The Sequencing of a Peroxidase Encoding Gene from *Bacillus* sp. PHS155

Supreya Pongprerachok

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Thesis Title	The Sequencing of a Peroxidase Encoding Gene from *Bacillus* sp. PHS155
Author	Miss Supreya Pongprerachok
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K. Chantrapromma .....

(Assoc. Prof. Dr.Kan Chantrapromma)
Dean, Graduate School

ชื่อวิทยานิพนธ์	การเรียงลำดับเบสของยีนเปอร์ออกซิเดสจากเชื้อ *Bacillus* sp. PHS155.
ผู้เขียน	นางสาว สุปรียา พงศ์พีรโชค
สาขา	ชีวเคมี
ปีการศึกษา	2540

บทคัดย่อ

เปอร์ออกซิเดสที่ผลิตได้จากพืช เช่น ฮอสเรดิสสามารถนำไปใช้ในการพัฒนาขบวนการเทคโนโลยีและการวินิจฉัยโรคต่างๆ ได้มีการศึกษาเอนไซม์เปอร์ออกซิเดสในเชื้อจุลินทรีย์พบว่าจะมีคุณสมบัติเปอร์ออกซิเดสร่วมกับคาทาเลส อย่างไรก็ตามในประเทศไทยได้แยกเชื้อ *Bacillus* sp. PHS155 ซึ่งสามารถผลิตเปอร์ออกซิเดสที่ปราศจากการรบกวนของคาทาเลสและทนต่ออุณหภูมิสูงได้ เมื่อศึกษายีนเปอร์ออกซิเดสพบว่ามีขนาด 9.0 กิโลเบสที่ถูกโคลนเข้าสู่ λ GEM-11 โดยในงานวิจัยนี้ได้ทำการสับโคลนนิ่งยีนที่ผลิตเปอร์ออกซิเดสของเชื้อ *Bacillus* sp. PHS155 ซึ่งมีขนาด 2.7 กิโลเบสและ open reading frame ยาว 2,187 คู่เบส เมื่อแปลรหัสทางพันธุกรรมจะให้สายโปรตีนที่ประกอบด้วยกรดอะมิโน 728 ตัว น้ำหนักโมเลกุล 82,321.84 ดาลตัน, ประกอบด้วยเบส G + C 48.46% ค่า pI เท่ากับ 5.31 ตำแหน่งของ Shine-Dalgarno sequence อยู่ที่เบสตัวที่ 9 ถัดจากจุดเริ่มต้นของสายโปรตีน ทางด้าน upstream เมื่อเปรียบเทียบความเหมือนกับสายโปรตีนของเอนไซม์คาทาเลส I (ไฮโดรเปอร์ออกซิเดส) จากเชื้อ *Bacillus stearothermophilus* จะมีค่า 85.3%

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Abstract

Extracting peroxidase from plants such as the horseradish has been extensively studied and it is widely used for the development of many biotechnological proceses and diagnotic assays. This enzyme can be found in various microorganisms and a few studies on microbial have been published. Most of the microorganisms produce peroxidase which have peroxidase and catalase activity. However, *Bacillus* sp. PHS155 which was isolated in Thailand, produces peroxidase without catalase activity and tolerated high temperature. The encoding gene is considered to have a size of 15.0 kb has been cloned in λ-GEM-11. In this report, we subcloned the peroxidase gene from *Bacillus* sp. PHS155 in *Escherichia coli* UM228. The nucleotide sequence of 2.7 kb *EcoR*I fragment containing the peroxidase gene (*perA*) consisted of an open reading frame 2,187 base pairs encoding a peroxidase of 728 amino acid residues. This enzyme shows molecular weight of 82,321.84 dalton. The G+C content of the peroxidase gene is 48.46% and isoelectric point is 5.31. A Shine-Dalgarno sequence was found 9 base pair upstream from the translational starting site. Comparison of the deduced amino acid sequence peroxidase

of *Bacillus* sp. PHS155 shows 85.3% homology in the amino acid sequence to the catalase I (hydroperoxidase) of *B. stearothermophilus.*

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I would like to express my sincere gratitude and deepest appreciation to my advisor, Assistant Professor Dr. Chokchai Wanapu for his kindness, valuable advice, encouragement, suggestions, constructive and intensive supervision throughout the laboratory work and the preparation of this thesis. I am indebted to a member of the thesis examination committee, Assistant Professor Dr. Amonrat Phongdara for providing constructive comments and suggestions, and also for proof reading the thesis.

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Supreya Pongprerachok

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List of Abbreviations

°C	= degree celcius
dATP	= deoxyadenosine 5' -triphosphate
dCTP	= deoxycytidine 5' -triphosphate
dGTP	= deoxyguanosine 5' -triphosphate
dNTP	= deoxynucleotide 5' -triphosphate
dTTP	= deoxythymidine 5' -triphosphate
DNA	= deoxyrybonucleic acid
EDTA	= ethylenediamine tetra acetic acid
fig.	= figure
g	= gram
mg	= miligram
µg	= microgram
ng	= nanogram
nm	= nanometer
hr	= hour
bp	= base pair
kb	= kilobase pair
M	= molar
mM	= millimolar
pmole	= picomole
l	= litre
ml	= millilitre

µl	= microlitre
min	= minute
IPTG	= isopropylthiogalactoside
OD	= optical density
PEG	= polyethyleneglycol
%	= percentage
rpm	= round per minute
sec	= second
SDS	= sodium dodecyl sulfate
TEMED	= tetramethyl ethylenediamine
TE	= Tris-EDTA buffer

Chapter 1

Introduction

Enzymes play a variety of important roles in plants, animals and also the microbial environment. Some enzymes have been widely used in the development of many biotechnological processes both in clinical diagnosis and in some industrial applications and one of these enzymes is peroxidase. This enzyme can be found in higher plants, animals and various microorganisms. Currently, peroxidase is of interest to biological researchers, because it has been widely used in the clinical field for the colorimeteric measurement of biological materials for diagnosis and also for various laboratory experiments (Kim and Yoo, 1996). In addition, some scientists have also used this enzyme to detoxify phenols and aromatic substances from industrial waste water (Alberti, *et al.*, 1981).

Peroxidase from plants has been extensively studied, such as the horseradish (*Armoracia rusticana*) peroxidase group, which consists of more than 40 components (Intrapruk, *et al.*, 1994). However, they have very low specificity and exist as a multitude of isoenzyme which makes it difficult to ascertain their actual functions (Kawaoka, *et al.*, 1994). Because of the limited supply of plant peroxidase , horseradish and especially its high cost in production, there are many problems in the production of peroxidase using a suspension cell culture system. These problems include 1). slow growth rate, 2). sheer sensitivity and 3). low yield of product (Kim and Yoo, 1996). Thus, identifying for a microbial peroxidase, which can perform the same activity as horseradish enzyme,

but which can be made cheaper and in unlimited quantities if necessary (Loprasert, *et al.*, 1989), would be useful. A microbial peroxidase is sought to which will make the method more commercially attractive because of their biochemical diversities and these enzyme concentrations can be increased by environmental and genetic engineering. The peroxidase from microorganisms usually acts as a catalase-peroxidase enzyme that can catalyze both catalase and peroxidase reactions at significant rates.

Loprasert and colleagues (1988) purified a thermostable catalase-peroxidase from a thermophilic bacterium, *Bacillus stearothermophilus* IAM 11001, and the enzyme was found to have catalytic activity. The peroxidase gene (*perA*) was cloned and sequenced. The deduced amino acid sequence showed 48% homology to that of *Escherischia coli* catalase HPI (Loprasert. *et al.*, 1989). Because of its remarkable heat stability, the enzyme is potentially useful for practical application.

Recently, *Bacillus* sp. PHS155 has been isolated from hot ponds in Southern Thailand. This bacteria can produce peroxidase without catalase activity and which tolerates high temperature. The peroxidase produced from this strain has been purified and characterized (Thongma, 1997). In addition, a 9.0 kb *BamH*I fragment of *Bacillus* sp. PHS155 DNA containing the peroxidase encoding gene was cloned into Lambda GEM-11 (Na Pathaluing, 1996).

The objective of this work was to study the subcloning of the peroxidase encoding gene of *Bacillus* sp. PHS155 in *E. coli* and to determine the nucleotide sequences. The homology of the amino acid

sequence with that of the catalase I of *B. stearothermophilus* IAM 11001 was also discussed.

Literature Review

1. Structure of peroxidase

Peroxidases (donor : hydrogen-peroxide oxidoreductase, EC 1.11. 1.7). The feature of a common active site or prosthetic group (heme) of enzyme is composed of an iron-porphyrin complex (Loew, 1983).

Porphyrin is formed by the condensation of glycine and succinyl CoA into δ-aminolevulinate. Two molecules of δ-aminolevulinate then condense to from porphybilinogen. Four porphybilinogen condense head-to-tail to form a linear tetrapyrrole, which remains bound to the enzyme. The linear tetrapyrrole cyclizes by losing the ammonium ion. The cyclic product is uroporphyrinogen III ; an asymmetric arrangement of side chain. The cosynthetase is essential for isomerizing one of the pyrrole ring to yield uroporphyrinogen III. 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 porphyrin ring and conversion of two of the propionate side chains into vinyl groups yield protoporohyrin IX. Chelation of iron finally gives heme (Fig. 1) (Stryer, 1981).

2. Enzyme reactions

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 an organic hydroperoxide. The interaction of the resting enzyme ferriperoxidase (PFe^{3+}) with H_2O_2 results in the formation of an unstable intermediate. This intermediate

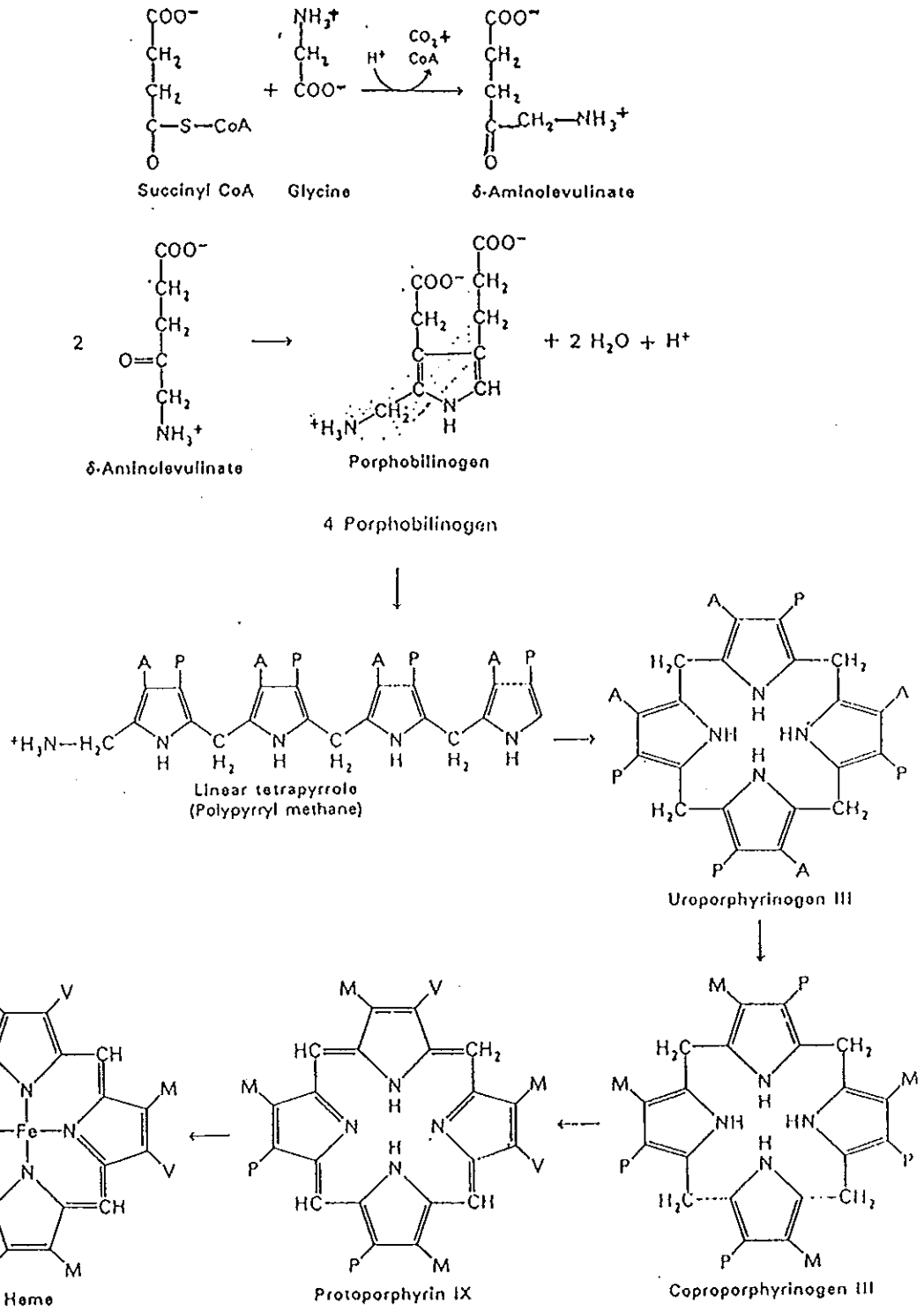


Fig. 1 Pathway for the synthesis of heme

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

source : Stryer (1981)

known as compound I, reacts with an electron donor (HDH), to from compound II. The compound II removes an additional electron and return to the resting from.

Schematically :

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

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

compound II + HDH $\rightarrow$ 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, peroxidase use H_2O_2 to oxidize a variety of compounds whereas catalase is usually defined as the enzyme catalyzing the dismutation of H_2O_2 to O_2 and H_2O. The study of the peroxidase reaction is frustrated by the ability of enzyme to act as a catalase and decompose H_2O_2 (Loprasert, *et al.*, 1988). Rehm and Reed (1987) have found a reaction to be useful to improve the removal of stain.

Reactions :

$$2\,HA + H_2O_2 \xrightarrow{\text{Peroxidase}} 2\,H_2O + 2A \quad (A : \text{a color product})$$

$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2$$

Peroxidase catalyzes the dehydrogenation of a large number of organic compounds for example phenol hydroquinones and hydroquinoid amines, especially benzidine derivatives: *o*-toluidine, *o*-cresol, *o*- or *p*-phenylenediamine, *p*-hydroquinone, guaiacol, pyrogallol, homovanillnic, leucomalacthite green, reduced *o*-toluidine, *o*-dianisdine, benzidine, 4,4-diaminodiphenylamine, 2,6-dichlorophenoliphenol and some azo dyes

derived from these. 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 ($SH + O_2 \rightarrow S + H_2O_2$) and mono-oxygenases ($S\text{-}H + O_2 + NADPH \rightarrow S\text{-}OH + NADP^+ + OH^-$). On the other hand, reaction of peroxidase can be catalyzed by catalase with certain substrates, e.g. alcohol, phenol and several inorganic compounds, as well as by haemoglobin and some of its break-down products with typical peroxidase substrates. Oxidase can in many cases react like peroxidase and use H_2O_2 as a source of oxygen. 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 (Putter, 1974).

The term peroxidase in its widest sense includes a group of specific enzymes such as NAD peroxidase, NADH peroxidase, fatty acid peroxidase, cytochrome peroxidase and glutathione peroxidase as well as a group of non-specific enzymes from different sources, which are simply known as peroxidase such as horseradish peroxidase.

