lingens, 1984

UD; undertermine

peroxidase gene. The results revealed that amino acid sequences of plant peroxidases showed 36-50% identical, whereas these 5 HRPs showed lower homology with microbial peroxidase sequences. Black and Reddy (1991) isolated six putative lignin peroxidase (LIP) gene from a λEMBL3 phage library of the white-rot fungi, *Trametes versicolor*, using the *Phanerochaete chrysosporium* LIP cDNA CLG5 as the probe. Sequence analysis of one of the genes, VLG1, showed that its coding region was interrupted by six small introns and that it encoded a mature LIP protein (341 aa; MW 36,714) that was preceded by a 25 aa signal sequence. This protein had a relatively high degree of aa homology to the LIP proteins purified from *T. versicolor* and had an aa homology of 55-60% to the LIP proteins of *P. chrysosporium*, which was comparable to that found between *P. chrysosporium* and *Phlebia radiata* LIP proteins. Ritch and colleague (1992) isolated and characterizatied the 5'-untranslated region of the genomic clone, LG2, encoding LIP2, the major LIP isozyme from *Phanerochaete chrysosporium* strain OGC101. Dhawale (1993) analyzed the upstream sequences of all published lignin peroxidase and manganese peroxidase genomic clones from *Phanerochaete chrysosporium*. This analysis revealed the presence of putative activator protein-2 (AP-2) recognition sequences in 11 of 15 lignin peroxidase genes. This lignin peroxidase clone GLG6 and the manganese peroxidase gene (*mnp*-1) had two copies of the putative AP-2 sequence in the upstream region. Interestingly, the lignin peroxidase gene VLG4 of another white rot fungus, *Trametes versicolor*, and the *nit*-2 gene of *Neurospora crassa* also contain putative AP-2-binding sequences. Since all of these genes were regulated by nutrient nitrogen. This led to the hypothesis that an AP-

2-like transcription factor might be involved in inducing gene expression during nitrogen limitation in fungi. Parsonage and colleague (1993) used the T7 RNA polymerase expression system for developing a modified plasmid vector which gives reliable, high level expression in *E. coli* of the gene encoding streotococcal NADH peroxidase. The recombinant enzyme has been purified to homogeneity which yields over 35 mg of pure protein per liter of culture. Recombinant NADH peroxidase was fully active and exhibits spectroscopic and redox properties identical to those for the enzyme purified from *Streptococcus faecalis* 10C1. Heym and colleagues (1993) studied the chromosomal location of *Kat*G which encoded the heme-containing enzyme catalase-peroxidase and its nucleotide sequence of *Mycobacterium tuberculosis* for predicting the primary structure of enzyme. They reported that *M. tuberculosis* enzyme was an 80 kDa protein containing several motifs characteristic of peroxidases and showed strong similarity to other bacterial catalase-peroxidase.

Objectives

1. To purify peroxidase from *Bacillus* sp. PHS 155.
2. To study enzyme kinetics and characteristics of the purified proxidase.
3. To determine the size of the peroxidase gene.
4. To study the expression of peroxidase gene encoding from *Bacillus* sp. PHS 155.

Chapter 2

Materials and Methods

Materials

1. Instruments

Instruments	Model	Company
Analytical balance	Junior 2000C	Precisa
Autoclave	HA-300 MII	Hireyama
Fraction collector	2100	BIO-RAD
Hot plate stirrer	Nuova II	Sybron
Incubator	Im 550 R	Clayson
Larminar air flow	Nu 425-400 E	Nuaire
Lyophilizer		BIO-RAD
Microconcentrator		Lab-line instruments
Microscope	CH-2	Olympus Amicon
pH meter	109	Activon
Refrigerated centrifuge	Z 382 K	TLG
UV-visible Spectrophotometer	Ultrospec-3	Pharmacia
Ultrasonicator		MSE
Vortex mixer	G-560E	Scientific industries Inc.
Water bath		Lab-line instruments

2. Chemicals

Chemicals	Company
Acetic acid	Merck
Acrylamide	Merck
Albumin	Pharmacia
Aminoantipyrine	Fluka
Ammonium chloride	Fluka
Ammonium persulfate	Analyticals
Blue dextran	BHD
Bromophenol blue	Sigma
Bovine serum albumin (BSA)	Analyticals
Catalase	Sigma
Catechol	Sigma
Coomassie blue G-250	Sigma
Coomassie blue R-250	Sigma
Cupric sulphate	Merck
DEAE-Sephacel	Merck
2,4-Dichlorophenol	Fluka
Di-potassiumhydrogen phosphate	Fluka
Ethanol (absolute)	Merck
Ferric phosphate	Sigma
Ferritin	Sigma
Ferrous sulphate	Fluka
Glycine	Merck
Guaiacol	Sigma

Chemicals	Company
Hydrochloric acid	Merck
Hydrogen peroxide	Merck
2-mercaptoethanol	Sigma
Methanol (absolute)	Baker analyed
N-methylenebisacrylamide	Sigma
N,N,N',N'-tetramethylenediamine	
(TEMED)	Sigma
O-aminophenol	Aldrich
O-dianisidine	Sigma
Ovalbumin	Sigma
Potassium chloride	Fluka
Potassium dihydrogen phosphate	Merck
Pyrogallol	Sigma
Sephadex G 50-150	Whatman
Sephadex G-200	Whatman
Sephacryl S-300	Whatman
Sodium acetate trihydrate	Analyticals
Sodium chloride	Analyticals
Sodium dodecyl sulfate	Sigma
Sodium hydroxide	Eav Noble
Thyroglobulin	Sigma
Tris-hydroxymethylaminomethane	Fluka
Zinc sulphate	Fluka

3. Microorganisms

The microorganism used in this thesis was *Bacillus* sp. PHS 155. This strain was isolated by Assist. Dr. Amornrat Phongdara and Assist. Dr. Rattana Roengrairatanaroj, Department of Biochemistry, Faculty of Science, Prince of Songkla University. It was isolated from a hot pond in the south of Thailand and was stored at 4°C in 2YT semisolid medium as a stock culture and subcultured every month. The 2YT broth in 1 L culture consisted of 10 g yeast extract, 10 g tryptone and 5 g NaCl. 1.75% agar was added for semisolid medium.

The catalase-deficient mutant, *Escherichia coli* UM228 was grown in LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L of distilled water) containing 50 μg/ml of ampicillin at 37°C. The *E. coli* UM228 was used for the expression of *Bacillus* sp. PHS 155 peroxidase gene (*perA*).

4. Plasmid vector

Plasmid pOD68 was provided by Dr. Savitr Trakunaleamsai. It was a pUC19 derivative containing the *perA* of *Bacillus stearothermophilus* IAM 11001 (ATCC 8005). The pBluescript II SK+ was purchased from Stratagene, USA.

Methods

1. Cell cultivation

The *Bacillus* sp. PHS 155 was grown under aerobic conditions. A full loop of stock culture was inoculated into 10 ml 2YT broth as a starter. This culture was incubated on a rotary shaker (250 rpm) at 55°C for

12 hrs. A two percent (v/v) inoculum was transferred to 500 ml of medium and incubated under the same conditions. Growth of the bacterium was monitored spectrophotometrically at 600 nm. The activity of peroxidase and catalase from cell-free extracts (crude enzyme) were assayed.

2. Preparation of crude enzyme

The bacterial cells were harvested by centrifugation at 5,000 rpm for 20 min. The pellet was washed twice with 0.1 M Tris-HCl, pH 8.0 and then suspended in 2 ml of the same buffer. The suspended cells were disrupted using sonication at 24 kHz for 25 min. The crude enzyme solution was separated from the cell debris by centrifugation at 12,000 rpm at 4°C for 20 min.

3. Enzyme assays

3.1 Peroxidase assay

The method used for determining peroxidase activity was a modification of the method of Shannon and colleagues (1966). The reaction mixture consisted of 100 µl of 0.1 M H_2O_2, 50 µl of 0.5% (w/v) *o*-dianisidine as the substrates, 2.84 ml of 50 mM sodium acetate buffer, pH 5.4 and 10 µl of the enzyme solution. The blank contained by the reaction mixture without H_2O_2. The reaction was started by the addition of H_2O_2 to the mixture and was determined spectrophotometrically using the absorbance measurement at 460 nm at 60°C. The absorption value was recorded each minute within the linear relationship between optical density (OD) and time. The extinction coefficient for *o*-dianisidine at 460 nm is 11,300 M^{-1} cm^{-1}. One unit of peroxidase activity was defined as the

amount of enzyme which converted 1 μmol of *o*-dianisidine per min (Mliki and Zimmermann, 1992).

3.2 Catalase assay

The catalase assay used was a modified from the method of Hildebrandt and Roots (1975). The reaction mixture contained 100 μl of 0.1 M H_2O_2, 2.84 ml of 50 mM sodium acetate buffer, pH 5.4 and 10 μl of the enzyme solution. The buffer was set as blank. The reaction was started by the addition of H_2O_2 and the enzyme solution. Catalase activity was determined spectrophotometrically by measuring the decrease in the absorbance at 240 nm at 60°C. The reaction was recorded as described for the peroxidase assay. One unit of catalase was defined as the disappearance of 1 μmole of H_2O_2 per min. ($\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$) (Mliki and Zimmermann, 1992).

4. Protein determination

Protein was determined by the method of Bradford (Bradford, 1976), (see Appendix). During the chromatography, the protein in each fraction was monitored sepectrophotometrically at the OD_{280}.

5. Purification of peroxidase

All the purification steps were carried out at 4°C.

5.1 DEAE-Sephacel

Crude enzyme from a 10 L culture was applied to the first column (3.6 x 24 cm) of DEAE-Sephacel, which had been equilibrated with 0.1 M Tris-HCl, pH 8.0. The flow rate was 10 ml/hr and a volume of 2 ml was collected for each fraction. The protein in the eluent was monitored at 280

nm by spectrophotometry. When the absorbance became zero, the column was eluted with 500 ml of a 0 to 1 M NaCl linear gradient in the same buffer. The fractions of each protein peak were pooled and dialysed against the same buffer for 24 hrs at 4°C. All dialysates were concentrated by lyophilization. The pellets were dissolved in a minimal amount of the same buffer. Peroxidase activity and total protein were determined. The protein peak containing high enzyme activity were transferred to the second column.

5.2 Sephadex G-200

The enzyme mixtures separated from the first column were layered onto the second column (1.4 x 46.7 cm.) of Sephadex G-200 previously equilibrated and eluted with 5 mM potassium phosphate buffer, pH 7.0 Fractions of 2 ml were collected at a flow rate 4 ml/hr. Protein fractions were assayed for peroxidase activity and total protein. The fractions showing high specific enzyme activity were pooled and applied to the next column.

5.3 Sephadex G 50-150

The pooled fractions from the Sephadex G-200 column exhibiting high specific enzyme activity were loaded onto the third column (1.2 x 116 cm.) of Sephadex G 50-150. This column had been equilibrated and eluted with the same buffer as Sephadex G-200. For the proteins peak 2, the fractions of 1 ml at a flow rate 6 ml/hr was collected. For the proteins peak 3, the fractions of 1.5 ml at a flow rate 12 ml/hr was collected. The protein peaks were assayed for peroxidase activity and for total protein as in 5.2. Fractions containing high specific enzyme activity were pooled and concentrated in a microconcentrator with a molecular weight cut off

30 kDa filter. Finally the concentrated enzyme was loaded onto the next column.

5.4 Sephacryl S-300

The concentrated enzyme was applied to the final column (1.8 x 24.5 cm.) of Sephacryl S-300 which had been equilibrated and eluted with the previous buffer. Flow rate was 6.4 ml/hr and fractions of 1 ml were collected. The fractions contained proteins were further assayed for peroxidase activity. The purified enzyme from this column was used to determine homogeneity, characteristics and kinetics.

6. Electrophoresis

Sodium dodecyl sulfate polyacrylamide (SDS-PAGE) in 10% gel was carried out according to the method of Weber and Osborn (1969),(see Appendix). After electrophoresis, the protein was stained with Coomassie Brillant Blue R-250. Native-PAGE was performed at 4°C. The peroxidase band was detected using a modified method of Herzog and Fahimi (1973). The native gel was incubated in a mixture of 2.3 mM 3,3'-diaminobenzidine (DAB) and 50 mM potassium phosphate, pH 7.0. The peroxidase band was detected by adding 10 mM H_2O_2 into the mixture, and appeared as brown band.

7. Characterization of the purified enzyme

7.1. Determination of MW

The standard proteins and the purified enzyme were chromatographed on a Sephacryl S-300 column (1.8 X 24.5 cm), (see 5.4). The flow rate of this column was 6.4 ml/hr and 1 ml was collected for each

fraction using by a fraction collector. The molecular weight was determined using ovalbumin (43 kDa), albumin (67 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) as the standard proteins. The molecular weight was evaluated using the relationship between log molecular weight and partition coefficient value (K_{av}) of the standard proteins. The molecular weight was also determined using SDS-PAGE in 10% gel with the following standard protein marker : carbonic anhydrase (30 kDa), ovalbumin (43 kDa), albumin (67 kDa) and phosphorylase b (94 kDa). The molecular weight was evaluated using the relationship between the log molecular weight and relative mobility of the standard proteins.

7.2 pH optimum

The effect of pH on peroxidase activity was measured as in 3.1 but different buffers were used instead. (the pH ranges of 4 to 5.4 in 50 mM sodium acetate, 6 to 8 in 50 mM potassium phosphate buffer, 8 to 9 in 50 mM Tris-HCl buffer and 9 to 10 in 50 mM glycine-NaOH buffer)

7.3 Optimum temperature

Optimum temperature of the peroxidase activity was assayed as described in 3.1 but at the determined temperature (40, 50, 55, 60, 65, 70 and 80°C).

7.4 Temperature stability

For the determination of heat stability of the purified enzyme, the enzyme solution was incubated at 50, 60 and 70°C. After incubation at each temperature for the certain period (0, 0.5, 1, 1.5, 2, 2.5, 3, 4 and 5 hrs), the enzyme solution was rapidly cooled and centrifuged at 3,000 rpm

for 5 min and 4°C to remove the denatured protein. The enzyme assay was performed as described in 3.1.

7.5 Substrate specificity

The substrate specificity of peroxidase was determined as described in 3.1 at the optimum pH and temperature but the different substrates and OD for monitoring were used as shown in Table 4.

Table 4 The substrates and optical density (OD) for testing

substrates	OD (nm)
20 mM *o*-dianisidine	460
42 mM *o*-aminophenol	480
42 mM catechol	480
42 mM guaiacol	430
42 mM pyrogallol	420
4.1 mM dichlorophenol	500
0.67 mM aminoantipyrine	500
4.1 mM dichlorophenol and 0.67 mM aminoantipyrine	500

source : Loprasert and colleagues (1988)

7.6 Enzyme inhibition

Peroxidase assay was carried out as described previously (see 3.1) in the presence of the ions given in Table 5.

Table 5 The kinds and concentration of the ions for testing

The kinds of ions	concentration
Fe_2SO_4	10^{-3} M
Fe_3PO_4	10^{-3} M
KCl	10^{-3} M
NaCl	10^{-3} M
$MgCl_2$	10^{-3} M
$CaCl_2$	10^{-3} M
$ZnSO_4$	10^{-3} M
EDTA	10^{-3} M
SDS	1%
Ethanol	5%
Methanol	5%

8. Kinetics of the peroxidatic reaction

The Michaelis-Menten constant of the purified enzyme was determined by varying the concentration of the single substrate (*o*-dianisidine 12.3-28.7 mM or H_2O_2 25-150 mM) in the presence of a fixed saturating concentration of the other. The kinetics of the purified enzyme was determined using the Lineweaver-Burk plot of enzyme activity against varied substrate concentration.

9. Characterization of peroxidase gene

9.1 Size determination of peroxidase gene

The genomic fragments of the positive Lambda GEM-11 *perA* clones (obtained from Ms. Kintkand Na Pathalung) was deleted by digesting of *Cla*I restriction enzyme. Six fragments of peroxidase gene were separated by 1% agarose gel electrophoresis and subcloned into pBluescript II SK+ plasmid vector. For isolation the smallest size of the peroxidase gene, the DNA fragments and pBluescript II SK+ harboring peroxidase gene were blotted to Nylon STD, .4UM membrane (Pharmacia, USA) and fixed the DNA by UV crosslinking for 90 second. After that the membrane was hybridized with Flouorescein-labeled pOD68 probes at 68°C overnight followed by the method of Sambrook and colleagues (1989). Before labeling, pOD68 was denatured by heat to 90°C for 5 min and quickly chilled the denatured DNA on ice. The DNA was labeled with Random Primer Fluorescein Labeling Kit (Dupont NEN, USA) consisted of 5 µl random primers and reaction buffer mix, 5 µl of Fluorescein nucleotide mix, and 1 µl Klenow fragment polymerase. The mixture was incubated overnight at room temperature, and the reaction was stopped by adding 5 µl of 0.1 M EDTA (pH 8.0). Any unhybridized probe can be removed by washing the membrane in the mixture of 2.0xSSC, 1.0%SDS and the mixture of 0.2xSSC, 0.1%SDS for 15 min each at 68°C. The hybridized DNA on the nylon membrane was detected by CDP-*star*TM Nucleic Acid chemiluminescence reagent (Dupont NEN, USA),(see Appendix) and then autoradiographed with X-rays film (Fuji Rx, Japan).

9.2 The expression of peroxidase gene

The positive plasmid which had the smallest size of peroxidase gene was transformed into host (*E. coli* UM228) followed by the method of Sambrook and colleagues (1989). Colonies of the transformants containing pBluescript II SK+ harboring peroxidase gene would be white when plating on LB/ampicillin agar plates which spreaded with 40 µl of 2%X-gal and 100 µl of 100 mM IPTG 30 min prior to plating, after incubation for 12 hrs. at 37°C. For colonies of the transformants without insertion would remain blue. The mutant *E. coli* UM228 cultured in 2YT medium at 37°C for 24 hrs. under the aerobic condition by using *E. coli* UM228 wild type as a control.

Chapter 3

Result and Discussion

1. Cell cultivation

Bacillus sp. PHS 155 was grown in 2YT broth medium under the condition described previously. Growth and enzyme production are shown in Fig. 2. Maximum growth was obtained after 20 hrs. cultivation. The peroxidase enzyme was observed during the exponential phase and reached its maximum in the stationary phase after 30 hrs. It was of interest to note that no catalase activity was observed in the cells throughout the cultivation period. Hence this result confirmed that *Bacillus* sp. PHS 155 was a catalase-negative strain. Peroxidase activity appeared after a lag period of 5 hrs and showed highest activity with 0.22 U/ml after 30 hrs. The expression of peroxidase began at the late exponential phase and reached a maximum level during stationary phase. H_2O_2, which is generated during respiration or the oxidation of nutrients to obtain energy, plays a significant role in aging cells. It is capable of damaging DNA, proteins and the lipid membrane in photosynthetic organisms (Elstner, 1982). Therefore, it is possible that the peroxidase is produced to function as a main protection against cellular damage from H_2O_2 There was the report on the physiological functions of hydroperoxidase in *Rhodobacter capsulatus* which had two hydroperoxidase : a catalase-peroxidase and a peroxidase. A mutant strain that was deficient in the catalase-peroxidase activity behaved similarly to the wild type, except that viable counts of its aerobic cultures declined much faster after reaching the stationary phase. It was inferred that the peroxidase provides a protection against H_2O_2 in log-

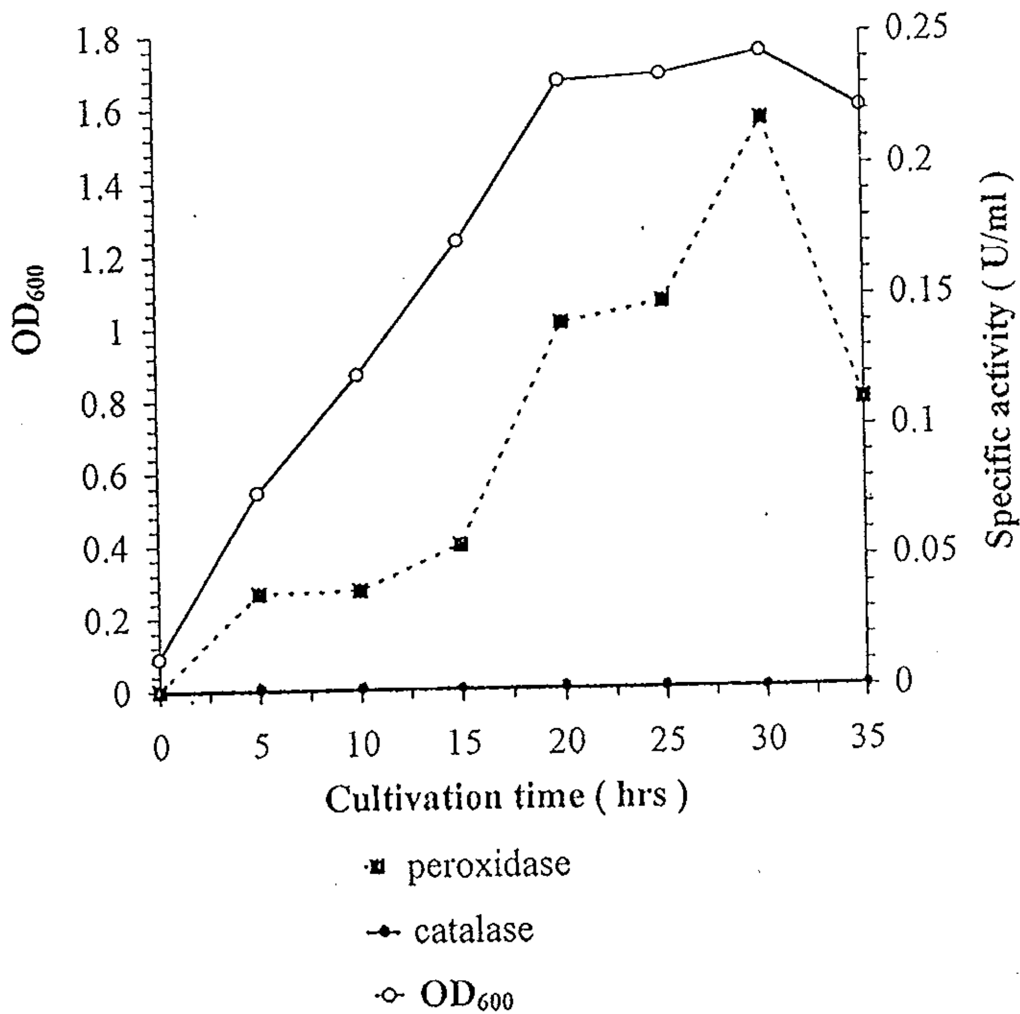


Fig. 2 Time course of growth, catalase, and peroxidase productions of *Bacillus* sp. PHS 155.

Bacillus sp. PHS 155 was grown in 2YT broth medium containing 10 g yeast extract, 10 g tryptone and 5 g NaCl with shaking at 250 rpm, 55°C.

phase cells and that the catalase-peroxidase provides protection under the oxidative conditions that prevail in aging cultures (Hochman, *et al.*, 1992).

However, there were reports that suggested a distinct function of hydroperoxidase. *E. coli* synthesized two different enzymes with catalase activity, a catalase-peroxidase (Claiborne and Fridovich, 1979) and a typical catalase (Loewen and Switala, 1986), which were regulated differently. The typical catalase functions in nongrowing cells, whereas catalase-peroxidase had a general antioxidant role in log-phase cells. So the real function of peroxidase in the oxidative stress response in cells is still in question.

2. Purification of peroxidase

The cell-free extract obtained from a 10 l culture of *Bacillus* sp. PHS 155 was processed through a series of chromatography columns. The result of a typical purification procedure is summarized in Table 6. The enzyme solution could bind to the anion exchangers DEAE-Sephacel, which suggests that the enzyme has a negative charge. The enzyme was eluted from the exchanger by increasing the ionic strength of the elution buffer, thereby weakening the electrostatic interactions between the adsorbed molecule and the exchanger. The use of gel filtration in step 2 was very effective for the removal of a large amount of contaminating proteins. However, the loss of the peroxidase activity after the final column might be the effect of the loss of heme during the purification because heme was tightly bound to the enzyme (Hochman and Shemesh, 1987). The final preparation from step 4 showed an approximately 8.76 fold increase in purity with a recovery of 0.05% of the original activity.

Table 6 Purification of peroxidase from *Bacillus* sp. PHS 155

step	peak	volume(ml)	total protein (mg)	activity (U)	specific activity(U/mg)	yield (%)	purity(fold)
crude		42.5	318.75	121.86	0.38	100	1
DEAE-Sephacel	peak 1	1	13.5	3.35	0.25	4.24	0.66
	peak 2	2	100	8.28	0.08	31.37	0.21
	peak 3	3	141	10.59	0.08	44.24	0.21
	total	**6**	**254.50**	**22.22**	**0.41**	**79.85**	**1.08**
Sephadex G-200	peak 1	2	0.33	0.96	2.91	0.10	7.66
	peak 2	6	8.40	7.19	0.86	2.64	2.26
	peak 3	6	11.58	4.32	0.37	3.63	0.97
	total	**14**	**20.31**	**12.47**	**4.14**	**6.37**	**10.89**
Sephadex G 50-150*	peak 2	1.5	0.64	1.08	1.69	0.20	4.45
	peak 3	0.75	0.25	1.01	4.04	0.08	10.63
	total	**2.25**	**0.89**	**2.09**	**5.73**	**0.28**	**15.08**
Sephacryl S-300*	peak 2	1	0.09	0.09	1.00	0.03	2.63
	peak 3	1	0.06	0.14	2.33	0.02	6.13
	total	**2**	**0.15**	**0.23**	**3.33**	**0.05**	**8.76**

U : The amount of enzyme which converted 1 μM of *o*-dianisidine per min.

* : non determined peak 1

The final specific activity was 3.33 U/mg protein. Details of the chromatographic profiles of the purification steps are shown in Fig. 3-6. The degree of purification of the final preparation of enzyme was examined by non-denaturing PAGE (Fig. 7).