3. Enzyme properties

Metabolism in an oxygen-enriched environment often results in the generation of reactive oxygen species such as superoxide, hydroxyl radical and hydrogen peroxide. Among these systems in the group of heme proteins designated hydroperoxidases. The group consists of three classes of proteins : 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). Catalases isolated from higher organism are similar to each other in that they have molecular weights in range of 225-270 kDa, contain four equally sized subunits each containing one ferric heme prosthetic group (photoporhyrin IX) show a broad pH range of 5 to 10.5, and are specifically inhibited by 3-amino-1, 2, 4-triazole (Brown- Peterson and Salin, 1993). Other characteristics of these typical catalases are hydrophobic properties which are revealed by extractability in ethanol-chloroform and binds to phynyl-Sepharose (Hochman and Shemesh, 1987). The typical peroxidases catalyzes the oxidation of H_2O_2 by a large variety of substrates. It also may function in the detoxifying of hydrogen peroxide as well as in various cellular activities of biosynthesis and degradation (Elstner, 1982). The peroxidases, like catalases, are heme enzymes, but they are monomeric proteins which show diversity in their molecular weight. Moreover, the heme iron of catalases is not reducible, whereas the heme iron of the peroxidases can be reduced with dithionite (Brown-Peterson and Salin, 1993). The peroxidases constitute a heterogeneous group of enzymes that share with catalases a number of structural and physicochemical properties (Hochman and Shemesh, 1987). Morishima and his colleagues (1986) have reported that the catalytic property of horesradish peroxidase (HRP) is regulated by the binding of 2 mol of endogeneous Ca^{2+} to the enzyme.

Brown-Peterson and Salin (1993) reported the catalase-peroxidases were only recently classified as a distinct group of enzymes. The catalase-peroxidases have been identified in various genera of microorganisms.

They exhibit both catalase and peroxidase activities. Like that of typical peroxidases, the heme component of these enzymes can be readily reduced with dithionite. They are also similar to typical catalase in that they exhibit a tetrameric molecular weight in the range of 240 kDa with equally sized subunits. However, they possess narrow pH ranges for maximal activity and increased sensitivity to temperature, are inactivated by H_2O_2, and are not inhibited by 3-amino-1, 2, 4-triazole. In addition, the enzyme can oxidize the pyridine nucletides NADH , NADPH and cytochrome c (Hochman and Goldberg, 1991). Table 1 summarized the reports available on the characteristic of peroxidases.

4. Sources of Enzymes

The peroxidases are widely distributed and have been isolated from plants, animals, as well as from microorganisms. One of these three sources may be favored for a given enzyme.

4.1 Plant Enzymes

The source of plant peroxidase can be found in the guttation fluid from *Helianthus annuus*, xylem sap (Magwa, *et al.*, 1993), carrot hairy root cell culture (Kim and Yoo, 1996), barley leaves (Saeki, *et al.*, 1986), barley seed (Theilade and Rasmussen, 1992), and *Ipomoea batanas* seedlings (Floris, *et al.*, 1984). However, Plant peroxidases occur in the cytoplasm and on membranes, oxidatively degrade the important plant hormone indole acetic acid (IAA) (Gasper, *et al.*, 1982). They play an integral role in secondary cell wall biosynthesis by catalyzing the polymerization of cinnamyl alcohol into lignin and by forming rigid cross-links between

Table 1 characteristics of peroxidase from different microorganisms

strains	MW (kDa)	feature	optimum		stability		pI	K_m for H_2O_2 (mM)	references
			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	Claibone and Fridovich, 1979
Halobacterium halobium	110	mono	UD	UD	UD	UD	UD	7.7μM	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

Table 1 (continue)

strains	MW (kDa)	feature	optimum		stability		pI	K_m for H_2O_2 (mM)	references
			pH	temp. (°C)	pH	temp. (°C)			
Rhodopseudomonas capsulata	236	tetra	5-5.3	UD	UD	UD	UD	UD	Hochman and Shemesh, 1987
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	UD	UD	Van P'ee and Lingens, 1984

UD : undetermine

cellulose, pectin, hydroxyproline-rich glycoproteins, and lignin (Fry, 1986 and Grisebach, 1981). The peroxidases are also implicated in phenol oxidation (Lagrimini, *et al.*, 1987), wound healing (Espelie *et al.*, 1986), defense against pathogens (Hammerschmidt *et al.*, 1982), and the regulation of cell elongation (Goldberg, *et al.*, 1986). Moreover, 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), sex expression (Ghosh and Basu, 1984), and organogenesis of vegetation and floral buds (Kay and Basile, 1987). Although peroxidases are highly catalytic enzymes, they have very little specificity and exist in a multitude of isoenzyme forms, making it difficult to ascertain their actual functions.

A general investigation of the distribution of peroxidase activity in 60 species of higher plants (covering 31 families) was carried out in leaves of *Ipomoea cairica*. They were selected as a novel source for peroxidase preparation. It could be an alternative commercial source of high activity peroxidase enzyme (Lin, *et al.*, 1996). Most higher plants exhibit a large numbers of peroxidase isoenzymes. Hoyle (1977) found 42 isozymes or isoforms in commercial preparation of horseradish (*Armoracia ruticana*) peroxidase (HRP), usually classified acidic, neutral and basic isoenzymes by their isoelectric points or elution profiles during ion-exchange column chromatography. In 1990, Yeung and Cavey detected the peroxidases from bean (*Phaseolus vulgaris*) during seed development also. 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 and callus culture are different.

Moreover, in cell suspension culture, peroxidases found in the medium are different from those inside the cell (Mader, 1992). However, the distribution and function of each isoenzyme is still unclear. In fact other peroxidase isoenzymes have a regulatory role in plant senescence (Floris, *et al.*, 1984).

4.2 Animal Enzymes

The most serious problems in animal enzyme production are related to the quantity, stability and purification costs. 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 (TPO) involves the biosynthesis of thyroid hormones, and is purified by monoclonal antibody assited chromatography. In addition TPO has been shown to be a major component of the thyroid microsomal antigen involved in autoimmune thyroid diseases (Kimura, *et al.*, 1989). Mammalian glutathione peroxidase have lengths of approximately 200 amino acid and a selenocysteine residue at the active site, protect haemoglobin in erythrocytes from oxidative breakdown, catalyzing the reduction of H_2O_2 by glutathione (Tyson, 1992). However, glutathione peroxidase is shown to be chromatographically different from that of catalase. Because its enzymatic activity is not inhibited by neither azide or cyanide, nor is a band found, it was concluded that glutathione peroxidase is not a heme containing peroxidase. It is further determined that glutathione is the prefered thiol of glutathione peroxidase in the reduction of H_2O_2. The cytosolic

selenoenzyme glutathione peroxidase is first identified in rat erythrocytes and has since been widely found in many mammalian and mammalian species (Spallholz and Boylana, 1991).

Mammalian blood cells contain specific peroxidases such as myeloperoxidase, eosinophil peroxidase, and platelet peroxidase. They are heme-containing glycoproteins and are 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 the human defense against microorganisms in neutrophils and eosinophils (Sakamaki, *et al.*, 1989). The presenec 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 century (Klebanoff, 1991).

4.3 Microbial Enzymes

Microorganisms have become increasingly important as producers of industrial enzymes and in fact most enzymes used in industry today are of microbial origin. Microbial cells have short fermentation times, in expensive media, ease of developing simple screening procedures, and the existence of distinct proteins from different strains which catalyze the same reaction. This allows flexibility in choice of fermentation conditions since these different enzymes may have different stabilities and different pH and temperature optima.

In addition, microbes may be manipulated by genetic engineering.

The construction of strains 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).

Several peroxidases from microbial origin which act as both peroxidases and catalases have been described in enzyme properties. Table 2 shows the strains of microorganisms which produce peroxidase enzyme.

5. Application of Peroxidase

5.1 Enzymatic removal of hazardous organics from industrial waste water.

Various aromatic compounds, phenols and aromatic amines are present in waste waters of numerous industries which involve coal conversion, petroleum refining, organic chemicals and dyes, resins and plastic. In fact all phenol and aromatic amines are particularly toxic to marine life. Phenol is toxic to fish in concentrations as low as 5 ppm. Moreover, many phenol and aromatic amines are mutagenic and carcinogenic. Many more are referred to as "priority pollutants" (e.g. chlorophenol, nitrophenols, phenol and pentachlorophenol). Therefore, removal of such chemicals from industrial aqueous effluents is of great environmental significance.

Current methods for the removal of phenols and aromatic amines from waste waters include microbial degradation, absorption on activated carbon, chemical oxidation, solvent extraction memberane processes, and irradiation (Alberti and Klibanov, 1981). Although these methods are effective, they suffer from such shortcomings as high cost,

Table 2 The strains of microorganisms producing the peroxidase enzyme

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

incompleteness of purification, formation of hazardous by-products, and applicability to only a limited concentration range. A new method has been elaborated for the removal of toxic organics from industrial waste waters. It involves the treatment of solution with hydrogen peroxide. In the course of this oxidation, the corresponding phenolic and aromatic amine radicals are generated. These free radicals diffuse from the active center of the enzyme into solution, there they react with phenol molecules, forming polyaromatic products. These insoluble polymers then precipitate out of solution and can be separated by simple filtration (Klibanov *et al.*, 1980).

This approach is used to remove over 40 different phenols and aromatic amines from waste water. For many pollutants, the efficiencies of the enzymatic removal are very high (exceeding 99%). Table 3 gives the removal efficiencies for some of the phenols and aromatic amines from water reported by Alberti and Klibanov (1981). The removal of chlorophenols from waste water is immobilized by horseradish peroxidase. It was discovered that horseradish peroxidase was selectively absorbed by magnetite in the crosslinking method (Tatsumi *et al.*, 1996).

5.2 The degradation of lignin

Lignin occurs as one of the major components of woody and herbaceous plants and is the most abundant renewable aromatic material on earth. Biological degradation of lignin has aroused interest in its potential application to industrial processes such as biomechanical pulping, bleaching of paper, production of feedstock from wood and agricultural wastes and conversion of ligno-cellulosic materials to fuel and chemicals. (Kirk and Farrell, 1987).

Table 3 Removal of aromatic amines and phenols from water by horseradish peroxidase and hydroperoxide

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-Dimethylnaphthylamine	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
2,6-Dimethylphenol	82.3
Aniline	72.9

Table 3 (continue)

Pollutant	Removal Efficiency
4-Chloroaniline	62.5
4-Bromoaniline	84.5
4-Fluoroaniline	68.4
1,3-Diaminophenol	98.6
Diphenylamine	80.5
1-Naphthol	99.6
2-Nitroso-1-naphthol	98.6
4-Phenylphenol	99.9
8-Hydroxyquinoline	99.8

source : Alberti and Klibanov (1981)

In 1986, Hammel and colleagues demonstrated the one-electron mechanism of ligninase action by showing that the key reaction of biologically stable C-C and other linkages in the molecule, which protects the organic material from the degradative enzyme of microorganisms (Alfani and Cantarella, 1987). The recent discovery of several enzymes that are believed to play similar roles has stimulated lignin biodegradation research by many biochemists. Kirk and Farrell (1987) discovered that the white-rot fungi, *Phanerochaete chrysosporium* produces two extracellular heme peroxidases of manganese peroxidase (MnP) and lignin peroxidase (LiP) which, along with an H_2O_2-generating system, constitute the major components of its lignin-degrading system. The activity of ligninase has been detected in other white-rot fungi, including *Bjerkandera adusta*, *Coriolus versicolor*, *Panus tigrinus*, *Phlebia radiata* and *Lentinus edodes* (Crestinic, *et al.*, 1996).

5.3 The mechanism of lignification

Plants are required to adapt to the change and stresses of their environment in order to survive and they do so by altering their growth patterns. In 1991, Gasper and colleagues have proposed a two-step mechanism in peroxidase induction, where growth control may occur through the basic and acidic peroxidases. 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 lesione, growth and morphogenetic changes in various stresses. The stresses are known to mediate rapid bursts in 1-aminocyclopropane-1-

carboxylic acid (ACC), ACC-oxidase and ethylene. The early burst in ACC level results in a rapid increase in basic peroxidases functioning as indole-3-acetic oxidases (IAA-oxidases). The reduction of IAA content could enhance the ACC-synthase system and hence the ACC-level. Membrane-bound basic peroxidases and/or the soluble ones might in parallel function as ACC oxidases which convert ACC to ethylene. Since the ethylene is known to control the level of phenylalanine ammonialyase (PAL) and acidic peroxidases. The subsequent increase in activity of ACC-synthase, acidic peroxidases and PAL probably is the result of *de novo* protein synthesis in response to control lignification process.

5.4 Enzyme immunoassays

As a result of the widespread use of radioimmunoassays during the past two decades many markers have been introduced for the quantitative measurement of primary antigen-antibody interaction. One of these methods utilizes an enzyme as a lable 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. In 1985, Zmijeewski and Bellanti reported on the method using an enzyme-linked antibody, peroxidase and alkaline phosphatase, and the end point of measurement can be measured colorimetrically or visually. This method has clinical application particularly in viral infections, serodiagnosis of many diseases, rubella detection and including infectious diseases. Gonzalez and colleagues (1996) developed an indirect ELISA test for the detection of *Pseudomonas fluorescens* and related bacteria in

refrigerated meat. Two new detection systems for horseradish peroxidase have been for use developes in immunoassays. These detections use dimethyl or diethyl analogues of *p*-phenylenediamine with 4-chloro-1-naphthol to generate a blue product, or 3-methyl-2-benzothiazolinone hydrazone with 4-chloro-1-naphthol to generate a red product. These reagents have more sensitivity and lower background staining than current 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 nitroblue tetrazolium substrates.

6. Relationships among amino acid sequences of microbial, plant and animal peroxidases

Most peroxidase sequences from microbial, plant and animal sources have lengths of 300-400 amino acids. Many peroxidases are glycoproteins with a median molecular mass in the 40,000-50,000 kDa range, and all contain a heme group located at the active site (except the glutathione peroxidases, which have a selenocysteine residue at the active site).The range of functions displayed by peroxidases is largely dependent on the three dimensional orientation of the heme group at the active site, for example, in the case of yeast cytochrome c peroxidase and fungal ligninase. In addition functional modifications are brought about by the number, location, size and composition of the oligosaccharide chains attached through asparagine linkages to the polypeptide core.These chains may be post-translationally modified, thus opening up further possibilities for functional fine tuning. They do not, however, appear to contribute to

the correct folding of the protein (Smith. *et al*, 1991).

Henrissat and colleagues (1990) reported an alignment of eight peroxidases including five lignin-degrading peroxidases, two plant peroxidases, and a cytochrome c peroxidase by the multiple alignment program of Corpet (1988). However, the results are different from these reports in the alignment between the three enzyme groups.

Recently, *Bacillus stearothermophilus* catalase I (formerly peroxidase) was also confirmed to be closely related to catalase HPIs from *E. coli* and *Salmonella typhimurium* by the fact that the amino acid sequence deduced from the revised nucleotide sequence shows high homology to those of catalase HPIs throughout the whole sequence. In addition, these three bacterial catalases were found to be homologous to yeast cytochrome c peroxidase, whose tertiary structure is known. These four sequences were aligned, and it was found that almost all the amino acid residues surrounding and interacting with the heme group of cytochrome c peroxidase are conserved in all the catalase sequences. The regions including two conserved histidines were aligned for 23 sequences, includng three bacterial catalase sequences, a cytochrome c peroxidase sequence, nine lignin-degrading peroxidase sequences, and ten plant peroxidase sequences. Seven amino acid residues including the three important residues of Arg-48, His-52, and His-175 of cytochrome c peroxidase are conserved in all the sequences. In addition, amino acid residues conserved in a group-specific manner were identified. The phylogeny of this enzyme family was also obtained. The phylogenic tree is composed of three main branches each containing bacterial catalases, lignin-degrading peroxidases, or plant peroxidases, cytochrome c

peroxidase belongs to the catalase branch. (Fig. 2) (Trakunaleamsai, *et al.*, 1995).