2.1 DEAE-Sephacel

The peroxidase was adsorbed on the DEAE-Sephacel column that had been equilibrated with 0.1 M Tris-HCl pH 8.0 buffer. The column was washed with the same buffer, proteins were eluted gradually using a 0-1 M NaCl linear gradient. The eluted proteins were separated into nine peaks (Fig. 3). The eluted enzyme peaks overlapped with the eluted proteins. The three protein peaks which showed high activity were designated protein peak 1, 2, and 3. Finally, they were separately lyophilized and loaded onto the next column.

2.2 Sephadex G-200

The elution profiles of lyophilized peroxidase on Sephadex G-200 column was shown in Fig. 4. The separation patterns were almost the same from protein peak 1, 2 and 3. There were three protein peaks but the enzyme activity was only expressed in protein peak 2. In Fig. 4(A), the volume of eluted protein was very small, so it is not applied to the next column.

2.3 Sephadex G 50-150

In Fig. 5(A) and 5(B), the protein eluted as only one peak and the enzyme activity also corresponded to that peak. The fraction exhibiting high specific activity was checked by SDS-PAGE. They showed the same patterns and many protein bands could be detected (Fig. 9). This result indicated that peroxidase and contaminant proteins might not be separated

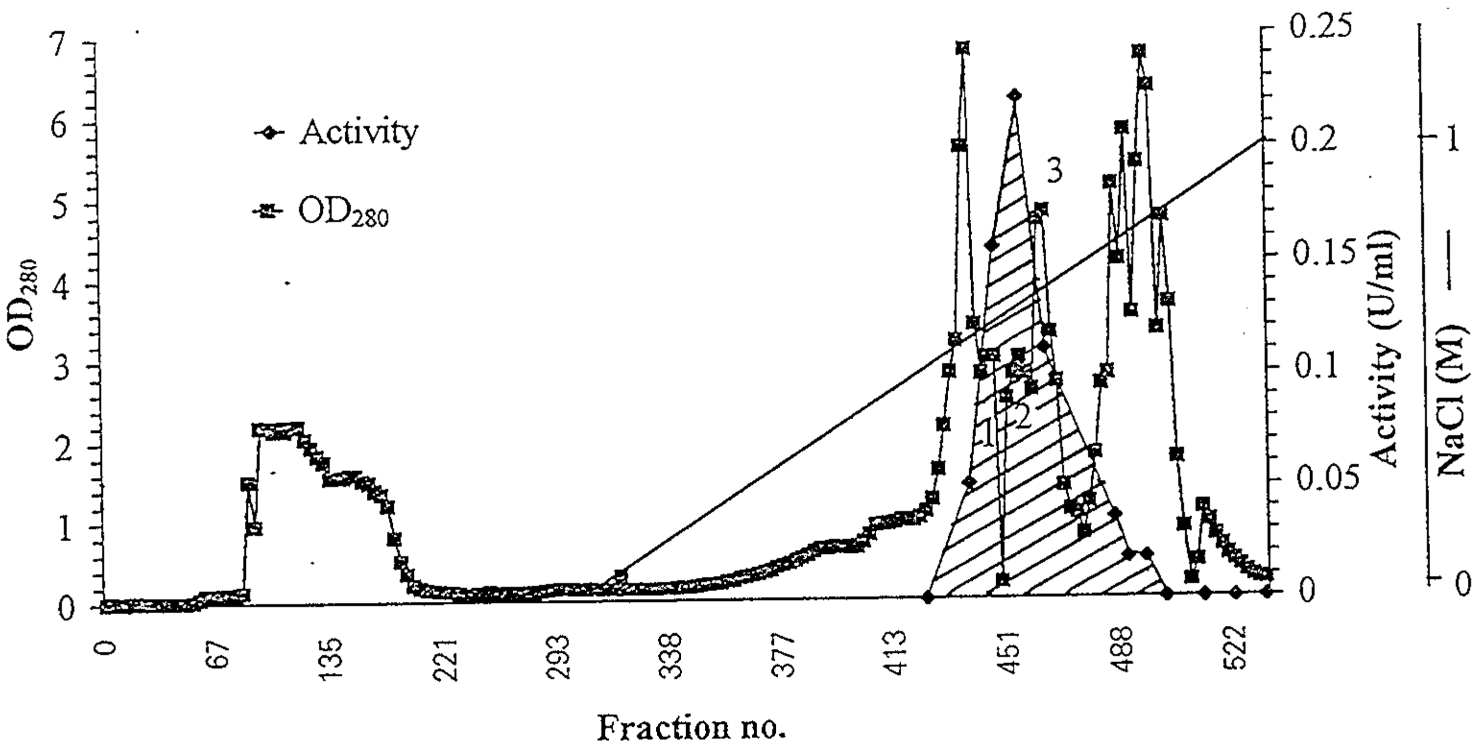


Fig. 3 Chromatographic profile of peroxidase on DEAE-Sephacel column. Crude enzyme from *Bacillus* sp. PHS 155 was loaded into DEAE-Sephacel column which was washed with 0.1 M Tris-HCl pH 8.0 until the absorption reached the base line, and then eluted with 500 ml of a 0 to 1 M NaCl linear gradient at a flow rate of 10 ml/hr. 2 ml fractions were collected.

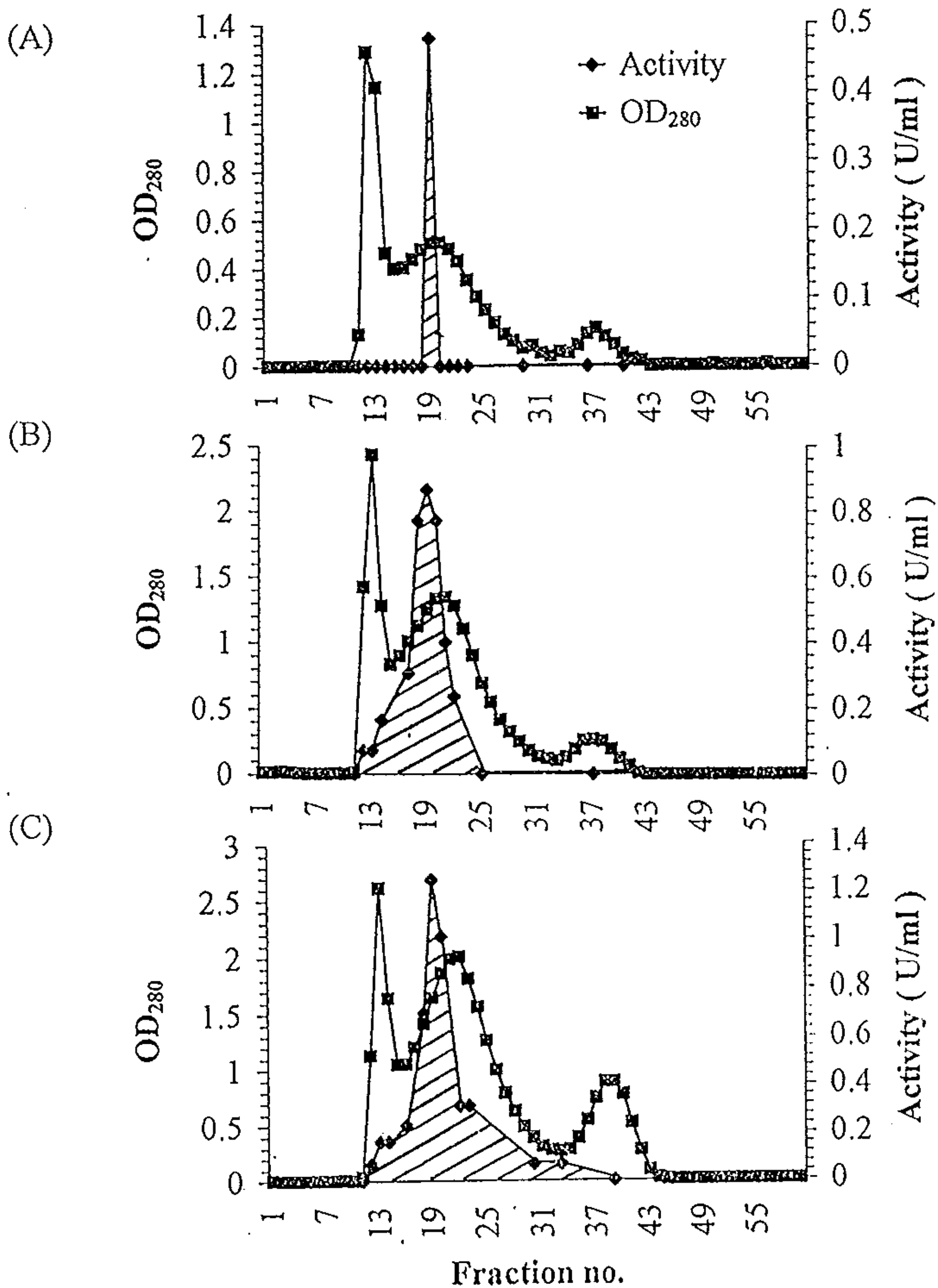


Fig. 4 Chromatographic profile of peroxidase on Sephadex G-200 column. The enzyme peak 1(A), 2(B), 3(C) were applied to Sephadex G-200 previously equilibrated and eluted with 50 mM potassium phosphate buffer, pH 7.0. Fractions of 2 ml were collected at a flow rate 4 ml/hr.

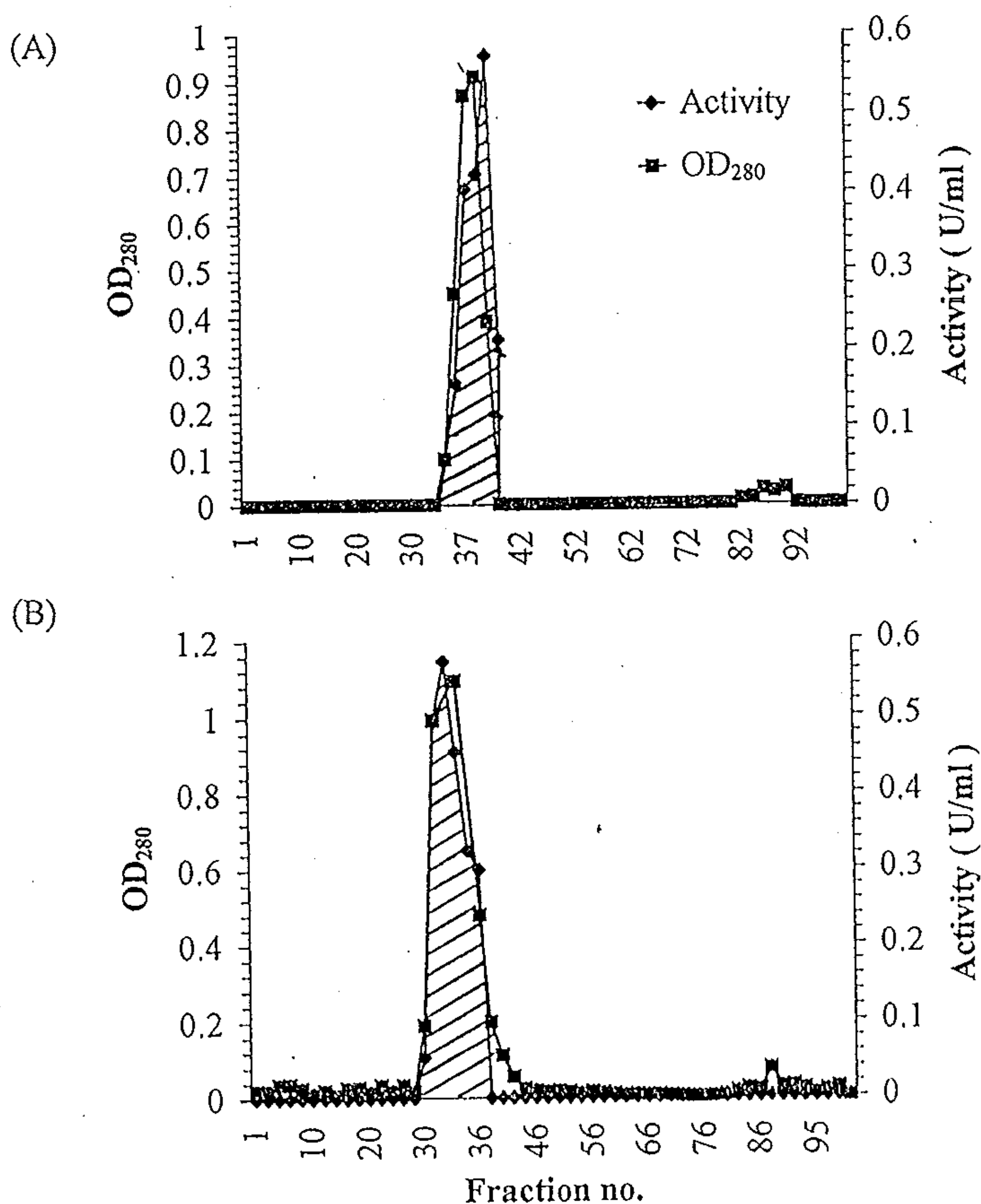


Fig. 5 Chromatographic profile of peroxidase on Sephadex G 50-150 column. The eluted enzyme from Sephadex G-200 were loaded to Sephadex G 50-150 column which equilibrated and eluted with the same buffer as in Sephadex G-200 column. In Fig 5(A) proteins peak 2, Fractions of 1 ml at flow rate 6 ml/hr was collected. In Fig 5(B) proteins peak 3, Fractions of 1.5 ml at flow rate 12 ml/hr was collected.

by this column. It was possible that the experimental design was not appropriate. The proteins which eluted from Sephadex G-200 column might not be separated in the separation capacity of this column. Because the separation range of this column (1.5 kDa-150 kDa) was narrower than Sephadex G-200 column (5 kDa-250 kDa). So the eluted activity peak was concentrated as described in methods before loading to the final column.