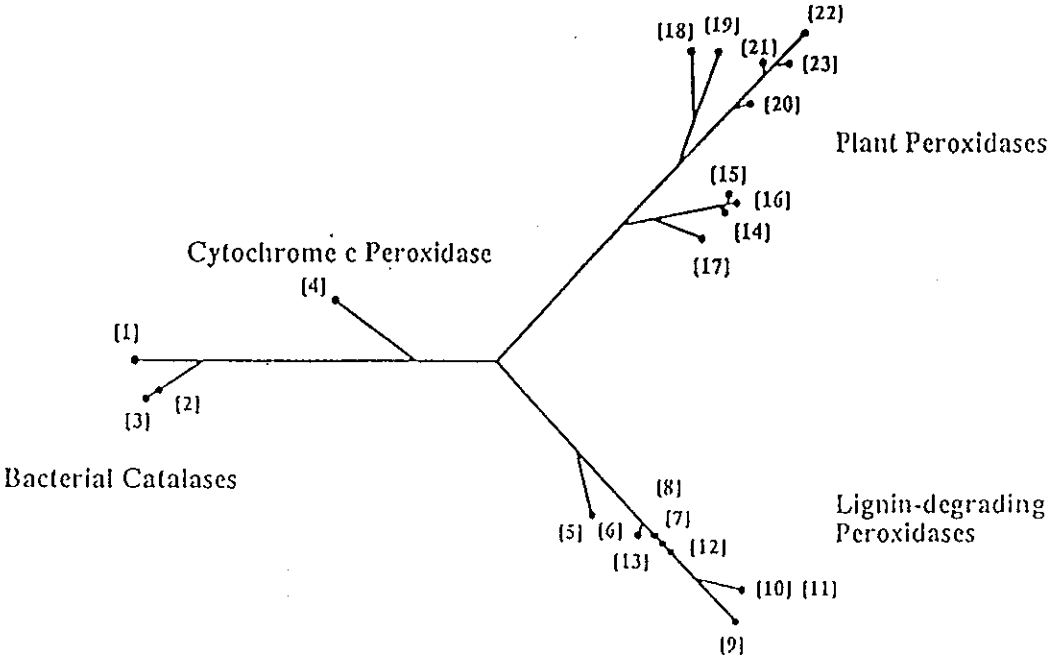


Fig. 2 phylogenic tree of the peroxidase family including bacterial catalases. The numbers in brackets correspond to the enzyme numbers in Table 4 The length of the lines corresponds to the relative distance calculated by maximum parsimony method
source : (Trakunaleamsai, *et al.*, 1995).

Table 4 List of the members of peroxidase family

Enzyme number	Enzyme	Species	GeneBank Accession number
1	catalase I	*B. stearothermophillus*	M29876
2	catalase HP I	*E. coli*	P13029
3	catalase HP I	*S. typhimurium*	P17750
4	cytochrome c peroxidase	*S. cerevisiae*	P00431
5	peroxidase H4 (Mn dependent)	*Phanerochaete chrysosporium*	A32630
6	peroxidase CLG5	*P. chrysosporium*	B29610
7	peroxidase H8	*P. chrysosporium*	P06181
8	peroxidase A27817	*P. chrysosporium*	A27817
9	peroxidase JQ0374	*Phlebia radiata*	JQ0374
10	peroxidase CLG4	*P. chrysosporium*	A29610
11	peroxidase LG4	*P. chrysosporium*	P14153
12	peroxidase LG3	*P. chrysosporium*	P21764
13	peroxidase SO1028	*P. chrysosporium*	SO1028
14	peroxidase TAP2	tomato	P15004
15	peroxidase TAP1	tomato	P15003
16	peroxidase	potato	P12437
17	peroxidase	turnip	P00434
18	peroxidase	cucumber	P19135
19	peroxidase	tobacco	P11965
20	peroxidase	horseradish	P17179
21	peroxidase	horseradish	P00433
22	peroxidase	horseradish	P17180
23	peroxidase	horseradish	P15232

source : (Trakunaleamsai, *et al.*, 1995).

Previously, Tyson (1992) reported a relationship among 18 peroxidases amino acid sequences of microbial, plant and animal origin using optimum alignment of all pairwise sequence combination to generate a total distance matrix. Some of these peroxidases contain signal peptides that were removed before pairwise alignment. In some species, for example *Arabidopsis thaliana*, horseradish, peanut, tomato and wheat an isozyme of peroxidase has been sequenced. Preliminary investigation of the horseradish, tomato and wheat isozyme showed that isozyme sequences within these are so similar that only one from each species need be taken as a representative of the two *Arabidopsis thaliana* isozymes. Both isozymes were included in the comparisons of the 18 sequences selected. The 18 sequences references are listed in Table 5, thus representing a sample to examine relationship and determine the essential sequence features of plant, animal and microbial peroxidases.

For the plant peroxidase shown in Fig. 3 at least four subgroups are suggested by the dendrogram branches. The marked length difference between the 2 peanut isozymes is reflected in the isolation of peanut 2 from sequences 2, 5, 7 (peanut 1), 8 and 10. The 2 *Arabidopsis* isozymes are much closer to one another. The extremely close relationship between tomato 1 and potato (Solanaceae), and between cucumber and tobacco, are detected in preliminary studies of the plant peroxidases that included all available isozyme sequences. In addition, among the animal and microbial peroxidase formed a fifth subgroup, a single subgroup (sequences 1, 6, 9, 13, 14, 17 and 18), in contrast to the plant sequences where four subgroups occur. These are sequences 3 and 4 (subgroup 1), 12, 15 and 16 (subgroup 2), 5 and 10 (subgroup 3), and 2, 7, 8 and 11 (subgroup 4). Subgroup 5

contains sequences 1, 6, 9, 13, 14, 17 and 18 . Therefore the microbial / animal peroxidases formed a fifth subgroup. (Fig. 3).

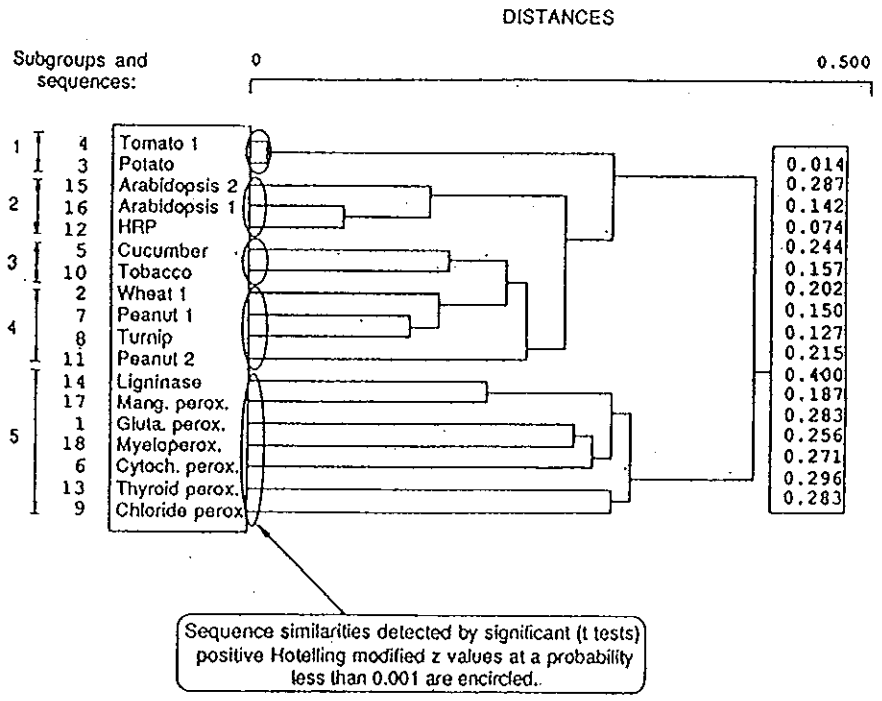


Fig. 3 Dendrogram showing relationships among 18 plant, animal and microbial peroxidase amino acid sequences. Sequence numbers as in Table 5, Branch distances shown in *box* at *right* of diagram. *Circled sequences* are related on basis of analysis of specific distances between sequences. source : (Tyson, 1992).

Table 5 References for the 18 peroxidase sequences, listed in order of increasing length. Sequence numbers, 1-18, are used throughout following tables. Wheat 1 tomato 1 are isozyme with in species. Name abbreviation shown in heading, e.g. Sequence # 1, i.e. glutathione peroxidase, is Sequence # 1 (Gluta. perox.)

Sequence #	Enzyme	Species	Amino acid number
1 (Gluta. perox.)	Glutathione peroxidase	*Bos primigeniustaurus*	205
2 (Wheat 1)	Wheat peroxidase 1	*Triticum aestivum*	289
3 (Potato)	Potato peroxidase	*Solanum tuberosum*	290
4 (Tomato)	Tomato peroxidase 1	*Lycopersicon esculentum*	290
5 (Cucumber)	Cucumber peroxidase	*Cucumis sativus*	293
6 (Cytoch. perox.)	Cytochrome c peroxidase	*Sacch. Cerevisiae*	294
7 (Peanut 1)	Peanut peroxidase 1	*Arachis hypogea*	294
8 (Turnip)	Turnip peroxidase	*Brassica napa*	296
9 (Chloride perox.)	Chloride peroxidase	*Caldariomyces fumago*	300
10 (Tobacco)	Tobacco peroxidase	*Nicotiana tabacum*	302
11 (Peanut 2)	Peanut peroxidase 2	*Arachis hypogea*	306
12 (HRP)	Horseradish peroxidase	*Armoracia rusticana*	308
13 (Thyroid perox.)	Thyroid peroxidase	*Sus scrofa*	332
14 (Ligninase)	Ligninase	*Phan. Chrysosporium*	344
15 (Arabidopsis 2)	Arabidopsis peroxidase 2	*Arabidopsis thaliana*	349
16 (Arabidopsis 1)	Arabidopsis peroxidase 1	*Arabidopsis thaliana*	354
17 (Mang. perox.)	Manganeseperoxidase	*Phan. Chrysosporium*	357
18 (Myelo. perox.)	Myeloperoxidase	*Homo sapiens*	467

source : (Tyson, 1992).

7. Cloning of peroxidases

It took about ten years to achieve success in the cloning of peroxidase gene into different organisms.

Fujiyama and colleagues (1988) cloned 3-cDNAs and genomic DNAs coding for HRP C (horseradish peroxidase C) and its derivatives based on the amino acid sequence of HRP C which was established by Welinder (1976).

Loprasert and colleagues (1989) cloned and sequenced peroxidases gene of *B. stearthermophilus*. The deduced amino acid sequence showed 48% homology to that *E. coli* catalase HPI. These results suggested that this peroxidase was a member of the bacterial catalases with broad-spectrum peroxidative activity.

Roberts and Kolattukudy (1989) cloned and sequenced peroxidases from a new family of wound-induced, suberization-associated acidic peroxidases from potato tuber and tomato fruit. The predicted amino acid alignment in mature peroxidases from the two species was 97% identical. Partial sequencing of acidic peroxidase HRP A1 showed about 80% identical to HRP A2.

Heym and colleagues (1993) reported on the *Kat G* gene of *Mycobacterium tuberculosis* which encoded the heme-containing enzyme catalase-peroxidase. In this study, the chromosomal location of *Kat G* and its nucleotide sequence was used to predict the primary structure. This enzyme is an 80,000 dalton protein containing several characteristics of peroxidases and shows strong similarity to other bacterial catalase-peroxidases.

Black and Reddy (1991) isolated six putative lignin peroxidase

(LIP) genes from a λEMBL 3 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 the coding region was interupted by six small introns and that it encoded a mature LIP protein (341 amino acid; MW 36,714) that was preceded by a 25 amino acid signal sequence. This protein had a relatively high degree of amino acid homology to the LIP proteins purified from *T. versicolor* and had an amino acid homology of 55-60% to the LIP proteins of *P. chrysosporium*, which was comparable to that found between *P. chrysosporium* and *Phlebia radiata* LIP protein.

Ritch and Gold (1992) studied the genomic clone, *LG2*, encoding Lip2, the major lignin peroxidase (LiP) isozyme was isolated and characterized from *P. chrysosporium* strain OGC101. The 5'-untranslated region of *LG2* contained sequence similar to *CRE* and *XRP* promoter elements. Comparison with genes encoding other LiP isozymes five related patterns of intron location, whose incidence coincides with described LiP structural subfamilies.

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. The lignin peroxidase gene VLG4 of another white-rot fungus, *Trametes versicolor*, and the nit-2 gene of *Neuruspra crassa* also contain putative AP-2-binding sequences. All of these genes were

regulated by nutrient nitrogen. This led to the hypothesis that an AP-2 transcription factor might be involved in inducing gene expression during nitrogen limitation in fungi.

Wolfframm and colleagues (1993) reported on the nucleotide sequence of a 1.5 kb fragment *Pseudomonas pyrrocinia* DNA containing the chloroperoxidase (CPO)-encoding gene (*cpo*) and its flanking region was determined. The *cpo* codes for a protein of 278 amino acid. The mature enzyme contains no N-terminal methionine, so that the CPO monomer consists of 277 amino acid with a calculated M_r of 30,304.Expression showed that the *cpo* from *P. pyrrocinia* is functionally expressed in *Escherichia coli* and *Streptomyces lividans*. Comparision with the amino acid sequence of the bromoperoxidase BPO-A2 from *S. aureofaciens* ATCC10762 revealed an identity of 38%.

Parsonage and colleagues (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 *Streptococcal* NADH peroxidase was fully active and exhibits spectroscopic and redox properties identical to those for the enzyme purified from *Streptococus faecalis* 10C1.

Na pathalung K. (1996) reported on the cloning of a 9.0 kb fragment chromosomal DNA of *Bacillus* sp. PHS155 containing the peroxidase gene (*perA*) which was digested with *BamHI* and directionally ligated into λ-GEM-11, which had been digested with the same enzyme to remove the central

stuffer region (λ-GEM-11 have left arm and right arm regions were 20.0 kb, 9.0 kb respectively) (Fig. 4). The ligation mixture was packaged *in vitro* with in Packagene Packaging System to construct genomic library (Promega, USA). The packaged phages finished, plaques from the resultant library were screened by colony hybridization with pOD68 probe and positive clone were selected , named λ-GEM-11-*perA* (Fig. 5).

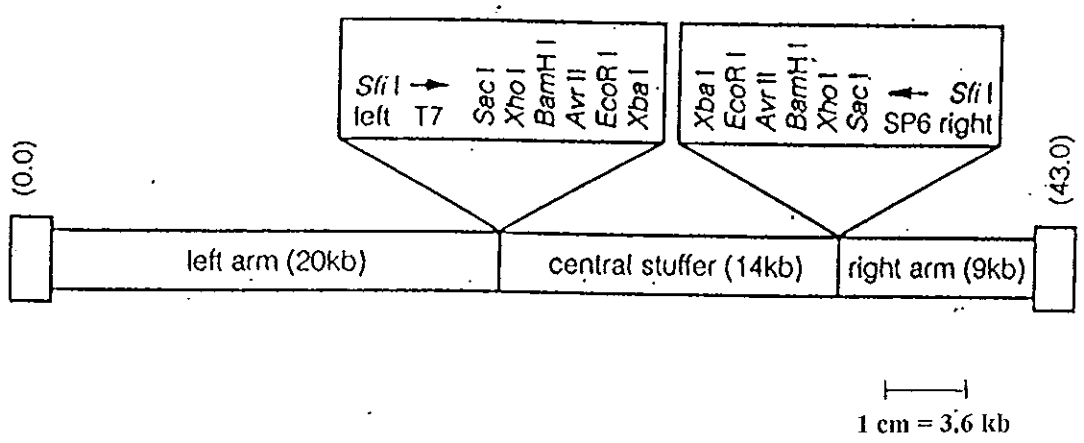


Fig. 4 Structural map of the λ-GEM-11 vector

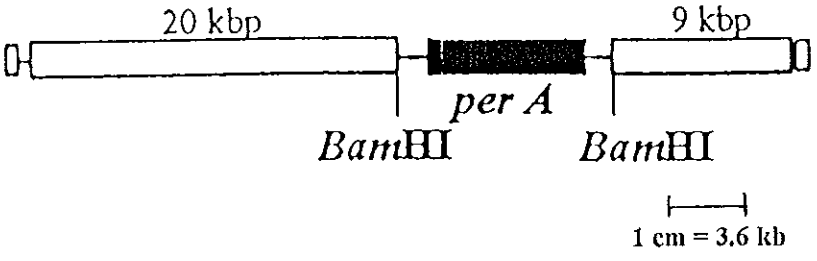


Fig. 5 Structural map of λ-GEM-11-*perA*

The 9.0 kb *perA* of *Bacillus* sp. PHS155 was cloned into λ-GEM-11 vector by *BamH*I restriction enzyme

Objectives

The objective of this study are:

1. To determine restriction map of λ-GEM-11-*perA*.
2. Subcloning of the λ-GEM-11-*perA* into pBluescript II SK+ vector.
3. To study the sequence of a peroxidase encoding gene from *Bacillus* sp. PHS155.
4. To study the homology of amino acid sequence of peroxidase gene with *Bacillus stearothermophilus* IAM11001.