2.4 Sephacryl S-300

Although the eluted proteins divided into 3 peaks and 5 peaks shown in Fig. 6(A) and 6(B), respectively, the enzyme peak was similarly eluted in protein peak 3. After purification steps of the enzyme, the enzyme peaks were eluted in the same pattern. So they were pooled together and checked homology by native PAGE.

After all steps of purification, the enzyme preparation was still not completely purified, as indicated by minor protein bands detectable on native PAGE gel (Fig. 7). Attempt to improve the chromatographic separation were unsuccessful because the enzyme rapidly lost activity at the final stage of purification. An activity staining of the active enzyme on native PAGE gel showed the major and minor bands. A brown-colored band was detected at the same position as the major band. This result could be used to confirm that the major band was peroxidase.

3. Characterization of peroxidase

3.1 Molecular weight determination

From a plot of log molecular weight *versus* the partition coefficient value (K_{av}) considered in Sephacryl S-300 column by $K_{av} = (V_e - V_0) / (V_t - V_0)$, when V_e = the elution volume = 37 ml, V_t = the packed bed volume =

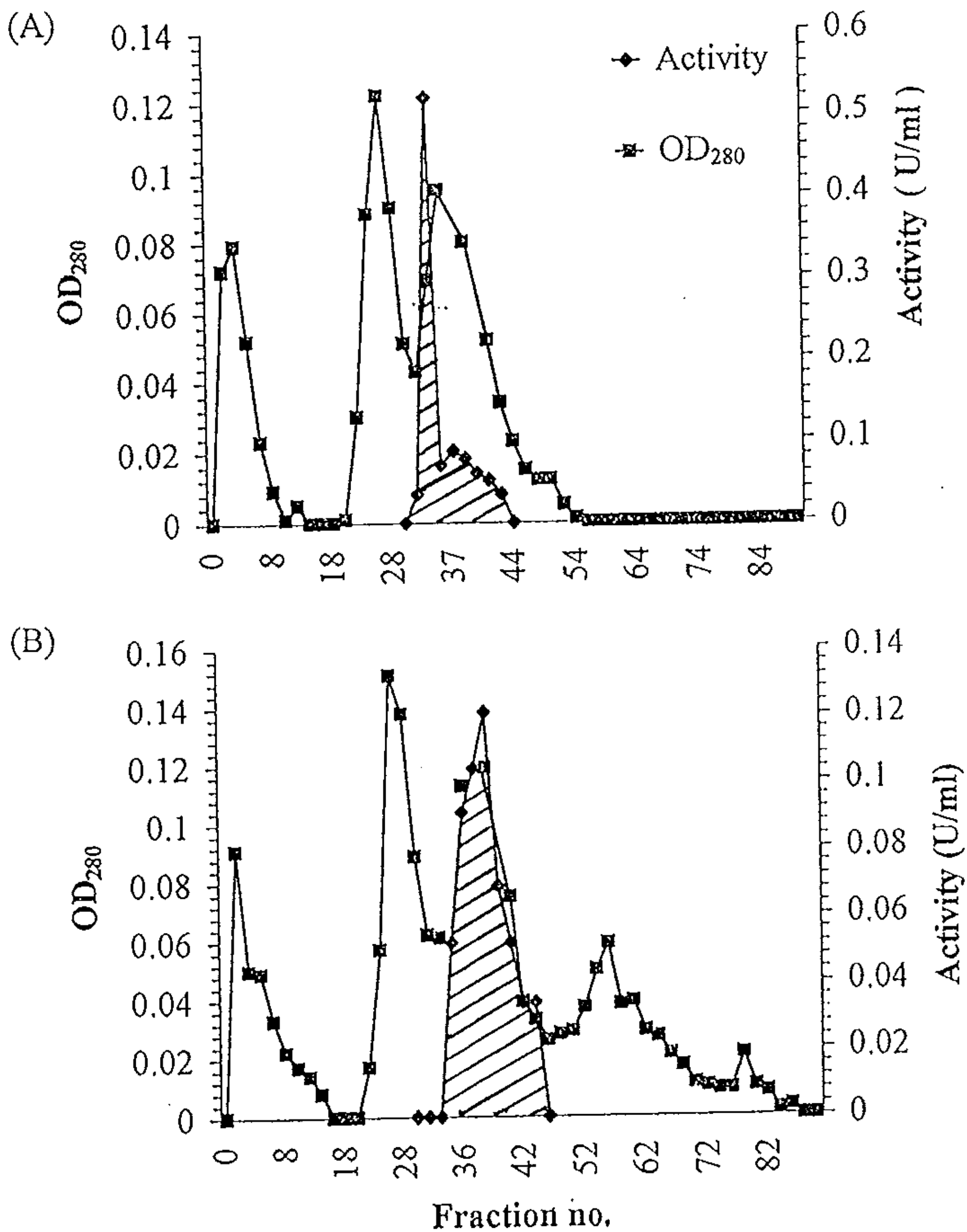


Fig. 6 Chromatographic profile of peroxidase on Sephacryl S-300 column. The concentrated enzyme were applied to Sephacryl S-300 column which equilibrated and eluted with the previous buffer. Flow rate was 6.4 ml/hr. Fractions of 1 ml were collected. Fig. 6(A), proteins peak 2 and Fig. 6(B), proteins peak 3.

Fig. 7 Polyacrylamide (non-denatured) 10% gel electrophoresis patterns of purified peroxidase. Peroxidase activity stained with 3,3'-diaminobenzidine (DAB) and H_2O_2. lane 1, marker proteins staining with coomassie brilliant blue; lane 2 and 3, protein staining from Sephacryl S-300; lane 4, protein staining from DEAE-Sephacel; lane 5, marker proteins staining with DAB and H_2O_2; lane 6, activity staining from Sephacryl S-300; lane 7, activity staining from DEAE-Sephacel. Arrow indicated the peroxidase band.

62.37 ml, V_o = the void volume 24 ml. K_{av} of the purified peroxidase was about 0.34 (Table 7). From this value, the molecular weight was estimated to be 67 kDa as shown in Fig 8. Next the partially purified peroxidase and reference proteins were estimated an apparent subunit by SDS-PAGE (Fig. 9 and 10). Although after the SDS-PAGE, the electrophoretic mobilities of the peroxidase showed many protein bands but there was one band corresponded to the MW of 67 kDa. Thus it was confirmed that the enzyme was 67 kDa (Table 8). Because of the equal MW of determination between SDS-PAGE and gel filtration, the result also elucidated the partially purified peroxidase might not contain intersubunit. However, peroxidases from other microorganisms such as *E. coli* (Claiborne and Fridovich, 1979), *H. halobium* (Fukumori, *et al.*, 1985) and *R. capsulata* were reported to be dimer or tetramer (Table 3). In addition, they had both peroxidase and catalase activity in single protein component in PAGE. So the peroxidase from *Bacillus* sp. PHS 155 distinguished from others because it had only peroxidase activity in the protein structure. The result was similar to peroxidase from plants which showed only peroxidase activity. Several plant peroxidase have been reported that peroxidase was a monomeric protein in the range of low MW such as 10.5 kDa of potato (Decedue, *et al.*, 1984), 42 kDa of *Ipomoea batatas* seeds (Floris, *et al.*, 1984), 39.5 kDa of *Ipomoea cairica* leaf (Lin, *et al.*, 1996), 45 kDa of tomato fruit, 44 kDa of Barley leaf, 48 kDa of *Euphorbia characias* latex and 50 kDa of *Hevea brasiliensis* (Sattayasevana, 1990).

Table 7 Partition coefficient value (K_{av}) of the standard proteins and the partially purified peroxidase in Sephacryl S-300 column

Proteins	K_{av}	Log MW	MW (kDa)
Thyroglubulin	0.10	5.82	669
ferritin	0.18	5.64	440
catalase	0.29	5.36	232
albumin	0.34	4.82	67
Ovalbumin	0.42	4.63	43
peroxidase	**0.34**	**4.82**	**67**

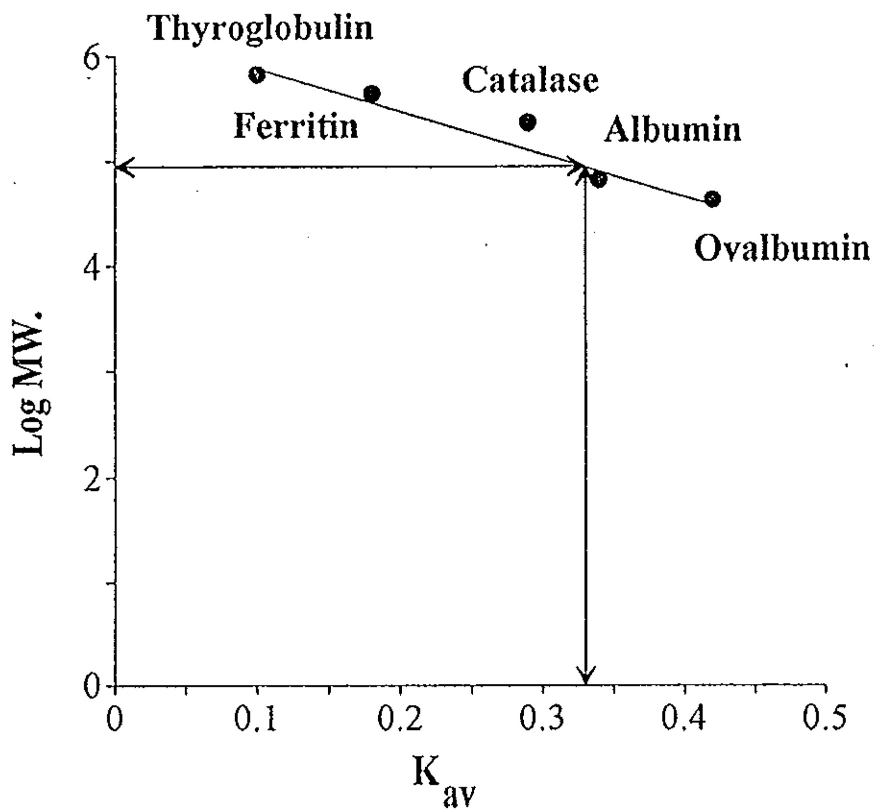


Fig. 8 Relationship between log MW and partition coefficient value (K_{av}) of standard proteins in Sephacryl S-300 column.

The standard proteins were loaded to the final column which equilibrated and eluted with the previous buffer. Arrow indicated the log MW and K_{av} of the partially purified peroxidase.

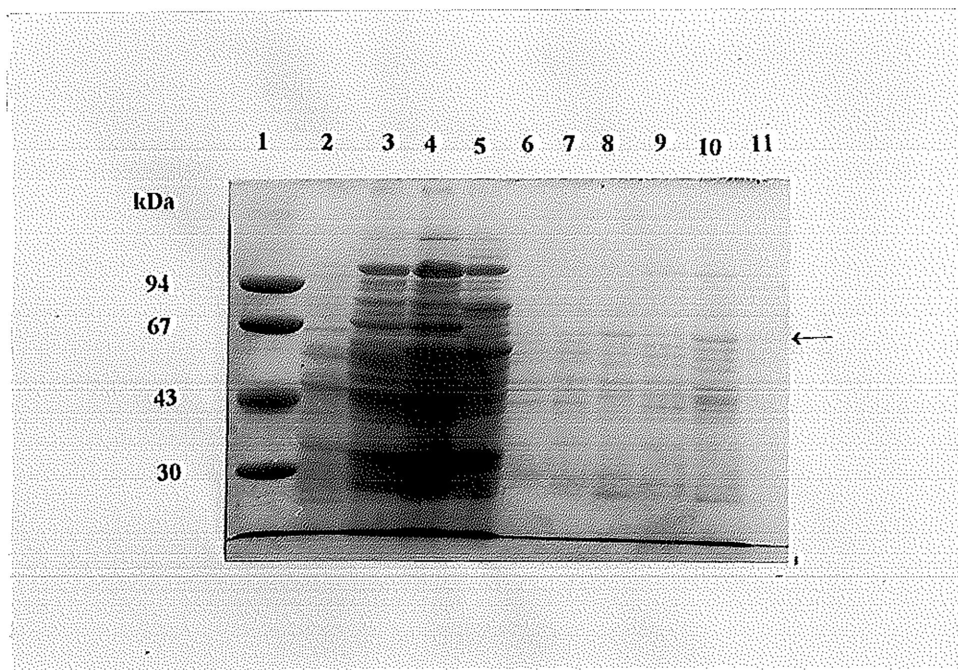


Fig. 9 SDS-PAGE of various stages of proteins purification.

Proteins were stained with coomassie brilliant blue. lane 1, marker proteins; lane 2, crude enzyme, lane 3-5, enzyme from DEAE-Sephacel peak 1, 2, 3; lane 6-8, enzyme from Sephadex G-200 peak 1, 2, 3; lane 9-10, enzyme from Sephadex G 50-150 peak 2, 3; lane 11, enzyme from Sephacryl S-300. Arrow indicated the peroxidase band.

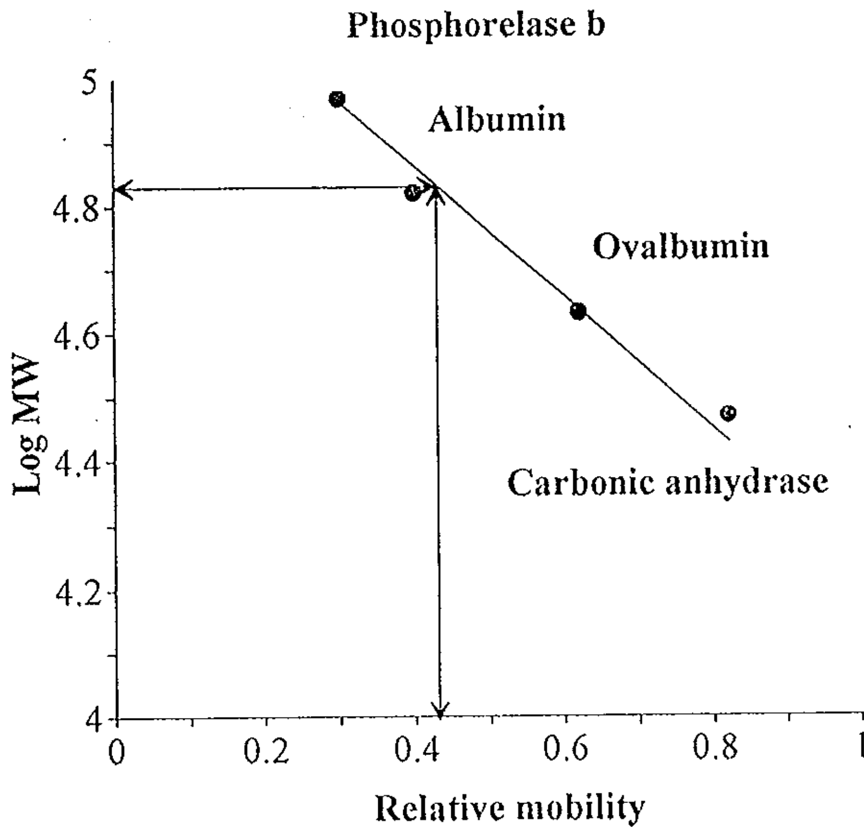


Fig. 10 Relationship between log MW and relative mobility of standard proteins by 10% SDS-PAGE gel.

Purified peroxidase was treated with SDS and 2-mercaptoethanol and electrophoresis in the presence of SDS. Protein standards of known MW were similarity treated. Log MW is here plotted as a function of mobility relative to the dye front (*R_f*). Arrow indicated the mobility relative and log MW of the partially purified peroxidase.

Table 8 Electrophoretic mobilities of the partially purified peroxidase and reference proteins on SDS-PAGE

Proteins	R_f	Log MW	MW (kDa)
Phosphorelase b	0.3	4.93	94
Albumin	0.4	4.83	67
Ovalbumin	0.62	4.63	43
Carbonic anhydrase	0.82	4.48	30
peroxidase	**0.41**	**4.8**	**67**

3.2 pH optimum

When the enzyme activity was assayed at different pH ranging from pH 4 to pH 10, was observed that the peroxidase had an optimum pH in the range of 5-7 (Fig. 11). At pH 5 to 7, the enzyme was highly active, but at pH values higher than 7 or lower than 5, activity decreased, which is similar to the effect of pH on the catalase-peroxidase from *R. capsulata* (Hochman and Shemesh, 1987). The thermostable peroxidase of *B. stearothermophilus was* reported to have a sharp pH optimum at a pH 6 (Loprasert, *et al.*, 1988). Peroxidase-catalase from *S. phaeochromogenes* (Van P'ee and Lingens, 1985) and from *E. coli* (Claiborne and Fridovich, 1979) showed a pH optimum at pH 5.0. This result showed that the pH optimum for the enzyme activity was slightly acid.

3.3 Optimum temperature

The effect of temperature on the rate of the reaction was studied over the range of 40-80°C under standard assay conditions, and the results were shown in Fig. 12. The optimum temperature which gave the highest activity was at 60°C. Above temperature of 65°C the enzyme activity was dropped off rapidly. However, there was little information about peroxidase from thermophilic bacteria. Loprasert and colleagues (1988) reported the apparent optimum temperature for the peroxidase activity was 70°C for *B. stearothermophilus*. This result suggests that this enzyme could work in high temperature.

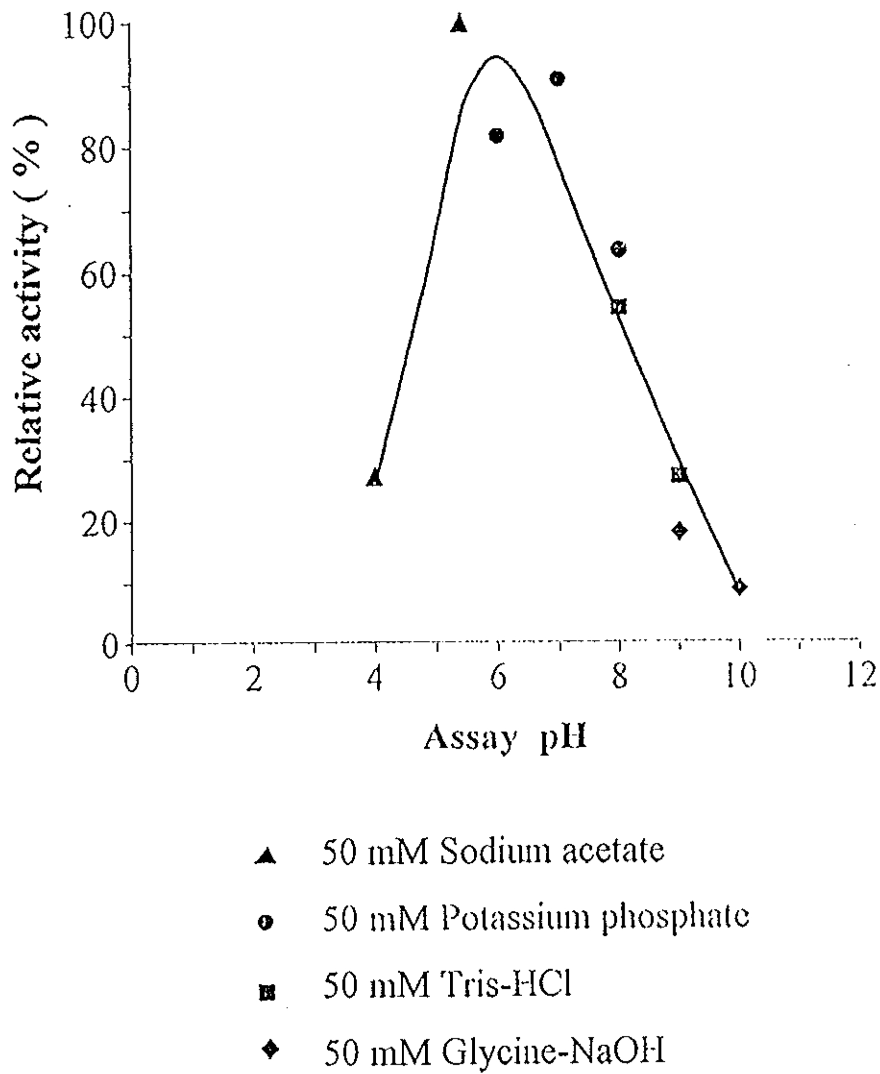


Fig. 11 The pH optimum of the peroxidase assay.

Peroxidase activity was assayed with *o*-dianisidine except that the pH of the buffer was adjusted to various values.

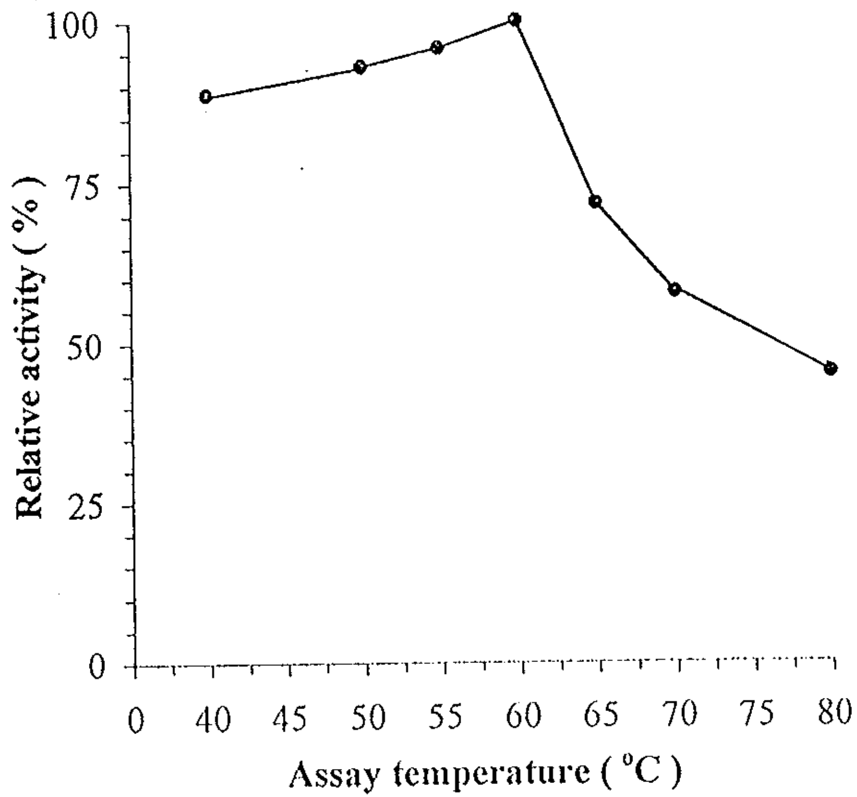


Fig. 12 The optimum temperature of the peroxidase assay.

The effect of temperature on the partially purified peroxidase activity was studied by determining the rate of reaction over the range 40°C-80°C under the standard assay condition.

3.1 Thermal stability

The stability of the *Bacillus* sp. PHS 155 peroxidase at elevated temperatures is noteworthy (Fig. 13). The thermal stability of the enzyme was rather high. When the enzyme was pre-incubated at 50°C and 60°C, the residual activity was still 100% after 3 hrs and 30 min, respectively. Pre-incubation at 70°C caused a rapid activity decrease to 40%. In contrast catalase-peroxidase from a mesophile (*E. coli*) and from a photosynthetic bacterium (*R. capsulata*) lost all activity after 5 min at 60 °C (Nadler, *et al.*, 1986). The enzymatic activity of catalase-peroxidase from halophilic bacterium (*H. halobium*) was completely inhibited at temperature above 50°C (Browm-Peterson and Salin, 1993). The result of Loprasert and colleagues (1988) showed that catalase-peroxidase from *B. stearothermophilus* retained 80% peroxidase activity after incubation at 70°C for 10 min.