Chapter 2

Materials and Methods

Materials

1. Chemicals

Chemical name	Company	Country
Absolute ethanol	B.D.H.	England
Acetic acid (glacial)	E. Merck	Germany
19:1 Acrylamide/Bis powder	Bio-rad	USA
Agarose type II	Sigma	USA
Ammonium persulfate	IBI	USA
β-mercaptoethnol	B.D.H.	England
Boric acid	Aldrich	USA
Bromophenol blue	B.D.H.	England
Chloroform	B.D.H.	England
Deoxyribonucleotide triphosphate(dNTPs)	Takara	Japan
Ethidium bromide	Sigma	USA
Formamide (deionized)	IBI	USA
8-Hydroxy-quinoline	B.D.H.	England
Isoamyl alcohol	E. Merck	Germany

Chemical name	Company	Country
Isopropyl β-D-Thiogalacto peranoside (IPTG)	E. Merck	Germany
Phenol	E. Merck	Germany
Potassium acetate	B.D.H.	England
Sodium carbonate	E. Merck	Germany
Sodium chloride	E. Merck	Germany
Sodium dodecylsulfate (SDS)	Sigma	USA
Sodium hydrogen carbonate	E. Merck	Germany
Sodium hydroxide	E. Merck	Germany
N,N,N', N',-tetra methy-ethylenediamine (TEMED)	Bio-rad	USA
Tris(hydroxymethy amino-methane (Trisma base)	E. Merck	Germany
Tris-sodium citrate	E. Merck	Germany
Urea	IBI	USA

These chemicals were of analytical grade.

2. Enzymes

Proteinase K, Bachringer-Mannheim,Germany

Taq DNA polymerase and reaction buffer, Gibco BRL, USA

3. Instruments

Instruments	Model	Company
Applied Biosystem DNA Sequencer	373A	ABI Inc., USA.
Autoclave	HA-300 MII	Hireyama
Hot plate stirrer	Nuova II	Sybron
Incubator	Im 550 R	Clayson
Larminar air flow	Nu 425-400 E	Nuaire
Micro high speed centrifuge	Tomy Sciko	Japan
pH meter	109	Activon
Polaroid camera	Fotodyne	USA
Refrigerated centrifuge	Z 382 K	TLG
UV-visible Spectrophotometer	Ultrospec-3	Pharmacia
Thermal cycling system	Touch Down	Hybaid
Vortex mixer	G-560E	Scientific industries Inc.
Water bath		Lab-line instruments

4. Miscellaneous

- Biodyne C nylon membrane, Pall Biosupport, New York.
- Pipette tip (for Pipetman P20, P100, P200), Treff, Switzerland.
- Pipette microcentrifuge tube, (0.2 ml), Robbins Scientific, USA.
- Pipette microcentrifuge tube, (0.6 ml), Robbins Scientific, USA.
- Pipette microcentrifuge tube, (1.5 ml), Robbins Scientific, USA.

- Polaroid films, Polaroid, England.

5. Bacterial strains and plasmid

Bacillus sp. PHS155, which produces thermostable peroxidase was isolated by Assistant Professor Dr. Amornrat Phongdara and Dr. Rattana Roengrairatanaroj, Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkla, Thailand. *E. coli* UM228 (see Appendix), a catalase HPI-deficient mutant (Trakunaleamsai, *et al.*, 1995), was kindly provided by Dr. Savitr Trakunaleamsai, Kasetsart University, Bangkok, Thailand and *E. coli* DH5α (see Appendix) (Stratagene, USA) were used as a host strain for transformation. The pBluescript II SK+ vector (see Appendix) (Stratagene, USA), was used in the subcloning mapping and sequencing of a *perA* gene. Bacterial strain *E. coli* LE392 (see Appendix) (Promega, Madison, WI, USA) was used for host strain of the λ-GEM-11 bacteriophage. The plasmid pOD68 (5.5 kb) was provided by Dr. Savitr Trakunaleamsai. The plasmid was a recombinant vector of pUC19 harboring a 1.7 kb *Cla*I-*Bst*EII fragment containing the *perA* of *Bacillus stearothermophilus* IAM 11001 and used as a probe for hybridization.

6. Media and culture conditions

The bacterial strain were grown in LB (Luria-Bertani) medium (see Appendix) at 55°C- 65°C for *Bacillus* sp. PHS155. LB medium could be modified with adding 0.2% maltose, 10 mM $MgSO_4.7H_2O$ was used as medium for culturing *E. coli* LE392, UM228, DH5α (see Appendix). When necessary, ampicilin (100 μg/ml) was added to the medium.

7. DNA manipulation

Plasmids pOD68 and pBluescript II SK+ were prepared by alkaline lysis (Sambrook, *et al.*, 1989). λ-GEM-11-*perA* DNA was prepared from the phage particle in the supernatant using PEG precipitation (Promega, Madison, WI, USA). Lambda DNA digested with *Hind*III were used as molecular weight markers for electrophoresis. Enzymes for DNA manipulation were purchased from Promega, Madison, WI, USA. Each DNA fragment for subcloning was excised from ethidium bromide stained gel, transferred to a microcentrifuge tube and followed by purification by procedure of Gene Cleane II Kit. (BIO 101). The prepared pOD68 probe was labelled with digoxigenin-dUTP (DIG-dUTP) by method of Boehringer mannheim.

Methods

1. Preparation of λ-GEM-11-*perA*

Preparation of λGEM-11 bacteriophage by bacterial culture for infection (host strain) was prepared by inoculating a single colony of LE392 which was then added into 3 ml modified LB broth and shaken and kept overnight at 37°C. Five hundreds microlitre of overnight culture was subcultured into 50 ml modified LB broth and shaken at 37°C for approximately 2.5 hrs or until the OD_{600} has reached 0.6-0.8. The culture was stored at 4°C for either 24 hrs or one week, and then the packaged phage was prepared by adding 5 µl lambda phage into 50 µl Packagene Extract (Promega, Madison, WI, USA) (thawed on ice), mixed by gently tapping the bottom of the tube serveral times and incubated at 22°C for

3 hrs. The mixed packaging was added with 455 µl phage buffer, 25 µl chloroform and gently mixed by inversion of the tube. After the packaged phages finished, 100 µl diluted packaged phage (100x) was added into 100 µl of bacterial culture LE392 and incubated at 37°C for 30 min to allow the phage to absorb. The mixture was mixed with 3 ml molten (45°C) LB top agar and immediately poured onto LB plate prewarmed at 37°C. The top agar was allowed to harden and incubated and inverted at 37°C overnight. After that lambda lysate was prepared to be used for extraction genomic lambda GEM-11 by inoculating a single plaque from agar plate into a microcentrifuge tube containing 100 µl of S buffer (see Appendix) and placed at 4°C overnight.

2. Amplification and extraction DNA from λ-GEM-11-*perA*

The mixture consisting of 70 µl of phage and 500 µl of bacterial culture LE392 OD_{600} = 0.5-0.6 was incubated at 37°C for 30 min. The mixture was added into 3 ml molten (45°C) LB top agar and immediately poured onto LB plate prewarmed at 37°C. The top agar was allowed to harden and incubated at 37°C overnight. After 3 ml of SM buffer (see Appendix) was added to the plaque and scraped the top agar with a spatula. The Lambda lysate was shaken at 37°C for 30 min and then the supernatant was centrifuged at 8,000 rpm at 4°C for 10 min. The supernatant (initial phage lysate) was added to 1 µg/ml of DNAase I and 1 µg/ml of RNAase A and incubated 37°C for 30 min. An equal volume of Phage precipitation (see Appendix) solution was added, mixed and chilled on ice for 1 hr. The precipitated phage particles were recovered by centrifugation at 10,000 rpm at 4°C for 20 min and then the supernatant

was removed by aspiration. The phage particles were resuspended with 1 ml of Phage buffer (see Appendix) per 10 ml of initial phage lysate by vortexing, centrifuged at 8,000 rpm at 4°C for 2 min to remove debris. The supernatant was transferred to a sterile 1.5 ml microcentrifuge tube. An equal volume of saturated phenol : chloroform : isoamyl alcohol (25:24:1) was added, gently vortexed and centrifuged at 20,000 rpm for 5 min at room temperature to separate the upper aqueous phase containing DNA from the organic phase. The upper aqueous phase was reextracted twice with one volume of chloroform : isoamyl alcohol (24:1) to remove the residual phenol. The upper aqueous phase was transferred to sterile 1.5 ml microcentrifuge tube and DNA was precipitated by adding 2.5 volume of cold absolute ethanol. The mixture was left at -70°C for 10 min to enhance DNA precipitation. The precipitated DNA was collected by centrifugation at 12,000 rpm 10 min and then ethanol was discared. The DNA pellet was washed in 1 ml cold 70% ethanol, recentrifuged at 12,000 rpm 10 min at room temperature and then the supernatant was removed. The DNA pellet was allowed to dry under vacuum at room temperature and finally dissolved in 40-50 µl of TE buffer (pH 8.0) (see Appendix). The concentration of DNA was estimated by determination of the absorbance at 260 nm of the 1:200 dilution assuming that A_{260} unit has the DNA concentration at 50 µg/ml.

3. Mapping and first subcloning of λ-GEM-11-*perA*

The recombinant of a positive clone which contained a 9.0 kb *Bam*HI fragment was amplified and the DNA was extracted by methods as described in DNA manipulation (Promega, Madison, WI, USA).

For mapping, the recombinant λ DNA was digested with *Bam*HI, *Bgl*I, *Cla*I, *Kpn*I, *Pst*I, *Sac*I, *Sma*I, *Xho*I and then subcloning, restriction fragment with a *perA* gene were Southern-hybridized by using pOD68 as a probe, and 5.5 kb *Cla*I fragment from positive clone was ligated with pBluescript II SK+ vector restricted with the same enzyme. The recombinant pBluescript II SK+ vector harboring 5.5 kb *perA* gene were then used for transformation into *E.coli* UM228. The transformants were plated on LB medium supplemented with ampicilin (100 μg/ml), 110 μl of 100 mM IPTG, 45 μl of 2% X-Gal and incubated at 37°C overnight (14-16 hrs). The transformants without insertion would remain blue. The mutant *E.coli* UM228 cultured in medium at 37°C for 24 hrs under the aerobic condition by using *E.coli* UM228 wild type as a control. For colonies of the plasmid DNA of a positive clone was named pBPHS5.5.

4. The Expression of *perA* gene in *E.coli* UM228 having peroxidase activity

4.1 Cell cultivation

The pPBHS5.5 was grown under acrobic conditions. A full loop of stock culture was inoculated into 10 ml LB broth as a starter. This culture was incubated on a rotary shake (250 rpm) at 37 °C overnight. 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 from cell-free extracts (crude enzyme) was assayed.

4.2 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 resuspended in 2 ml of the same buffer. The cells were disrupted by sonicated at 24 kHz for 25 min. The crude enzyme solution was separated from the cell debries by centrifugation at 12,000 rpm at 4 °C for 20 min.

4.3 Peroxidase assay

The method used for determining peroxidase activity was modified from 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 into the mixture and which was incubated at 60°C and the O.D. at 460 nm was followed. 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).

5. Second subcloning of the DNA fragment harboring a *perA* gene for sequencing

The plasmid pBPHS5.5 was analyzed by single digestion (Fig. 9) and double digestion (Fig. 10-11). After that a 5.5 kb *Cla*I fragment from

pBPHS5.5 was digested with restriction enzymes (*Bam*HI, *EcoR*I, *Hinc*II, *Hind*III, *Kpn*I, *Pst*I and *Sal*I). (Fig. 12). The restriction fragments were Southern-hybridized by using pOD68 as a probe (Fig 13). The resulting 2.6 kb *Cla*I-*EcoR*I fragment was subcloned into the pBluescript II SK+ vector and named pBPHS2.6 (Fig. 14). The pBPHS2.6 was digested with *EcoR*I . After that 2.7 kb *EcoR*I fragment was ligated with pBluescript II SK+. The plasmid was named pBPHS2.7 (Fig. 15). The plasmid pBPHS2.6 contained 0.5 kb *EcoR*I-*Sal*I fragment and the 1.5 kb *Pst*I-*Sal*I fragment were each subclone at the appropriate site of pBluescript II SK+. The resultant plasmid was designated as pBPHS0.5 and pBPHS1.5, respectively (Fig. 15).

6. Analysis of peroxidase gene by Flurescence-based automated DNA sequencing

6.1 Preparation of templates

The plasmid DNA pellet from clones pBPHS2.7, pBPHS2.6, pBPHS1.5 and pBPHS0.5 was dissolved in 50 µl of TE buffer; pH 8.0 and then the concentrated DNA was determined by 1 OD_{260} (A_{260}) unit has the concentration at 50 µg/ml. For plasmid DNA pellet from clone pBPHS2.6 was amplified by PCR (polymerase chain reaction) using M13 primer and R1 primer. The sequences of primers was shown in table 6 and position of R1 primer were shown in Fig. 17. The cycling reaction was done using a programmable Touch Down Thermal cycling system. Reactions comprised denaturation at 92°C for 5 min, 50°C for 1:30 min and 72°C for 2 min at the first cycle. The subsequent 35 cycles were performed at 92°C for 1:30

min, 50°C for 1:30 min and 72°C for 2 min. and then the first cycle extension at 92°C for 1:30 min, 50°C for 1:30 min and 72°C for 10 min. After the cycling reaction was finished, PCR product was quantitated by running 1 µl of the sample on 1.5% agarose gel beside a lane of standard concentration.

6.2 Cycle sequencing reaction

The purified plasmid of cloned pBPHS2.7, pBPHS2.6, pBPHS1.5, pBPHS0.5 and the purified PCR products of pBPHS2.6 were used as a template for automated sequence analysis with *Taq* DNA polymerase using Dye Deoxy Terminator Cycle Sequencing Kit (PRISM™ Ready Reaction, Applied Biosystems) (see Appendix). The purified template (approximately 500 ng) was mixed with 8.0 µl of Terminator premix (see Appendix), 32 pmole of appropriated sequencing primer and deionized water to a total volume of 20 µl. The sequence of primers was shown in Table 6 and the position of primers was shown in Fig. 17. Thermal cycling was performed on Touch Down Thermal cycling system model at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min for 25 cycles. The extension product was precipitated by adding 2 µl of 3M sodium acetate; pH 5.2 and 50 µl precooled absolute ethanol and centrifuged at 12,000 rpm for 15 min at room temperature. The pellet was rinsed by adding 250 µl of 70% ethanol and allowed to air dry at room temperature, then stored at -20°C until sequencing electrophoresis gel.

Table 6 The sequence of oligonucleotide primers used in a peroxidase gene amplification and DNA sequencing

Primers	Sequence	Site of annealing in a peroxidase gene region*
pBluescript II SK+ vector		
M13-20	5'GTAAAACGACGGCCAGT3'	600-616
T7	5'AATACGACTCACTATAG3'	627-643
T3	5'ATTAACCCTCACTAAAG 3'	774-790
M12*	5'GGTTCGTTCACTTCCCA3'	1791-1807
M11	5'GTCGTCAACATCATCGG3'	1405-1421
M10	5'GTTTAAACGTCTCGCGA3'	1022-1038
T1	5'TATACAGGCGACCGCGA3'	906-921
T2	5'CGCCACACCTACGCAA 3'	1243-1260
T4	5'CAG GGTTAACGATTAGC3'	1672-1688
R1	5'AACTGTTTAACGGGGCG3'	662-678
R2	5'GGATTCGTTTTTCGGTCAT3'	427-445*

The number in this column indicate the position of nucleotides in the peroxidase gene region

6.3 Preparation of 6% polyacrylamide sequencing gel

Thirty-five g of urea and 10.5 ml of 40% acrylamide stock solution were combined in a 100 ml flask and deionized water was added to bring the volume up to 50 ml. The mixture was stirred and heated until the crystals were dissolved completely, then 7 ml of 10xTBE stock solution was added and was increased to 70 ml with deionized water. The acrylamide solution was vacuum filtered using 0.2 μm millipore unit for 5 min. Three hundred and fifty μl of 10% ammonium persulfate and 39.9 μl of TEMED were added to the acrylamide solution and immediately poured into the glass plates until the mold was full, and then the edges of the plates were clamped after inserting the shark tooth comb. The gel was left nearly horizontal to polymerize for at least 2 hrs at room temperature.

6.4 Loading the sample and automated DNA sequence analysis

Dried pellet of pBPHS2.7, pBPHS2.6, pBPHS1.5, pBPHS0.5 and dried PCR products of pBPHS2.6 were resuspended in 4 μl of loading buffer and heated at 90°C for 2 min to denature the DNA, then transferred immediately onto ice.The samples were loaded on a 6% polyacrylamide sequencing gel using an ABI 373 automated DNA sequencer for 14 hrs.

7. Analysis sequences of pBPHS2.6

Emission data analyzed by Macintosh-based Software (version 1.02). The sequence obtained from each run was compared and aligned with DNA sequences of the original catalase I gene with the help DNASIS version, a softwere program. Peroxidase gene coded on all fragments was named pBPHS2.6.

Chapter 3

Results

1. First subcloning of the peroxidases gene

The λ-GEM-11-*perA* DNA (cloned by from Ms. Kintkand Na Pathalung, 1996) (Fig. 5) was digested with various restriction enzymes and the result of digestion pattern obtained by eight restriction enzymes was shown in Fig. 6. The DNA fragment having a *perA* gene was detected by Southern hybridization using pOD68 as a detection probe (Fig. 7). The resulting 5.5 kb *Cla*I, a smallest DNA fragment containing *perA* gene was subcloned into pBluescript II SK+ vector restricted with the same enzyme and transformed into *E. coli* UM228. The plasmid of a peroxidase containing clone was named pBPHS5.5 (Fig. 8) for further study of the sequencing. The expression of peroxidase gene in *E. coli* UM228 with pBPHS5.5 was shown in Table 7.

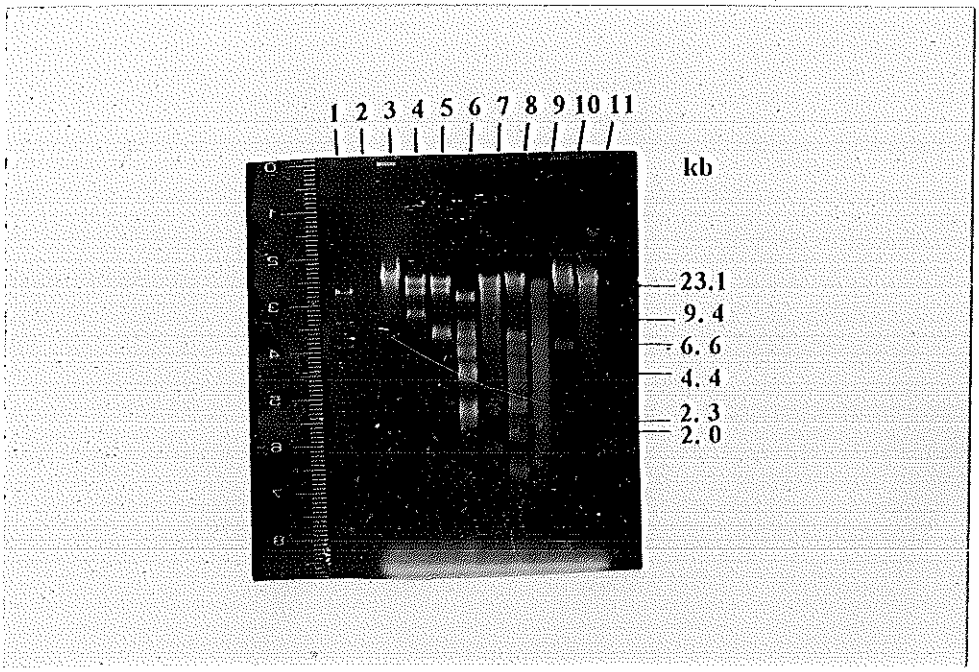


Fig. 6 Analysis of λ-GEM-11-*perA* gene

The λ-GEM-11-*perA* (10 μg) was digested with various restriction enzyme separated by 1% agarose gel electrophoresis and stain with ethidium bromide. Lane 1 : λ-*Hind*III (marker), lane 2 : pOD68 (undigested), lane 3 : λ-GEM-11-*perA* (undigested), lane 4, 5, 6, 7, 8, 9, 10, and 11 : λ-GEM-11-*perA* were digested with *BamH*I, *Bgl*II, *Cla*I, *Kpn*I, *Pst*I, *Sac*I, *Sma*I and *Xho*I, respectively.

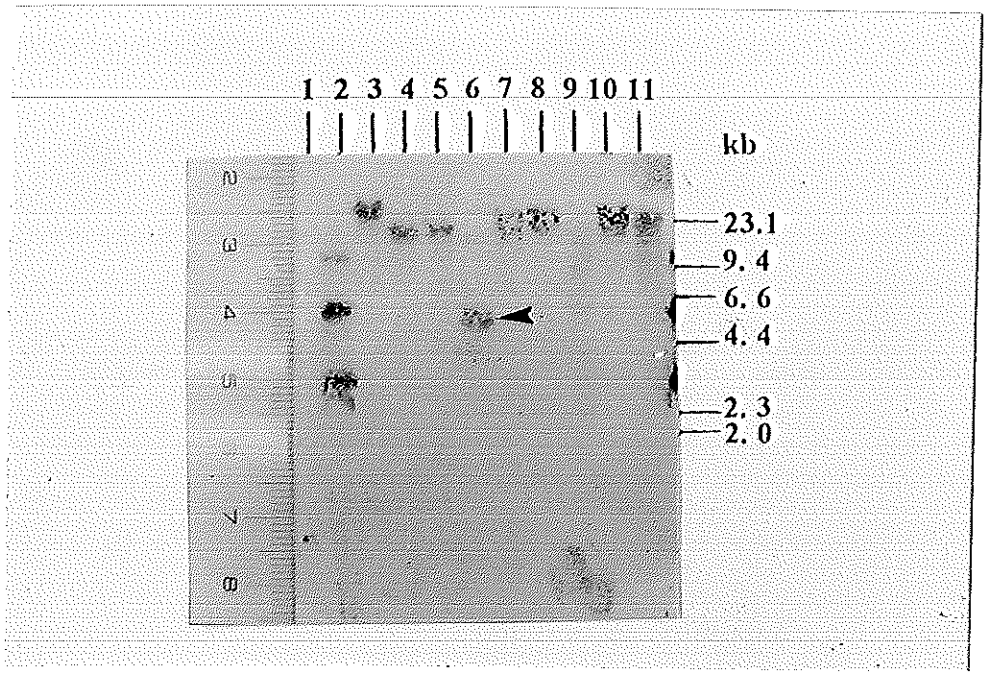


Fig. 7 Southern blot analysis of λ-GEM-11-*perA* gene

Southern hybridization of the DNA with Anti-DIG labeled, pOD68 probe a 1.7 kb *Cla*I-*BstE*II containing *perA* gene fragment. Arrow-head to right of sixth lane indicated the position of 5.5 kb-*Cla*I fragment which was smallest fragment containing *perA* gene. Lane 1 : λ-*Hind*III (marker), lane 2 : pOD68 (undigested), lane 3 : λ-GEM-11-*perA* (undigested), lane 4, 5, 6, 7, 8, 9, 10, and 11 : λ-GEM-11-*perA* were digested with *BamH*I, *Bgl*II, *Cla*I, *Kpn*I, *Pst*I, *Sac*I, *Sma*I and *Xho*I, respectively.

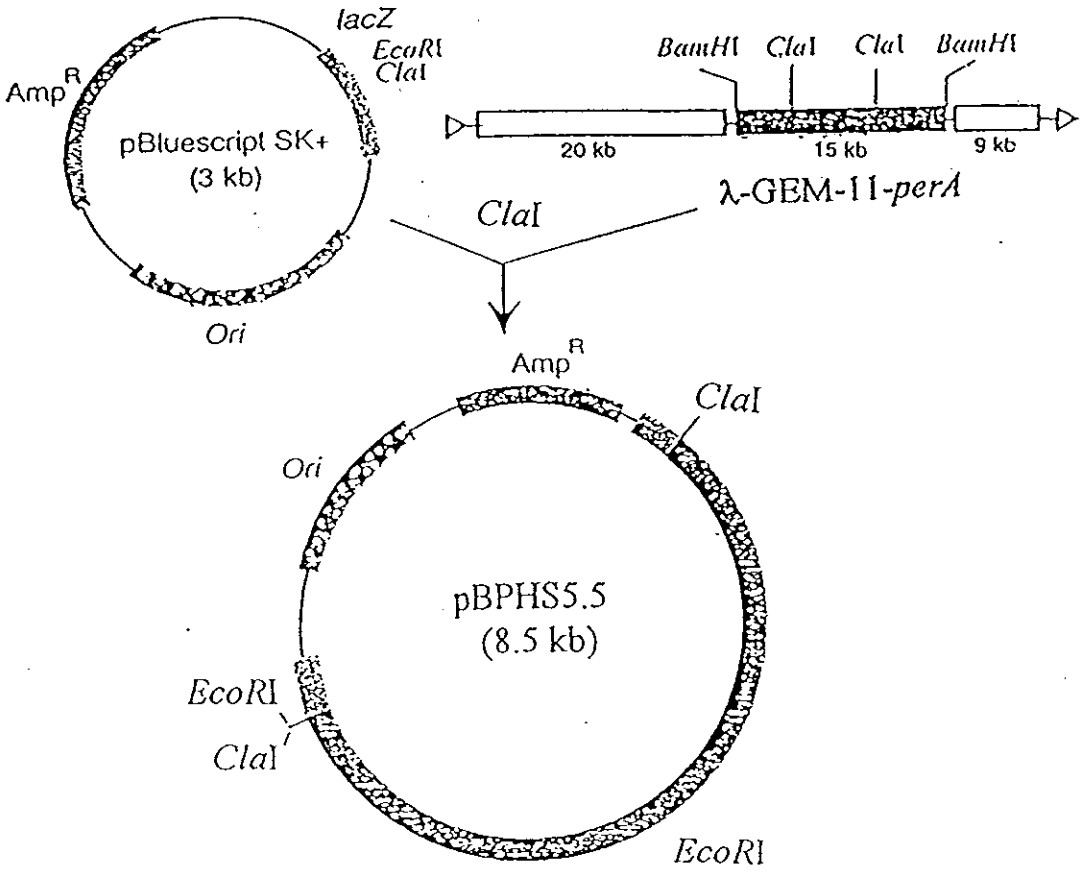


Fig. 8 The construction of plasmid pBPHS5.5

Plasmid pBPHS5.5 was a hybrid plasmid consisting of pBluescript II SK+ vector (3.0 kb) and the 5.5 *Cla*I fragment containing the peroxidase gene (*perA*) of *Bacillus* sp. PHS155

Table 7 The expression of peroxidase gene

Strains	Culture temp. (°C)	Specific activity* (U/mg)	Ratio**
Bacillus sp. PHS155	55	0.38	-
E. coli UM228	37	0.15	1
E. coli UM228 with pBPHS5.5	37	0.25	1.79

* The activity was assayed at 60°C.

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

2. Second subcloning of the DNA fragment harboring a *perA* gene for sequencing

The plasmid pBPHS5.5 was analyzed with 1% agarose gel electrophoresis to detect restriction map by single digestion (Fig. 9) and double digestion (Fig. 10-11). A 5.5 kb *Cla*I fragment of the plasmid pBPHS5.5 was digested with *Hind*III, *Pst*I, *EcoR*I, *Sal*I, *Hinc*II, *Kpn*I and *BamH*I, respectively and analyzed with 1% agarose gel electrophoresis (Fig. 12). The DNA fragment carrying a *perA* gene was detected size have a *perA* gene by Southern-hybridization by using pOD68 as a probe and found that the resulting 2.6 kb *Cla*I-*EcoR*I fragment, which contained a *perA* gene (Fig. 13). After that 2.6 kb *Cla*I-*EcoR*I fragment was subcloned into pBluescript II SK+ vector, named pBPHS2.6 and shown in Fig. 14.

The plasmid 2.7 kb *EcoR*I fragment was subcloned into pBluescript II SK+ vector, named pBPHS2.7 (Fig 15). The pBPHS2.7 containing 1.5 kb *Pst*I-*Sal*I fragment, and the 0.5 *EcoR*I-*Sal*I fragment were subcloned at the appropriate the size of pBluescript II SK+ vector. The resultant plasmid was named as pBPHS1.5 and pBPHS0.5, respectivly (Fig. 15). The clone pBPHS2.7, pBPHS2.6, pBPHS1.5 and pBPHS0.5 were used for sequencing. The position of restriction enzymes for the peroxidase gene of pBPHS2.6 are shown in Table 8.