3.5 Substrate specificity

From the reaction of peroxidase, compound I of peroxidase could be converted to compound II by the various substrates which served as its electron donors. Various kinds of hydrogen donors were examined as potential substrates for the peroxidase (Table 9). *O*-dianisidine was the best substrate giving the highest enzyme activity, *o*-aminophenol was also a hydrogen donor, giving 16% relative activity. The oxidation of *o*-dianisidine and *o*-aminophenol produced the bisazophenyl and the aminophenoxy radical as the primary product which was converted further into a stable oxidation product. The activity was negligible towards catechol, guaiacol, pyrogallol, dichlorophenol, aminoantipyrine and a mixture of dichlorophenol and aminoantipyrine. In contrast, the peroxidase

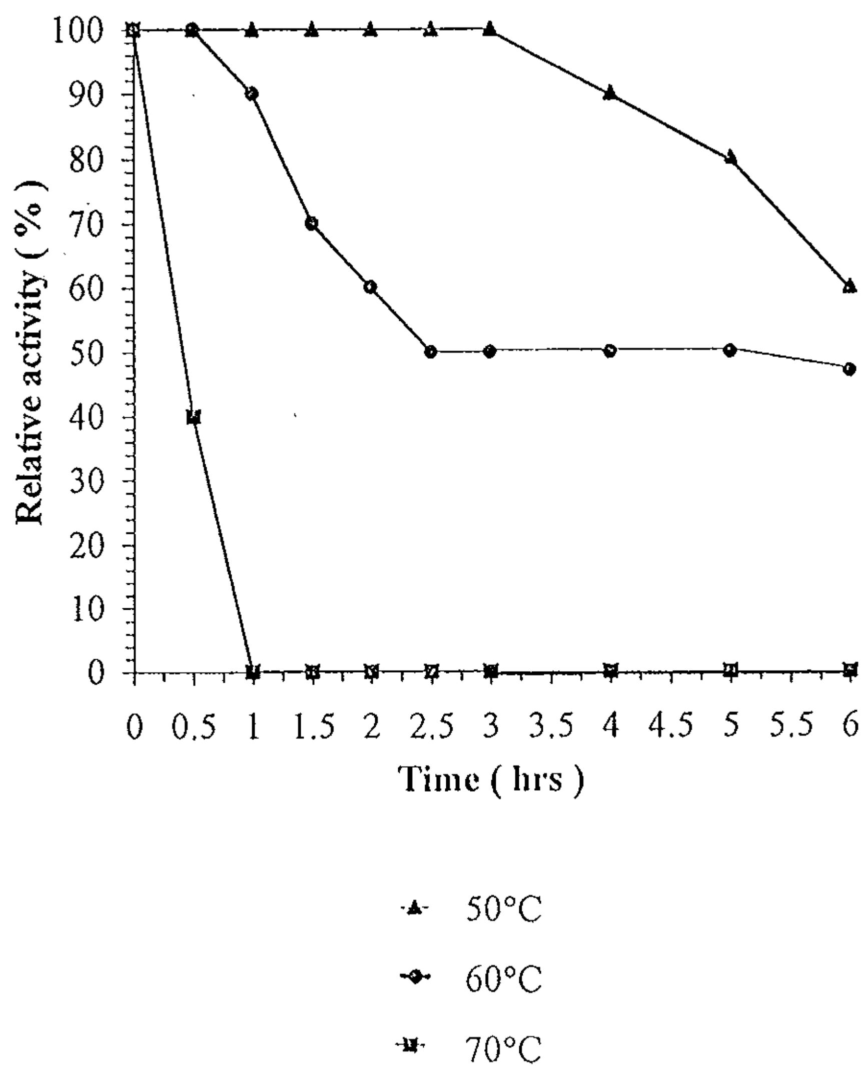


Fig. 13 Thermal stability of the peroxidase assay.

The enzyme was pre-incubated at 50°C, 60°C, 70°C at various times and then assayed for peroxidase activity.

Table 9 Substrate specificity of the partially purified peroxidase from *Bacillus* sp. PHS 155

Substrates	Relative activity (%)*
20 mM *o*-dianisidine	100
42 mM *o*-aminophenol	16
42 mM guaiacol	0
42 mM catechol	0
42 mM pyrogallol	0
4.1 mM dichlorophenol	0
0.67 mM aminoantipyrine	0
4.1 mM dichlorophenol + 0.67 mM aminoantipyrine	0

* The relative activity compared with 20 mM *o*-dianisidine.

from *Synechoccus* PCC-7942 was specific for *o*-dianisidine and pyrogallol (Mutsuda, *et al.,* 1996). However, the peroxidase from *B. stearothermophilus* was highly specific for a mixture of dichlorophenol and aminoantipyrine among the substrates examined. The activity toward *o*-aminophenol and pyrogallol was low, and activity was negligible towards catechol and guaiacol (Loprasert, *et al.*, 1988). This was similar to the substrate specificity of *Pellicularia filamentosa* peroxidase (Ichikawa, *et al.*, 1981). Haemoprotein *b*-590 from *E. coli* (Poole, *et al.*, 1986) and the catalase-peroxidase from *R. capsulata* (Hochman and Shemesh, 1987) also showed no detectable peroxidase activity toward guaiacol.

3.6 Enzyme inhibition

The effects of several metal ions on peroxidase were studied and the results are shown in Table 10. Of all monovalent and divalent cation tested (at 50 mM each) K^+, Na^+, Mg^+ and Ca^{2+} had no inhibitory effect but Zn^{2+} was inhibitory to peroxidase activity. EDTA, ethanol and methanol showed no inhibitory effect. Unlike the peroxidase from *Ipomoea cairica* (Lin, *et al.*, 1996), Fe^{2+} and Fe^{3+} did not inhibited the enzyme activity. It was markedly increased upon the addition of Fe^{2+}. This results might be due to the loss of heme during the purification step.

3.7 Kinetics of the peroxidase reaction

The partially purified enzyme determined with vary concentration of one substrate in the presence of a fixed saturating concentration of the second substrate in 50 mM sodium acetate buffer, pH 5.4. The Michaelis constant (K_m) and the maximum rate (V_{max}) of the enzyme derived from rates of catalysis measured at different substrate concentration. The results

Table 10 Effect of some metal ions on *Bacillus* sp. PHS 155 for peroxidase activity.

Inhibitors	Relative activity (%)*
no inhibitor	100
Fe_2SO_4**	>>100
Fe_3PO_4	92
KCl	100
NaCl	100
$MgCl_2$	100
$CaCl_2$	83
$ZnSO_4$	58
EDTA	108
SDS	25
Ethanol	117
Methanol	108

* The relative activity compared with no inhibitor.
** The reaction product showed the pellet particle.

in Table 11 and 12 were tabulated and calculated to obtain the K_m and V_{max} values for *o*-dianisidine (Fig. 14) and H_2O_2 (Fig. 15). A plot of 1/V versus 1/[S] (Lineweaver-burk plot) showed the K_m values for *o*-dianisidine and H_2O_2 were 50 mM. V_{max} values for *o*-dianisidine and H_2O_2 were 1.11 U/ml and 0.5 U/ml, respectively. This results suggested that the peroxidase had equal K_m values for both substrates. This is common for an enzyme that needs the presence of two substrates simultaneously to achieve half maximal activity (Sattayasevana, 1990). However, the K_m values for the peroxidase reaction was higher than the other microorganisms (Table 3). This result would certainly warrant further investigations.

4. Characterization of encoding gene peroxidase

4.1 Size determination

Previously, Ms. Kintkand Na Pathalung had cloned 9 kb chromosomal DNA from *Bacillus* sp. PHS 155. The peroxidase gene (*perA*) was digested with *Bam*HI and packaged into Lambda GEM^{-11} bacteriophage. After transfection of *E. coli* LE392 with this bacteriophage, peroxidase-producing clones were detected by hybridization assay with pOD68 (1.7 kb) as a probe. For characterization of *perA*, the DNA fragment was removed from the position Lambda GEM^{-11} clone by digestion with *Cla*I. The 9 kb *Bam*HI fragment was deleted and separated by 1% agarose gel electrophoresis, six fragments were obtained (Fig. 16 (A), 17). The result of southern blotting and hybridization to pOD68 probe shows that the gene can be located in a single restriction fragment (5.5 kb) by autoradiography (Fig 16(B)). Because pBluescript II SK+ (3 kb) are

Table 11 Kinetics of the partially purified peroxidase with *o*-dianisidine as substrate and 0.1 M H_2O_2 as co substrate.

O-dianisidine (mM)	U/ml	1/*o*-dianisidine	1/U/ml
12.30	0.212	0.08	4.72
16.40	0.265	0.06	3.77
20.00	0.318	0.05	3.14
24.50	0.360	0.04	2.78
28.70	0.425	0.03	2.35

$1/V_{max} = 0.90$, $V_{max} = 1.11$ U/ml

$-1/K_m = -0.02$, $K_m = 50$ mM

Table 12 Kinetics of the partially purified peroxidase with H_2O_2 as substrate and 20 mM *o*-dianisidine as co substrate.

H_2O_2 (M)	U/ml	$1/H_2O_2$	1/U/ml
0.050	0.239	20.00	4.17
0.075	0.318	13.33	3.14
0.100	0.318	10.00	3.14
0.125	0.356	8.00	2.84
0.150	0.371	6.67	2.70

$1/V_{max} = 2.00$, $V_{max} = 0.5$ U/ml

$-1/K_m = -18$, $K_m = 50$ mM

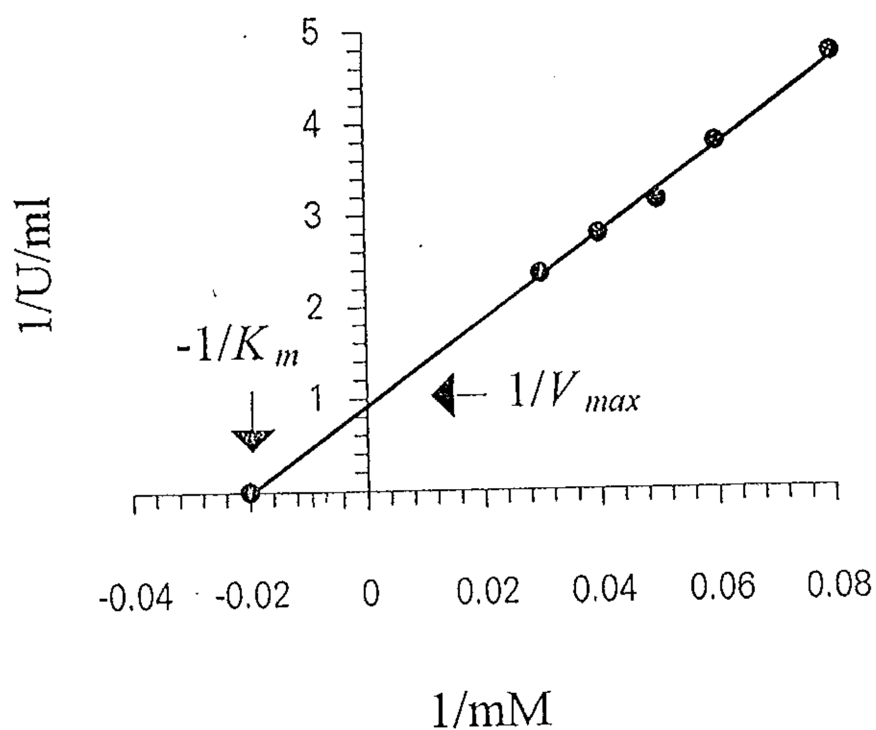


Fig. 14 Kinetics of the partially purified peroxidase with *o*-dianisidine as substrate.

The partially purified peroxidase was assayed with various concentrations of *o*-dianisidine (12.3-28.7 mM) in the presence of 0.1 M H_2O_2.

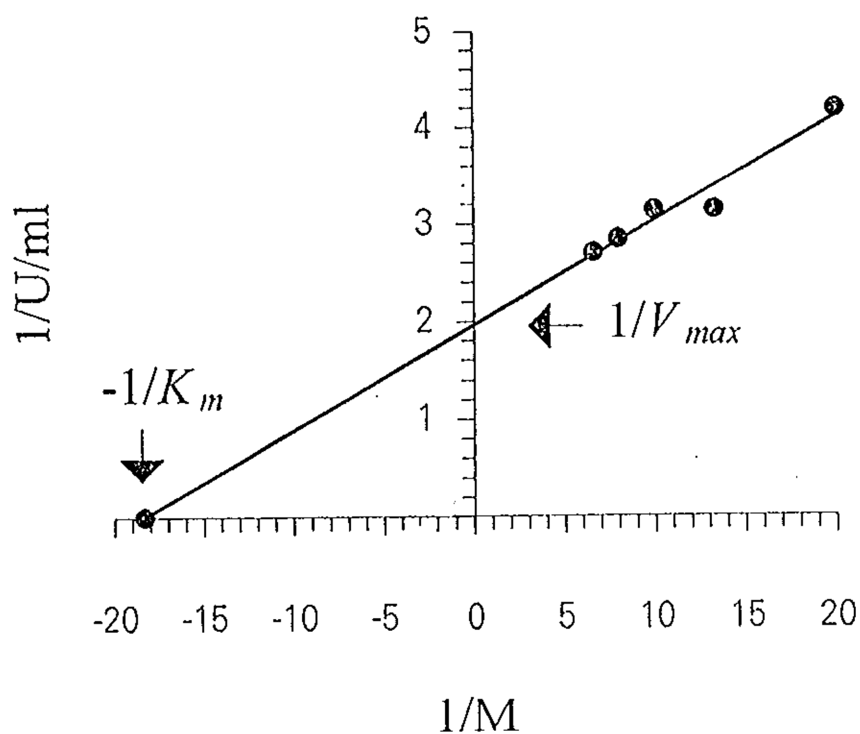


Fig. 15 Kinetics of The partially purified peroxidase with H_2O_2 as substrate.

The partially purified peroxidase was assayed with various concentrations of H_2O_2 (15-50 M) in the presence of 20 mM *o*-dianisidine.

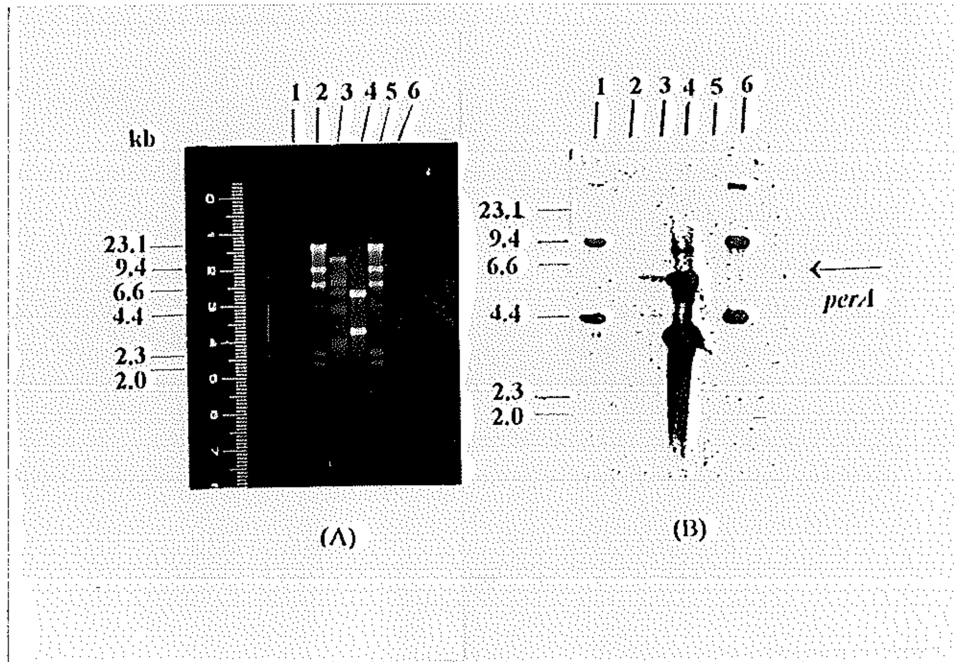
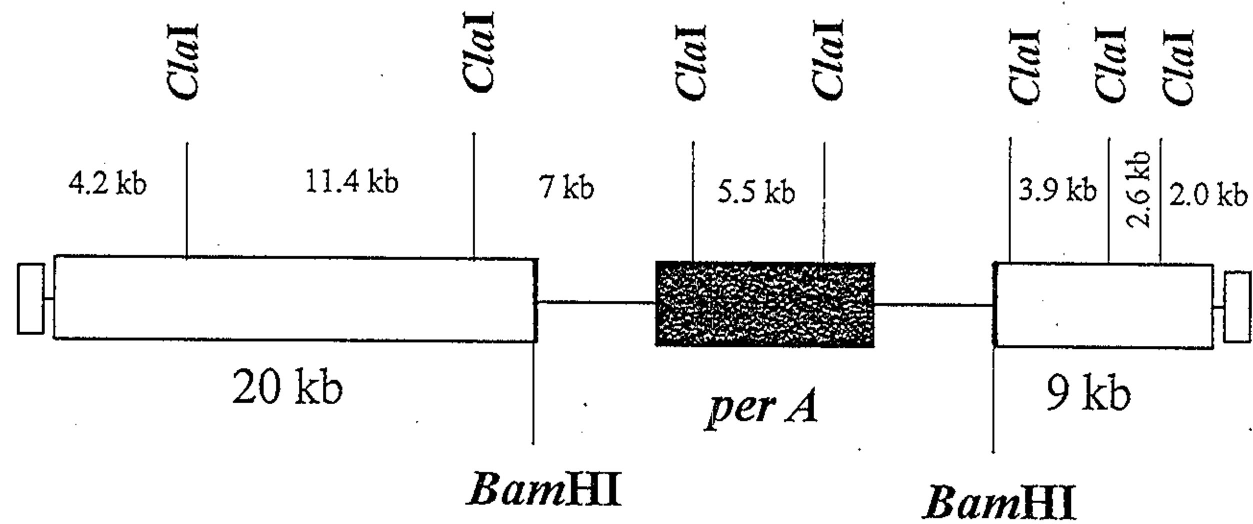


Fig. 16 Size determination of peroxidase gene

(A) The DNA fragment and pBluescript II SK+ contained *perA* were digested with *Cla*I, separated by 1% agarose gel electrophoresis and stained with ethidium bromide. (B) Autoradiography of the gel in panel A, after southern blotting, with pOD68 specific probe. lane 1 and 6, pOD68; lane 2 and 5, marker (λ-*Hind*III); lane 3, Lambda GEM^{-11} *perA* cut with *Cla*I; lane 4 pBluescript II SK+ harboring *perA* cut with *Cla*I. Arrow indicated the peroxidase band.



2 mm. = 1 kb

Lambda GEM-11-*per A*

Fig. 17 The structure of the position Lambda GEM-11 clone.

The 9 kb *perA* which packaged into Lambda GEM-11 was subcloned by *Cla*I restriction enzyme.

cloning vectors that have been designed to simplify commonly used cloning and sequencing procedures. These include construction of nested deletions for DNA sequencing, generation of RNA transcripts *in vitro,* site specific mutagenesis and gene mapping (Stratagene, USA). This fragment was then subcloned in pBluescript II SK+ before transformation to host (*E. coli* UM228). The pBluescript II SK+ harbored the *perA* was designated pBPHS9 (Fig. 18). It was analyzed in southern hybridization as the previous procedure in order to investigate whether the plasmid contained *perA* fragment or not. The exhibited bands of about 5.5 kb and 3 kb were observed (Fig. 16). This result reveals that the length of *perA* may be 5.5 kb. However, in case of 3 kb of pBluescript II SK+ could be detected by pOD68 probe. It describes that the pOD68 may be low specific for *perA* because of the long size of the fragment DNA probe.

4.2. Expression of the peroxidase gene in *E. coli*

One of the main advantages of working with a thermostable protein was the ease of isolation the heat-labile proteins by heat denaturation. Precipitation of all the heat-sensitive proteins by heat treatment effectively facilitates the purification of the thermostable peroxidase from *E. coli* (Loprasert, *et al.*, 1990). So purification of peroxidase was easy when heating a sample could remove a large portion of the contaminant proteins. This was the reason for transformation the *perA* from thermophilic *Bacillus* sp. PHS 155 into *E. coli*. In addition, the overproduction of many prokaryotic proteins in *E. coli* has been made possible by cloning the coding sequences into multiply plasmids. After transformation of *E. coli* UM228 (catalase-deficient mutant) with pBPHS9, the expression of the *perA* was determined by assaying peroxidase activity of the crude extracts

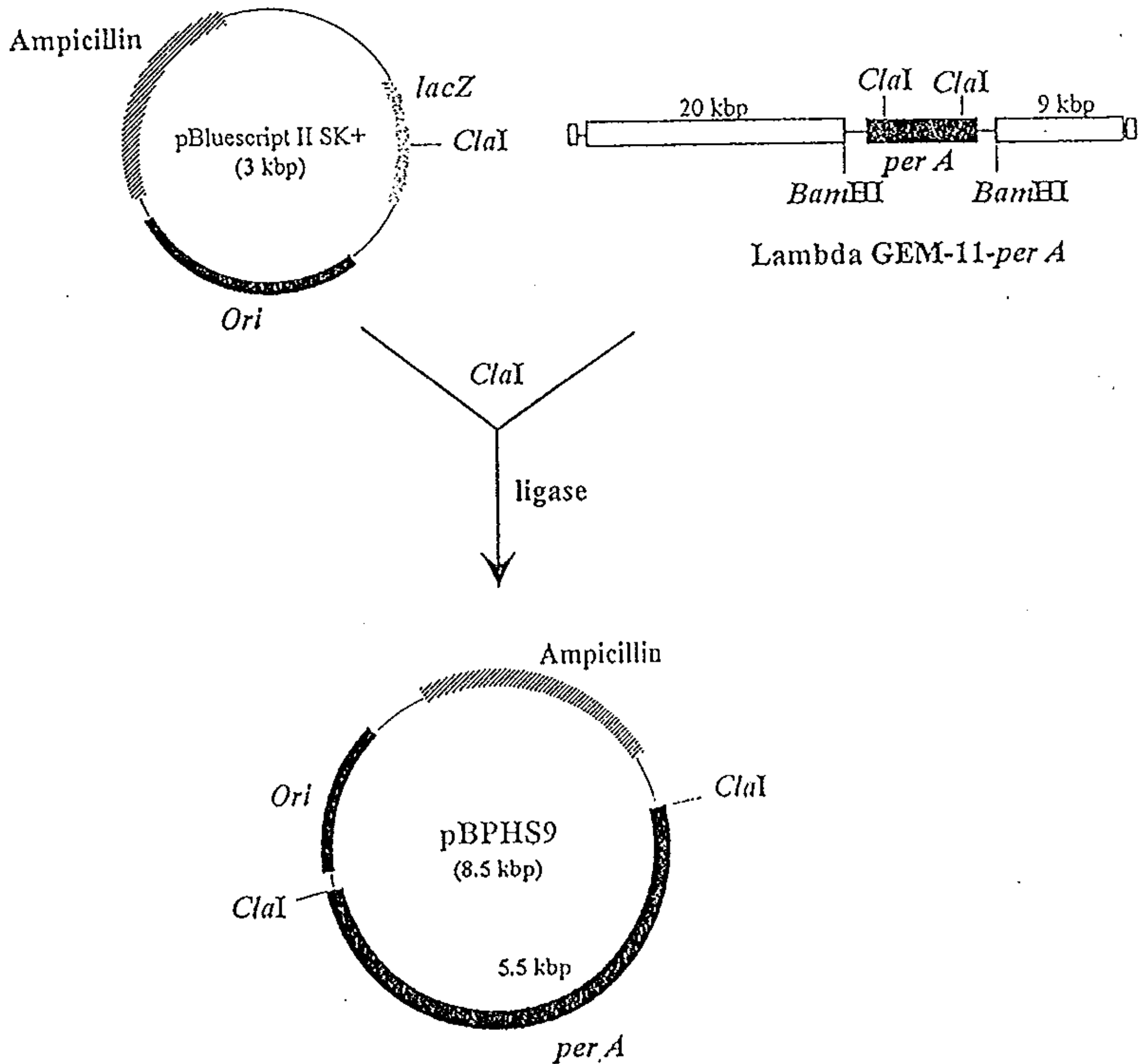


Fig. 18 The construction of pBPHS9.

Plasmid pBPSH9 was a hybrid plasmid consisting of pBluescript II SK+ (3 kb) and the *Cla*I fragment (5.5 kb) containing the peroxidase gene (*perA*) of *Bacillus* sp. PHS 155.

at 60°C. The *p*BPHS9 clone produced the enzyme more than the host *E. coli* UM228 but less than *Bacillus* sp. PHS 155 (Table 13). These results prove that the *perA* of *Bacillus* sp. PHS 155 is expressed in *E. coli.* The expressed product is enzymeically active. Similarly, the expressed product of peroxidase gene from *B. stearothermophilus* in *E. coli* UM228 (Loprasert, *et al.,* 1990). However, the level of activity was lower than wild type. These observation suggests that expression vector may required a gene that facilitates the stringent regulation of promoter activity. This regulatory element may be integrated either in the vector itself or in the host chromosome. Other elements that may be beneficial include transcription and translational "enchancers" as well as "minicistrons" in translationally coupled systems. These may be gene specific; therefore, their utility may be tested case by case (Makrides, 1996).

Table 13 The expression of peroxidase gene.

Strains	Culture temp. (°C)	Specific activity* (U/mg)	Ratio**
Bacillus sp. PHS 155	55	0.38	-
E. coli UM228	37	0.15	1
E. coli (pBPHS9)	37	0.25	1.79

* The activity was assayed at 60°C.

** The ratio compared with *E. coli* UM228.

Chapter 4

Summary

1. Cell cultivation

Intracellular peroxidase activity was detected in culture of *Bacillus* sp. PHS 155 in shaking 2YT medium at 55°C. A time course of cell cultivation demonstrated that this strain could produce peroxidase in exponential-phase growth and reached a maximum activity 0.22 U/ml at 30 hrs.

2. Purification of peroxidase

A peroxidase was purified from the crude extract of *Bacillus* sp. PHS 155 by ion exchange chromatography (DEAE-Sephacel) and gel filtration (Sephadex G-200, G 50-150, Sephacryl S-300) techniques. The enzyme was purified about 8.76 times with a recovery of 0.05%. The specific activity of enzyme was 3.33 U/mg. The purified peroxidase showed the major and minor bands on analytical PAGE. When strained for peroxidase activity with 3,3'-diaminobenzidine, a brown band become visible at a major band on PAGE.

3. Characterization of enzyme

When the partially purified enzyme was subjected to SDS-PAGE, the molecular weight of the peroxidase *Bacillus* sp. PHS 155 was 67 kDa. In addition, the molecular estimated from gel chromatography on Sephacryl S-300 was 67 kDa. For this result, peroxidase was thus revealed to be a monomeric enzyme. The pH optimum for the partially purified enzyme

showed high activity in the range 5-7. Under the assay conditions employed the optimum temperature of the enzyme was 60°C. The thermal stability of the enzyme demonstrated that a half life at 60°C and 70°C were 2 hrs 30 min and 30 min, respectively. To study the substrate specificity of the peroxidase, different kinds of hydrogen donor substrates were incubated with the purified enzyme. Among these compounds, only *o*-dianisidine and *o*-aminophenol gave relative activities of 100% and 16%, respectively. The activity was inhibited by Zn^{2+} and 1% SDS but was rapidly increased by addition of Fe^{2+}. For enzyme kinetic value, K_m and V_{max} for H_2O_2 were 50 mM and 0.5 U/ml as well as K_m and V_{max} for *o*-dianisidine were 50 mM and 1.11 U/ml.