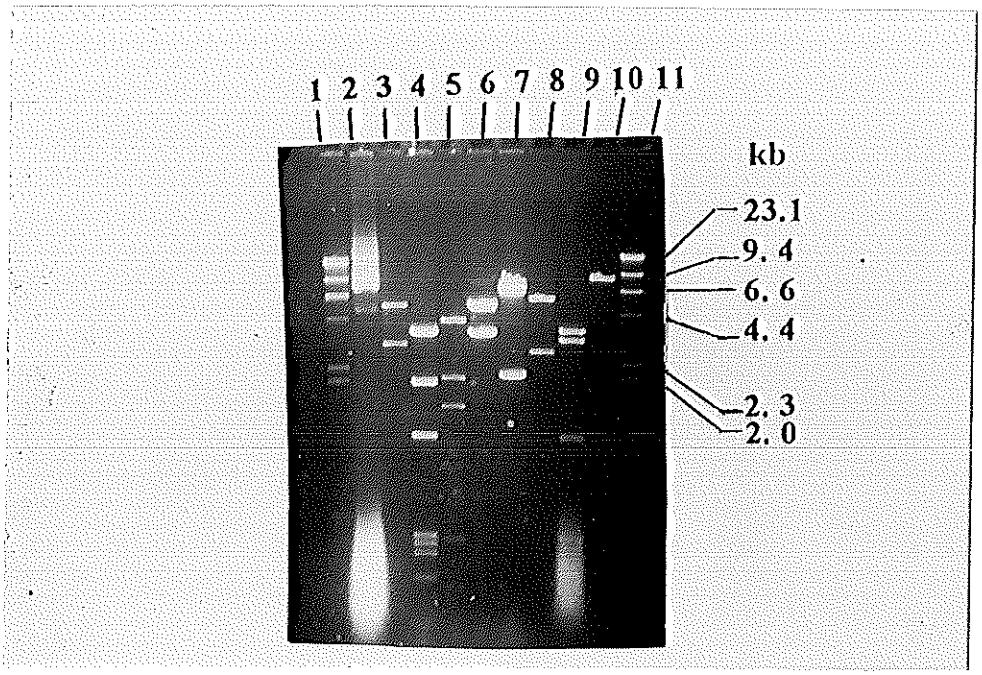


Fig. 9 Analysis pBPHS5.5 with single digestion

The pBPHS5.5 was digested with various restriction enzymes separated by 1% agarose gel electrophoresis and stained with ethidium bromide. Lane 1 and 11 : λ-*Hind*III (marker), lane 2 : pBPHS5.5 (undigested), lane 3, 4, 5, 6, 7, 8, 9 and 10 : pBPHS5.5 were digested with *Cla*I, *Hinc*II, *Kpn*I, *Pst*I, *Eco*RI, *Sal*I, and *BamH*I, respectively.

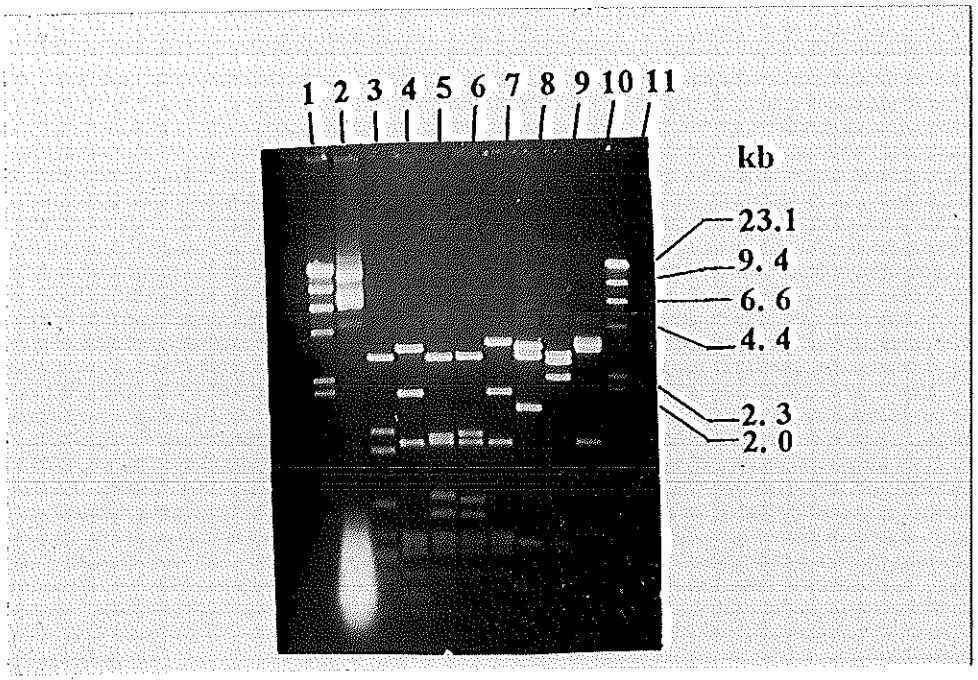


Fig. 10 Analysis pBPHS5.5 with double digestion

The pBPHS5.5 was digested with various restriction enzymes separated by 1% agarose gel electrophoresis and stained with ethidium bromide. Lane 1 and 11 : λ-*Hind*III (marker), lane 2 : pBPHS5.5 (undigested), lane 3, 4, 5, 6, 7, 8, 9 and 10 : pBPHS5.5 were digested with *Hinc*II/*Hind*III, *Hinc*II/*Kpn*I, *Hinc*II/*Pst*I, *Hinc*II/*EcoR*I, *Hinc*II/*Sal*I, *Kpn*I/*Pst*I, *Kpn*I/*EcoR*I, and *Kpn*I/*Sal*I, respectively.

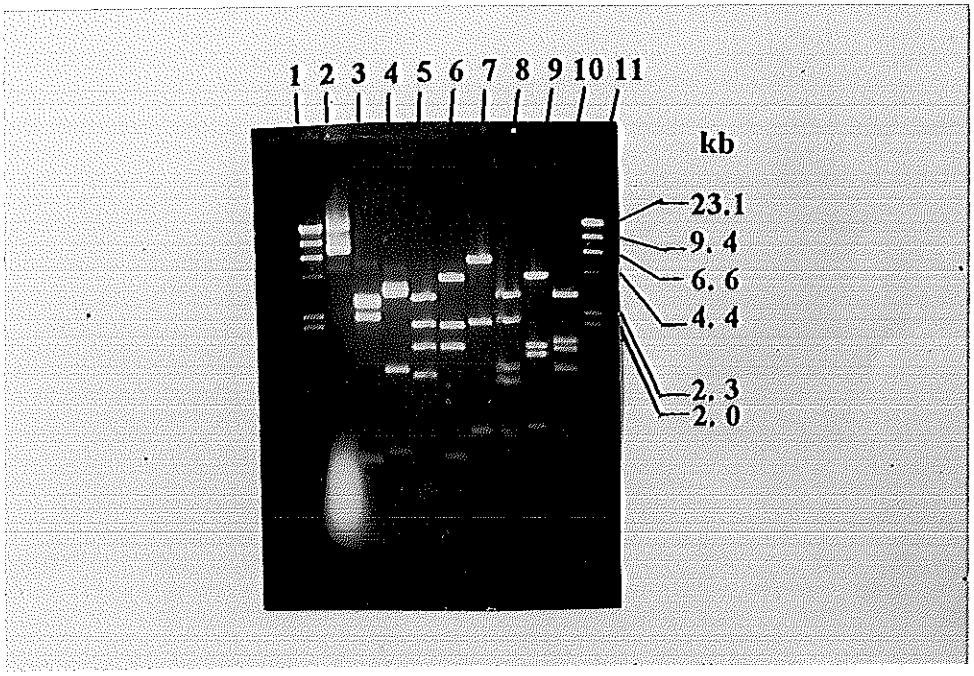


Fig. 11 Analysis pBPHS5.5 with double digestion

The pBPHS5.5 was digested with various restriction enzymes separated by 1% agarose gel electrophoresis and stained with ethidium bromide. Lane 1 and 11 : λ-*Hind*III (marker), lane 2 : pBPHS5.5 (undigested), lane 3, 4, 5, 6, 7, 8, 9 and 10 : pBPHS5.5 were digested with *Kpn*I/*Eco*RI, *Kpn*I/*Sal*I, *Kpn*I/*Hind*III, *Eco*RI /*Hind*III, *Eco*RI/*Pst*I, *Eco*RI/*Sal*I, *Eco*RI/*Hind*III, and *Pst*I/*Sal*I, respectively.

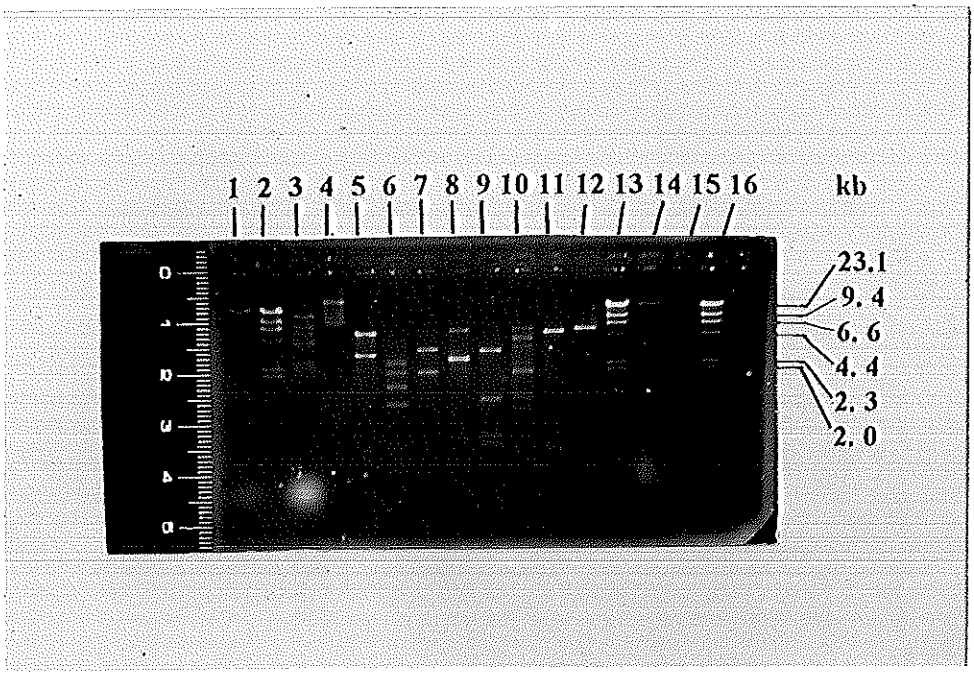


Fig. 12 Analysis 5.5 kb *Cla*I fragment from pBPHS5.5

The 5.5 kb *Cla*I fragment from pBPHS5.5 was digested with various restriction enzymes, separated by 1% agarose gel electrophoresis and stained with ethidium bromide. Lane 1 and 14 pOD68 (undigested), lane 2, 13 and 16 : λ-*Hind*III (marker), lane 3 : λ-GEM-11-*perA* cut with *Cla*I, lane 4 : pBPHS5.5(undigested), lane 5 : pBPHS5.5 cut with *Cla*I, lane 6 : 5.5 *Cla*I fragment cut with *Hind*III, lane 7, 8, 9, 10, 11 and 12 were digested with *Pst*I, *EcoR*I, *Sal*I, *Hinc*II, *Kpn*I and *BamH*I, respectively.

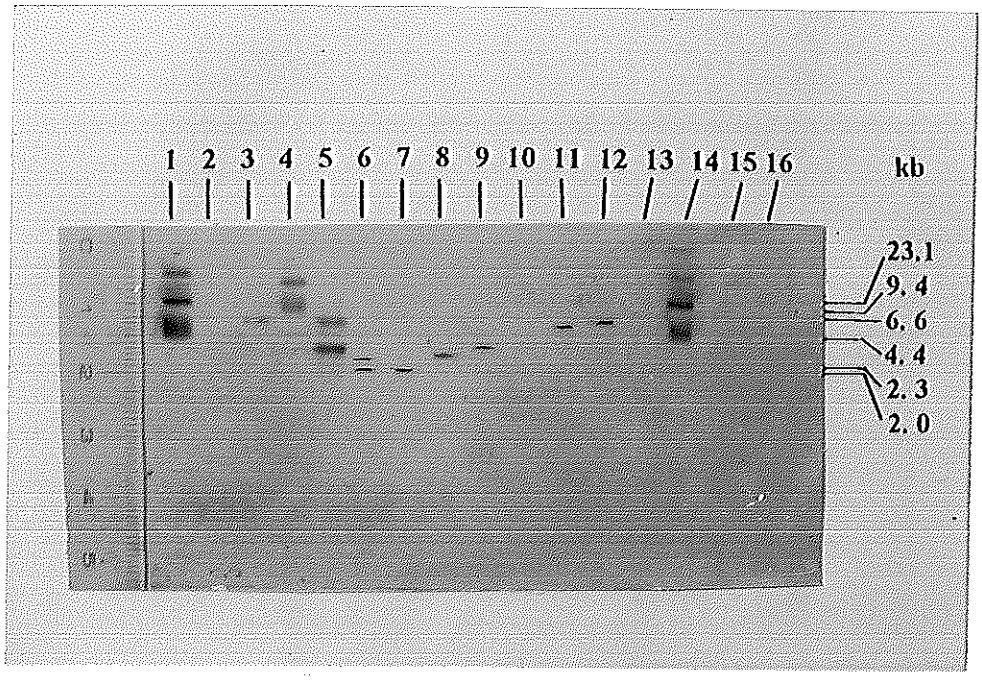


Fig. 13 Southern blot analysis 5.5 kb *Cla*I fragment from pBPHS5.5

The 5.5 kb *Cla*I fragment from pBPHS5.5 was digested with various restriction enzyme and hybridization with Anti-DIG labeled, pOD68 probe containing *perA* gene fragment. Lane 1 and 14 pOD68 (undigested), lane 2, 13 and 16 : λ-*Hind*III (marker), lane3 : λ-GEM-11-*perA* cut with *Cla*I, lane 4 : pBPHS5.5 (undigested), lane 5 : pBPHS5.5 cut with *Cla*I, lane 6 : 5.5 *Cla*I fragment cut with *Hind*III, lane 7, 8, 9, 10, 11 and 12 were digested with *Pst*I, *EcoR*I, *Sal*I, *Hinc*II, *Kpn*I and *BamH*I, respectively.

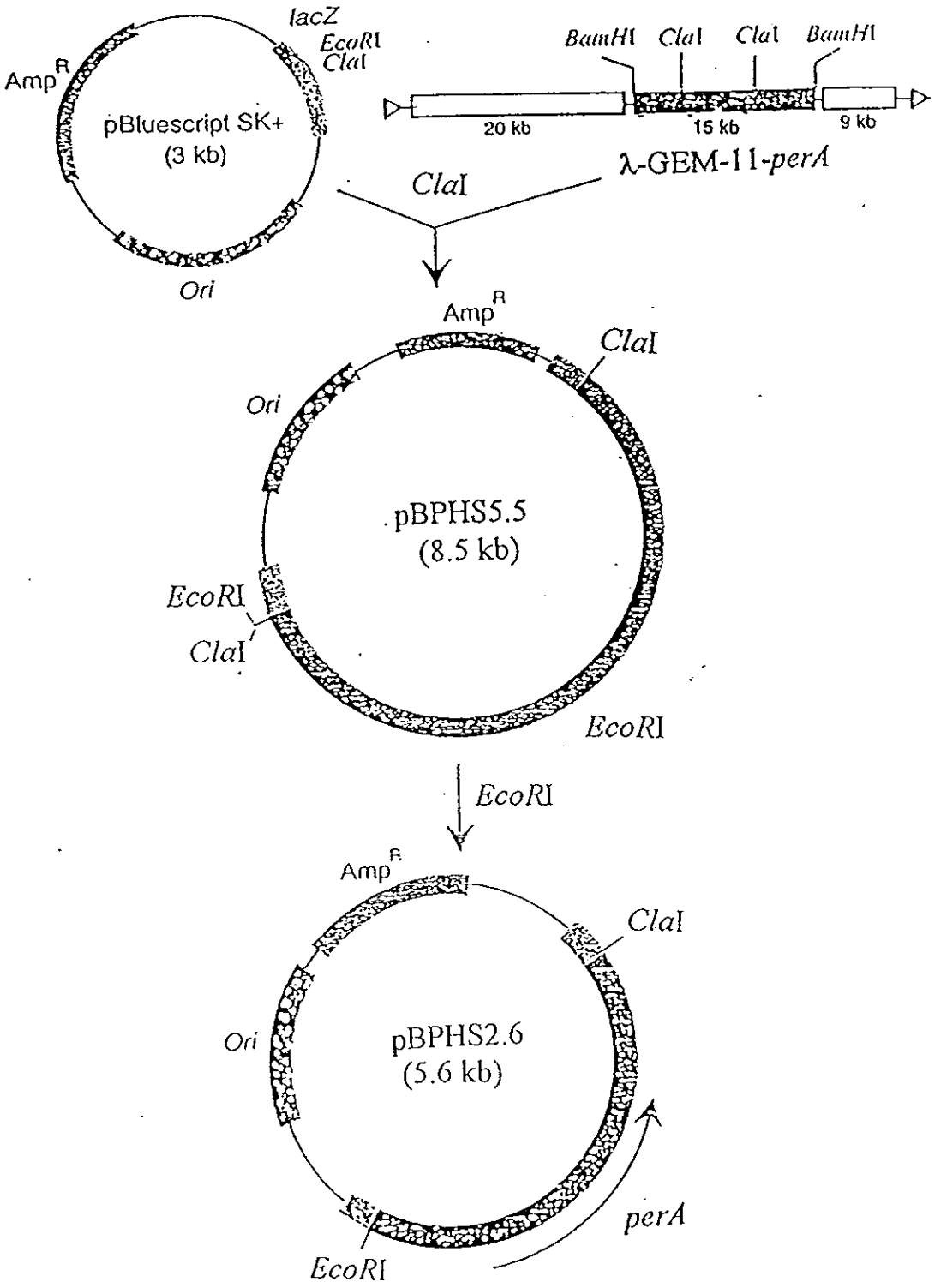


Fig. 14 The construction of plasmid pBPHS5.6

Plasmid pBPHS5.5 was digested *EcoR*I and subcloned into pBluescript II SK + (3.0 kb). The resulting of 2.6 kb *Cla*I-*EcoR*I fragment, which contained a *perA* gene.

Table 8 The position of restriction map enzymes for the peroxidase gene from pBPHS2.6

Enzyme Name	Sequence	Count	Start Position
*Cla*I	AT!CGAT	1	2649
*EcoR*I	G!AATTC	1	1
*EcoR*V	GAT!ATC	1	1555
*Hinc*II	GTA!GAC	4	1304, 1412, 1676, 2088
*Hind*III	A!AGCTT	2	2003, 2431
*Kpn*I	GGTAC!C	1	2261
*Nco*I	C!CATGG	1	338
*Pst*I	CTGCA!G	1	601
*Sal*I	G!TCGAC	1	2088

*Indication Mode : 5' terminal of the site

The position 1-2654

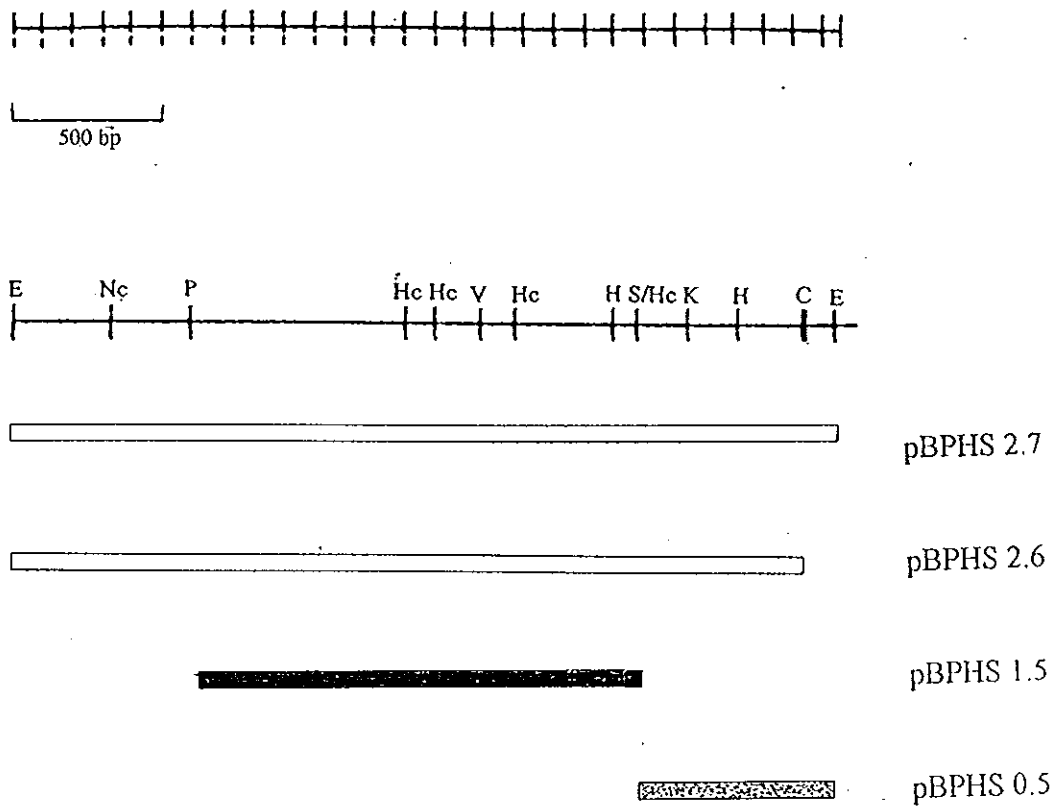


Fig. 15 Subcloning of pBPHS2.7 for sequencing

Plasmid pBPHS2.7 was a pBluescript II SK+ vector derived, *EcoR*I clone. : C-*Cla*I, E-*EcoR*I, H-*Hind*III, Hc-*Hinc*II, K-*Kpn*I, Nc-*Nco*I, P-*Pst*I, S/Hc-*Sal*I/*Hinc*II, V-*EcoR*V.

3. The DNA sequences of pBPHS2.6 peroxidase gene

The restriction map and the sequencing strategy of the pBPHS2.6 peroxidase gene are shown in Fig. 16. The nucleotide sequence and deduced amino acid are shown in Fig. 17. A 2,184 base-pair open reading frame starting at ATG (position 318) and terminating at TAA (position 2,504) which was capable of coding a peptide of 728 amino acid residues of 82,321.89 daltons was found.

A Shine-Dalgarno sequence, the ribosome-binding site GAAAGG-AG, was observed 9 base pairs upstream from the initiation codon. The possible promotor sequences, TATTTC at the -10 region was also observed upstream from the Shine-Dalgarno sequence (Fig. 17). The G+C content of the gene is 48.46%. The codon usage of this gene was shown in Table 9. For the amino acid which have four codons (Arg, Ala, Gly, and Pro), the codon with G or C as the third letter is preferred (Table 9). The hydropathy profiles of peroxidase was shown in Fig. 18. In addition the isoelectric point was calculated to be 5.31 (Fig. 19).

4. Comparison of amino acid of peroxidase

The deduced amino acid sequence of *Bacillus* sp. PHS155 peroxidase was compared with the sequence of *B. stearothermophilus* catalase I (shown in Fig. 20). Peroxidase gene consists of 728 amino acids, which is very close to the 731 amino acids of *B. stearothermophilus.* enzyme and show the overall homology of 85.3%.

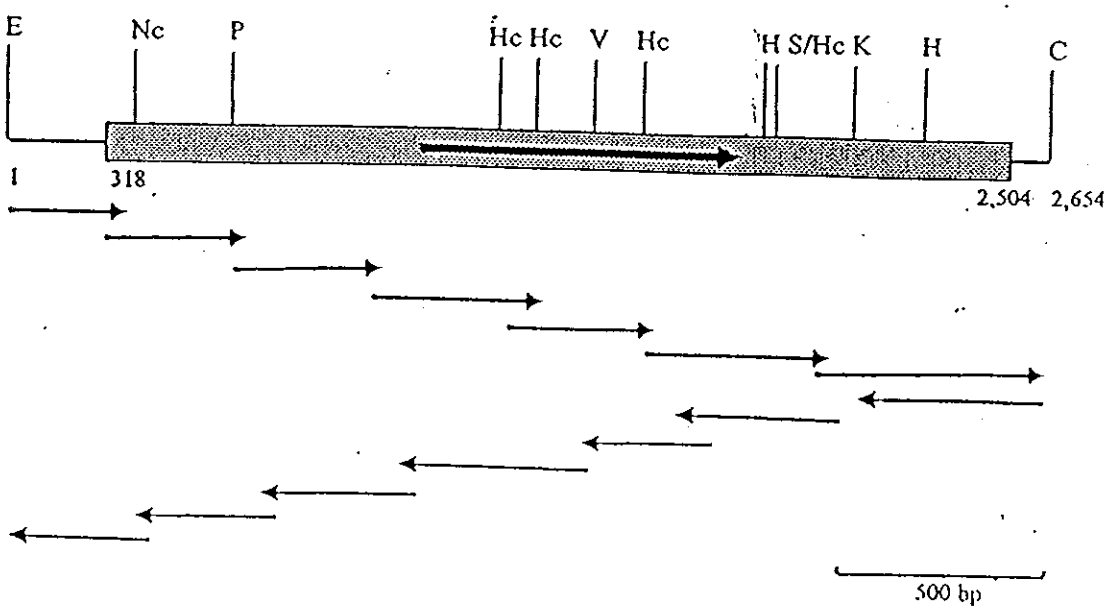


Fig. 16 Restriction map and Sequencing strategy for the peroxidase gene of pBPHS2.6

Start codon (318), Terminator codon (2504).

C-*Cla*I, E-*EcoR*I, H-*Hind*III, Hc-*Hinc*II, K-*Kpn*I, Nc-*Nco*I, P-*Pst*I, S/Hc-*Sal*I/*Hinc*II, V-*EcoR*V.