4. Characterization of encoding gene peroxidase

4.1. Size determination

Peroxidase gene of *Bacillus* sp. PHS 155 in the positive Lambda GEM^{-11} *perA* clones were deleted by *Cla*I restriction enzyme. The result showed 6 fragments in 1% agarose gel electrophoresis. Autoradiography of southern hybridization analysis of genomic DNA showed that the 5.5 kb *Cla*I restriction fragment carrying peroxidase gene. But the total length of peroxidase gene and its DNA sequence are currently unknown. It was inserted in pBluescript II SK+ vector for transforming into *E. coli* UM228.

4.2 The expression of encoding gene peroxidase

E. coli UM228 which carried peroxidase gene deletion, was incubated in shaking 2YT medium at 37°C. The extracts were assayed at 60°C for peroxidase activity found about 1.79 fold higher than the amount that found in *E. coli* UM228 wild type but less than *Bacillus* sp. PHS 155.

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Appendix

1. Protein determination (Bradford, 1976)

Materials

1. Reagent : The assay reagent is made by dissolving 100 mg of Coomassie Blue G250 in 50 ml of 95% ethanol. The solution is then mixed with 100 ml of 85% phosephoric acid and made up to 1 l with distilled water. The reagent should be filtered through Whatman No.1 filter paper brgore stroage in an amber bottle at room temperature. It is stable for several weeks, but slow precipitation of the dye will occur, so filtration of the storaged reagent is necessary brefore use.

2. Proteins standards : Albumin at a concentration of 1 mg/ml (1000 µl / ml for the microassay) in distrilled water is used as a stock solution. This should be stroaged frozen.

3. Plastic and glassware used in the assay should be absolutelt clean and detergent-free. Quartz (silica) spectrophotometer cuvetts should not be used, since the dye binds to thai material. Traces of dye bound to glassware or plastic can be removed with ethanol or detergent solution.

Method

1. Standard Method Pipet between 10 and 100 µl total volume into a test tube. If the approximated sample concentration is unknown, assay a rang of dilutions (1, 1/10, 1/100, 1/1000). Dupilcated each sample.

2. For the calibration curve, pipet duplicate volumes of 75, 152, 250, 500, 750 and 1000 µl albumin stock solution into test tube and make each up to 1000 µl with distilled water. Pipet 10 µl of distrilled water into a further tube for the reagent blank.

3. Added 3 ml of proteins reagent to each tube and mix well by inversion or gentle vortexing. Avoid foaming, which will lead to poor reproducibility.

4. Measure the OD_{595} of the samples and standards against the reagent blank after at least 2 min and within 1 hr. of mixture. The 100 µl standard should give an OD_{595} value of about 0.2. Since the calibration curve is not linear, it should be determined and plotted for each set of assays.

5. Pipet duplicate samples containing between 1 and 10 µg in a total volume of 10 µl into test tubes into test tubes or eppendorf tube. If the approximate sample concentration is unknown, assay a rang of dilutions.

6. Pipet 10 µl of distilled water into a tube for the reagent blenk.

7. Add 3 ml of protein reagent, mix and meaure the OD_{595} as in step 4 of the standard method. The 10 µg standard should give an OD_{595} value of about 0.15.

2. SDS-PAGE Electrophoresis (Weber and Osborn, 1969)

Material

1. 10% SDS-PAGE resolving gel

H_2O	7	ml
30%Acrylamide mixture	10	ml
1 M Tris-HCl (pH 8.8)	11.25	ml
10%SDS	0.3	ml
1.5%Ammonium persulphate	1.5	ml
TEMED	0.030	ml

2. SDS-PAGE stacking gel

H_2O	11.3 ml
30% Acrylamide mixture	2.5 ml
0.5 M Tris-HCl (pH 6.8)	5.0 ml
10%SDS	0.2 ml
1.5% Ammonium persulphate	1 ml
TEMED	0.030 ml

3. Coomassie Brilliant Blue R-250 staining

Coomassie blue R-250 0.1% in water	208 ml
Acetic acid	84 ml

Adjust with distilled water to 500 ml

4. 30% Acrylamide mixture

Acrylamide	30 g
Bisacrylamide	0.8 g

Adjust with distilled water to 100 ml

Stored in refrigerator

5. Electrophoresis buffer

25 mM Tris-HCl	3 g
19 mM Glycine	14.4 g
01%SDS	1 g

adjust with distilled water to 1 l

Method

1. The slab gel (9x5x0.1 cm) with a 10 % separating gel and stacking gal (9x2x0.1 cm) was used.

2. The protein sample was mixed with a sample buffer containing 1% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.4 mg of bromophenol

blue in 0.5 M Tris-HCl buffer, pH 6.8, and boiled for 5 min. Electrophoresis was carried out at room temperature. A constant voltage of 10 milliampere was supplied until the tracking dye approached the buttom of tha gel.

3. After the electrophoresis was finished, the gel was stainning with Coomassie Brilliant Blue R-250.

3. Southern Blotting and Hybridization (Sambrook, *et al.*, 1989)

3.1 Southern Blotting

Material

0.2 N HCL

Denaturation solution (1.5 M NaCl, 0.5 M NaOH)

Neutralization solution (3 M NaCl, 0.5 M Tris-HCl pH 8)

20XSSC (3 M NaCl, 0.3 Sodium citrate, adjust to pH 7)

2XSSC

0.1%SDS

Whatman 3 MM paper and nylon membrane

Method

1. After electrophoresis, transfer thagel to a glass baking dish and trim away any unused areas of the gel with a razor blade. Cut off the bottom left-hand corner of the gel; this serves to orient the gel during the succeeding operations.

2. Denature the DNA by soaking the gel for 45 min in several volumn of denaturation solution with constant, gentle agitaton.

3. Rinse the gel briefly in deionized water and then neutralize it by soaking for 30 min in several volumns of neutralization solution at room

temperature with constant, gentle agitation. Change the neutralizaton solution and continue soaking the gel for a further 15 min.

4. While the gel is in the neutralization solution, wrap a piece of Plexiglas or a stock of glass plates to form a support that is long and wider than gel. Place the wrapped support inside a large baking dish. Fill the dish with transfer buffer (10XSSC) until the level of the liquid reachs almost to the top of the support. When the 3 MM peper on the top of the support is throughly wet, smooth out all air bubbles with a glass rod.

5. Using a fresh scalpel or a paper cutter, cut a piece of nitrocelllose fliter about 1 mm larger than the gel in both dimensions. Use gloves and blunt-ended forceps to handle the flilter. A nitrocellutose filter that has been tonched by greasy hands will not wet.

6. Float the nitrocellulose filter on the surface of a dish of deionized water until it wets completely from beneath, and then immerse the filter in transfer buffer for at least 5 min. Using a clean scalpel blade, cut a corner from the ntrocellulose filter to match the corner cut from the gel.

7. Remove the gel from the neutralization solution and invert it so that its underside is now uppermost. Place the inverted gel on the support so that it is centered on the wet 3 MM papers. Make sure that there are no air blubbles between the 3 MM paper and the gel.

8. Surround, but donot cover, the gel with Saran wrap or Parafilm. This serves as a barrier to pervent loquid from flowing directly from the reservior to paper tomels placed on top of the gel. If these towels are not percisely stacked, they tend to deoop over the edge of the gel and may touch the support. This type of short-circuiting is a major cause of inefficient transfer of DNA from tha gel to filter.

9. Place the wet nitrocellulose filter on top of the gel so that the cut corners are aligned. One edge of the filter should extend just over tha edge of the line ofslots at the gel. Make sure that there are no air bubbles between the filter and gel.

10. Wet two pieces of 3MM paper (cut to extactly the same size as tha gel) in 2xSSC and place them on top of the wet nitrocellulose filter. Smooth out any air bubbles with a glass rod.

11. Cut a stack of paper towels (5-8 high) just smaller than the 3MM paper. Place the towels on the 3MM papers. Put a glass plates on top of the stack and weigh it down with a500-g weight. The objective is to set up s flow of liquid from the resrevior through the gel and the nitrocellulose filter, so that fragments of denatured DNA are eluted from thagel and are deposited on the nitrocellulose filter.

12. Allow the transfer of DNA to proceed for 8-24 hr. As the paper towels become wet, they should be replaced.

13. Remove tha paper towels and the 3MM papers above tha gel. Ture over the gel and the nitrocellulose filtre and lay them , gel side up, on a dry sheet of 3MM paper. Mark tha position of the gel slots on thafilter with a very-soft-lead pencil or ballpoint pen.

14. Peel the gel from thefilter and discard it. Soak thefilter in 2xSSC for 5 min atroom temperature. This removes any pieces of agarose sticking to the filter.

15. Remove the filter from the 2xSSC and allow excess fluid to drein away. Place the filter flat on a paper towel to dry for at least 30 min at room temperature.

16. Sandwich the filter between two sheets of dry 3MM paper. Fix the DNA to the membrane by UV crosslinking.

3.2. DNA hybridization precedure

Materials

Prehybrization and Hybridization buffer

2xSSC

0.5% (w/v) Blocking reagent

5% (w/v) Dextran sulphate

0.1% (w/v) SDS

Method

1. Eqilibrate the membrane with the crosslinked, denature target in 2xSSC. Ensure that the entire membrane is welled before proceeding.

2. Insert membrane into an appropriate hybridization pourch or tube. Add prehybridization buffer supplement with 50 µl/ml Carrier DNA. For standard heat sealable polyester pouchs, a minimum volume of 0.1 ml/cm^2 of membrane is recommended.

3. Incubate at 65°C, in a shaking water bath, for at least one hour.

4. Combine the probe with 200-300 µl of hybridization buffer and Carrier DNA. The final concentration of carrier DNA in the hybridization step should be 50 µg/ml. Heat 95°C for 3-5 min and immediatedly place on ice for 5 min.

5. Add hybridization buffer, equilibrated at 68°C, to the denatured probe mix. The recommened hybridization volume for standard polyester pouchse is 0.05-0.1 ml/cm^2 of membrane. The recommened concentration of labeled probe in hybridization buffer is 20 ng/ml.

6. Empty the prehydization buffer from the pouch or tube and add the hybrization buffer (with probe and carrier DNA).

7. For maximum probe to target base pairing, hybridize overnight at 68°C, in a shaking water bath. If the template DNA for the probe labeling reaction was less than 500 base pairs then, hybridize at 55-60°C instead of 68°C.

4. Detection-nucleic acid chemiluminescence reagent (DuPont NEN, USA)

All step are carried out at room temperature.

Material

1. Buffer 1 (0.10 M Tris-HCl, pH 7.5, 0.15 M NaCl) Note : To minimize bavkground, pass thriugh a 0.22 μm filter brfore use.

2. Buffer 2 (0.10 M Tris-HCl, pH 7.5, 0.15 M NaCl)Note : To minimize backgroung, pass through a 0.22 μm filter before use. Add Blocking reagent after filtration.) 0.5% (w/v) blocking reagent

3. Antibody conjugate solution (i.e.,Conjugate in Buffer 2)

0.10 M Tris, pH 7.5

0.15 M NaCl

0.5% (w/v) blocking reagent

1/1000 (v/v) Antifluorescein-AP Conjugate

4. Buffer 3 (0.10 M Tris-HCl, pH 9.5, 0.10 M NaCl)

Method

1. Immerse and vigorously agitate the membrane in buffer 1 for 5 min. Use at least 1 ml of buffer per cm^2 of membrane. Ensure that all SDS (

indicated by soap-like foam in wash buffer) has been washed from the membrane before proceeding.

2. Block the membrane in buffer 2 for 1 hr with gentel agitation. Use at least 0.1 ml of buffer per cm2 of membrane.

3. Place tha membrane in the antibody conjugate solution and incubate for 1 hr with gentle agitation. Use at least 0.1 ml of solution per cm2 of membrane.

4. Vigorously wash the membrane 4x5 min in buffer 1.

5. Vigrously wash the membrane 2x5 min in buffer 3.

5. Chemiluminiscence substrate incubation (DuPont NEN, USA)

Material

1. CDP-*Star*^{tm} Nucleic Acid Chemiluminescence Reagent {(Raedy-to-Use) NEL60]

2. Blotting paper

3. Polypropylene sheet protector or plastic wrap

Method

1. Transfer the membrane from the final antibody conjugate wash solution to a vlean container.

2. Completely cover the membrane with CDP-Startm(0.01-0.05 ml/cm2)

3. Incubate the substrate on the membrane for 5 min.

4. Gently remove excess solution with blotting peper.

5. Place the damp membrane inside a polypropylene sheet protector with the black interleaf remove. Plastic wrap can be used in plase of a sheet protector. The plastic sheet protects the film from the wet

membrane. Ensure that there is no moisture on the outside of the sheet protector before overlay with film.

6. Film Exposure (FujiRx, Japan)

Material

1. Autoradiography film.
2. Film Expose cassette

Method

1. Place the prepare membrane, DNA side up, in a film cassette.
2. Expose the flm for 5-10 min and then develop.
3. Repeat the film expose, varying the expose times as needed for optimum sensitivity. A 5-10 min expose results in adequate signal detection for many applicaton (e.g., single gene copy). However, greater signal detection can be achieved when film expose begin at least 1 hr after incubation with CDP-*Star*^{TM}.

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