*Eco*RI

1 5'GAATTC↓GGAATCG 13

14 AGGCGATTGACTTAATCGAATTTATTCGCAACTTGACAACTGAACAAGTCAAATATATGCT 74

75 GCAACATTAAAGATTGGAGTGAAACGATGGACGGAAATGCGGATGTGAACGTATAAATCC 134

135 CCCATTTTATTCTGCAAACAGAATGAAATGGGGGATTTTTATAATTTTTAAATAAAAATAT 195

196 CATTTTTACTTTACTATTAAATATTAATTTAGTATAATTCTAATGTGCTAATTATTATCACT 257

258 CTCAACAAGTGAACACCTAGCATCATATTTCATTTATATGTAGGAAAGGAGAATAAAAGA 317

318 ATGGAAAACAAATGCCCGTTCCATGGAAGTGTGACGAATCATACTTCAAACAAAACAACA 377

1 M E N K C P F H G S V T N H T S N K T T 20

R2<----------

378 AACAAAGACTGGTGGCCGAATCAACTGAATTTAAGCATTCTTCATCAACATGACCGAAAA 437

21 N K D W W P N Q L N L S I L H Q H D R K 40

----

438 ACGAATCCCCATGACGAAACGTTCGATTATGCGGAACAATTTCAAACACTTGACTATTGG 497

41 T N P H D E T F D Y A E Q F Q T L D Y W 60

498 GCGTTAAAAGAAGATTTGCGTAAACTCATGACGACAAGCCAGGATTGGTGGCCTGCCGAC 557

61 A L K E D L R K L M T T S Q D W W P A D 80

558 TACGGACATTACGGTCCACTATTCATCCGCATGGATTGGCATTCTGCAGGAACATATCGC 617

81 Y G H Y G P L F I R M D W H S A G T Y R 100

R1<----------

618 ATTGGCGACGGACGAGGCGGCGCTTCCACAGGTACGCAACGGTTCGCCCCGTTAAACAGT 677

101 I G D G R G G A S T G T Q R F A P L N S 120

678 TGGCCACACAACGCGAACTTAGATAAAGCACGTCGCCTGTTGTGGCCGATTAAACAAAAA 737

121 W P H N A N L D K A R R L L W P I K Q K 140

738 TACGGCAATAAAATTTCTTGGGCCGATTTAATTGTGTTAGCTGGCAACGTGGCGATTGAG 797

141 Y G N K I S W A D L I V L A G N V A I E 160

798 TCTATGGGTGGAAAAACGATCGGTTTTGGCGCTGGCAGAGAGGACGTATGGCACCCAAAA 857

161 S M G G K T I G F G A G R E D V W H P K 180

----

858 GAAGACATTTATTGGGGGGCGGAAAAAGAATGGCTCGCCTCCGAACGCTATACAGGCGAC 917

181 E D I Y W G A E K E W L A S E R Y T G D 200

------>T1

918 CGCGAATTAGAAAATCCGCTTGCCGCCGTTCAAATGGGGCTGATTTATGTCAATCCAGAA 977

201 R E L E N P L A A V Q M G L I Y V N P E 220

M10<----------

978 GGTCCTGATGGCAACCCAAATCCGCTCGCGGCGGCGCGTGACATTCGCGAGACGTTTAAA 1037
221 G P D G N P N P L A A A R D I R E T F K 240

1038 CGAATGGGAATGAACGACGAAGAAACTGTGGCGCTCATCGCAGGCGGCCATACGTTTGGG 1097
241 R M G M N D E E T V A L I A G G H T F G 260

1098 AAGGCGCATGGAGCTGGTGCTGCATCGCATGTCGGCCCTGAACCAGAAGCAGCTCCGATT 1157
261 K A H G A G A A S H V G P E P E A A P I 280

1158 GAAGCGCAAGGGTTAGGATGGCTTAGCTCGTACGGAAAAGGGAACGGACGCGATACGATT 1217
281 E A Q G L G W L S S Y G K G N G R D T I 300

---------->T2

1218 ACAAGCGGTCTGGAAGGTGCTTGGACGCCAACACCTACGCAATGGGATAATAGCTATTTA 1277
301 T S G L E G A W T P T P T Q W D N S Y L 320

1278 CAATTGCTTTTTGAATATGAATGGAAGTTGACGAAAAGTCCAGCTGGTGCGTATCAATGG 1337
321 Q L L F E Y E W K L T K S P A G A Y Q W 340

1338 GAAGCAGTGAATATAAAAGAAGAACATTTAGCGCCTGATGTCGAAGATGCCAATGTGAAA 1397
341 E A V N I K E E H L A P D V E D A N V K 360

M11<----------

1398 GTGCCGCCGATGATGTTGACGACCGATTTAGCGTTGCGCTTTGATCCAATATATGAAAAA 1457
361 V P P M M L T T D L A L R F D P I Y E K 380

1458 ATTGCTCGTCGCTTTTATGAACATCCAGAGGAATTTGCAGATGCGTTTGCGCGCGCTTGG 1517
381 I A R R F Y E H P E E F A D A F A R A W 400

1518 TTTAAGCTCATTCATCGCGACATGGGGCCGAAAACGAGATATCTTGGCCCAGAAGTGCCG 1577
401 F K L I H R D M G P K T R Y L G P E V P 420

1578 AAAGAAGATTTCATTTGGCAAGACCCGATTCCAGAAGCTGATTATGAATTAACGGATGCG 1637
421 K E D F I W Q D P I P E A D Y E L T D A 440

---------->T4

1638 GAAATCGAAGAGATCAAAACGGAAATTTTACATTCAGGGTTAACGATTAGCGAACTTGTG 1697
441 E I E E I K T E I L H S G L T I S E L V 460

1698 AAAACGGCTTGGGCATCCGCTAGCACGTTCCGTAACTCCGATAAGCGTGGCGGAGCGAAC 1757

461 K T A W A S A S T F R N S D K R G G A N 480

M12<----------

1758 GGTGCGCGGATTCGGCTCGCGCCACAAAAAGATTGGGAAGTGAACGAACCAGAGCGACTT 1817

481 G A R I R L A P Q K D W E V N E P E R L 500

1818 GCGAAAGTGCTTGCCGTTTATGAAGACATTCAACGCGCGCTCCCTAAAAAAGTAAGCATC 1877

501 A K V L A V Y E D I Q R A L P K K V S I 520

1878 GCTGATTTAATCGTACTCGGCGGCAGCGCGGCAGTCGAGAAAGCAGCCCGCGATGCCGGG 1937

521 A D L I V L G G S A A V E K A A R D A G 540

1938 TTTGACGTCCAAACACCGTTTATGCCTGGACGAGGGGATGCTACGGAGGAACAAACGGAT 1997

541 F D V Q T P F M P G R G D A T E E Q T D 560

1998 GTGGAAAGCTTTTCCGTATTAGAACCGTTCGCTGACGGATTCCGCAACTATGAAAAGAAA 2057

561 V E S F S V L E P F A D G F R N Y E K K 580

2058 AAATATCGCGTCGGCCCTGAGGAACTGCTTGTCGACAAAGCCCAATTGCTCGGCTTAACC 2117

581 K Y R V G P E E L L V D K A Q L L G L T 600

2118 GCCCCAGAGATGACGGTATTAGTCGGTGGCTTGCGTGTGTTAGGAGCAAACTATCGCGAT 2177

601 A P E M T V L V G G L R V L G A N Y R D 620

2178 CTTCCTCACGGTGTATTTACGGATCGCATCGGGGTGCTCACAAACGATTTCTTTGTTCAT 2237

621 L P H G V F T D R I G V L T N D F F V H 640

2238 CTTGTCGATATGAACTATGAATGGGTACCAAGGGAAGATGGCTTGTATGACATTCGCGAC 2297

641 L V D M N Y E W V P R E D G L Y D I R D 660

2298 CGGAAAACGAAGGCTGTTCGTTGGACCGCCACTCGTGTAGATTTAATTTTCGGATCAAAC 2357

661 R K T K A V R W T A T R V D L I F G S N 680

2358 TCGATCCTTCGCTCTTACGCTGAATTTTACGCCCAAGACGACAACAAAGAAAAATTTGTC 2417

681 S I L R S Y A E F Y A Q D D N K E K F V 700

2418 CGCGACTTTATTCAAGCTTGGGTAAAAGTCATGAACGCCGACCGATTCGACCTTCGGAAA 2477

701 R D F I Q A W V K V M N A D R F D L R K 720

2478 AAAACAAAAACATCGGTTACGATTTAAAGGAGAAAAAGCAGGTGGACGATCGTCTCCCCT 2537
721 K T K T S V T I * 728

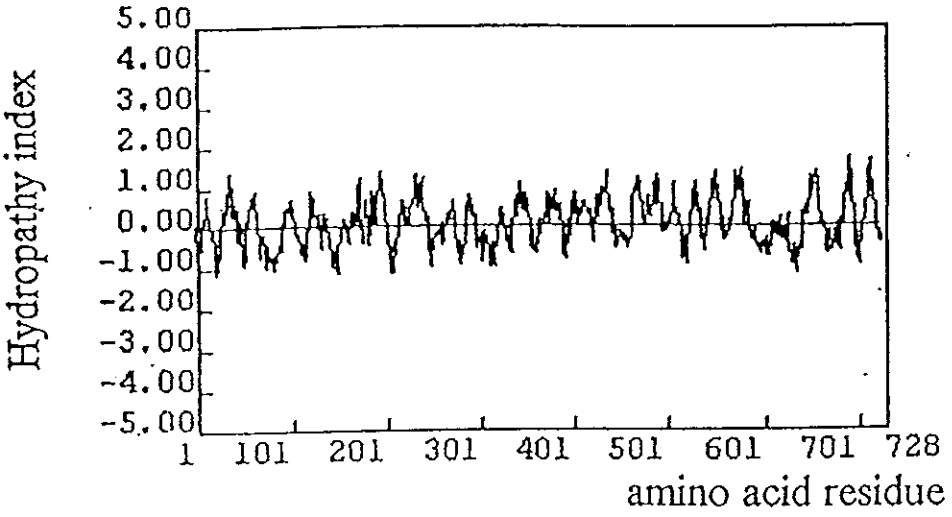
2538 GCTTTTTTATCTCTCATAAATAACGATTCCGTCACGCTCAATTTGCCGCGCGATCTCTTT 2597
2598 ATTCAATGAATCAAAAAACAGCACATCGAACGAATAAATGCCAACAATTTC↓ATCGAT 2654
Cla I

Fig.17 The nucleotide and amino acid sequence of peroxidase gene of *Bacillus* sp. PHS155. The nucleotide sequence was presented from position 1 to 2654. The possible Shine-Dalgarno sequence (SD) and promotor regions (-10 and -35 regions) were indicated by single underlining and double underlining, respectively. The nucleotide sequence was identified for both strands using the synthetic primers, indicated by broken arrows above the nucleotide sequence.

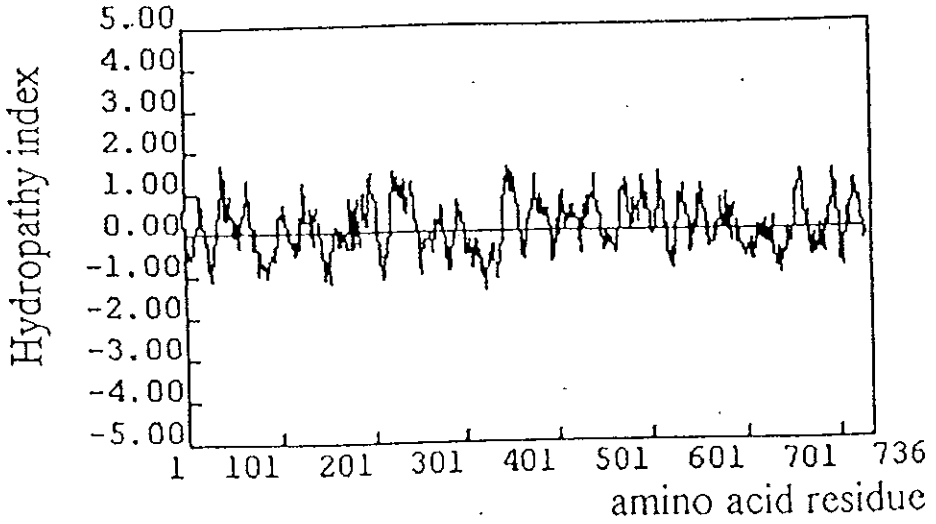
Table 9 The codon usage of peroxidase gene from pBPHS2.6.

Start codon : 318- Terminator codon : 2504

Codon	count	%	Codon	count	%	Codon	count	%	Codon	count	%
TTT-Phe	19	2.62	TCT-Ser	3	0.41	TAT-Tyr	19	2.62	TGT-Cys	0	0.00
TTC-Phe	9	1.24	TCC-Ser	5	0.69	TAC-Tyr	6	0.82	TGC-Cys	1	0.13
TTA-Leu	20	2.76	TCA-Ser	3	0.41	TAA-***	0	0.00	TGA-***	0	0.00
TTG-Leu	9	1.24	TCG-Ser	4	0.55	TAG-***	0	0.00	TGG-Trp	24	3.31
CTT-Leu	14	1.93	CCT-Pro	8	1.10	CAT-His	15	2.07	CGT-Arg	9	1.24
CTC-Leu	10	1.38	CCC-Pro	1	0.13	CAC-His	3	0.41	CGC-Arg	20	2.76
CTA-Leu	1	0.13	CCA-Pro	16	2.20	CAA-Gln	19	2.62	CGA-Arg	6	0.82
CTG-Leu	5	0.69	CCG-Pro	14	1.93	CAG-Gln	1	0.13	CGG-Arg	5	0.69
ATT-Ile	23	3.17	ACT-Thr	3	0.41	AAT-Asn	11	1.51	AGT-Ser	3	0.41
ATC-Ile	9	1.24	ACC-Thr	3	0.41	AAC-Asn	20	2.76	AGC-Ser	10	1.38
ATA-Ile	2	0.27	ACA-Thr	13	1.79	AAA-Lys	38	5.24	AGA-Arg	2	0.27
ATG-Met	14	1.93	ACG-Thr	25	3.45	AAG-Lys	6	0.82	AGG-Arg	1	0.13
GTT-Val	5	0.69	GCT-Ala	19	2.62	GAT-Asp	28	3.86	GGT-Gly	13	1.79
GTC-Val	11	1.51	GCC-Ala	15	2.07	GAC-Asp	24	3.31	GGC-Gly	20	2.76
GTA-Val	8	1.10	GCA-Ala	11	1.51	GAA-Glu	46	6.31	GGA-Gly	15	2.07
GTG-Val	14	1.93	GCG-Ala	23	3.17	GAG-Glu	10	1.38	GGG-Gly	10	1.38



A



B

Fig. 18 Aligned hydropathy profiles of *Bacillus* sp. PHS155 peroxidases (A) and *Bacillus stearothermophilus* catalase I (B)

Positive hydropathy indicates hydrophobicity.

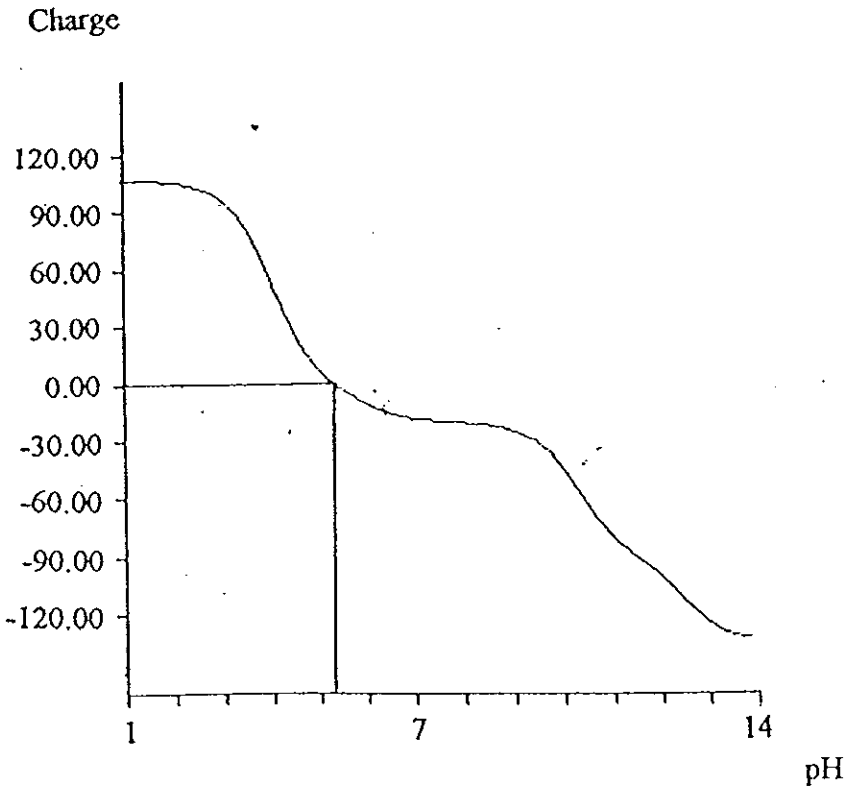


Fig. 19 Aligned isoelectric profiles of *Bacillus* sp. PHS155 peroxidases
The Isoelectric point was 5.31

Table 10 Characteristics of amino acid in the peroxidase gene

Amino acid	Number	pKa
: Positive :		
Arginine (Arg) (R)	43	12.5
Histidine (His) (H)	18	6.6
Lysine (Lys) (K)	44	10.5
: Nagative :		
Asparagine (Asp) (D)	52	3.9
Cysteine (Cys) (C)	1	8.3
Glutamic acid (Glu) (E)	56	4.3
Tyrosine (Tyr) (Y)	25	10.1

* N-terminal : Methionine (Met) (M), pKa = 9.3

* C-terminal : *** , pKa = 0.0

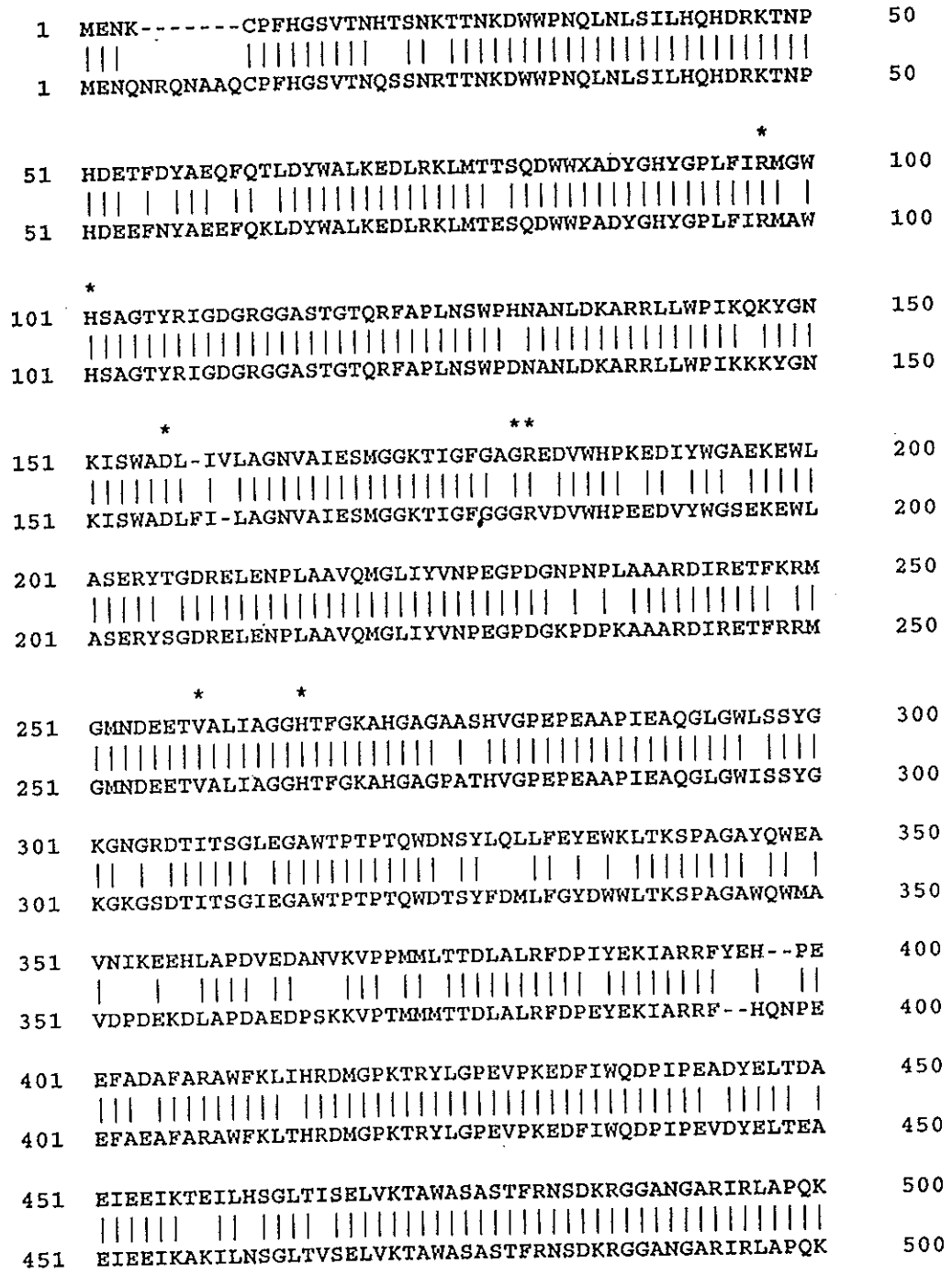


Fig.20 Comparison of the deduced amino acid sequences of *Bacillus* sp. PHS155 (upper) and *Bacillus stearothermophilus* (lower) Trakunaleamsai, *et al.*, 1995). Residues conserved sequences indicated with star above the top sequence. Matching percentage (Total Window : 85.3%, Alignment Window : 85.3%)

```
501  DWEVNEPERLAKVLAVYEDIQRALPKKVSIADLIVLGGSAAVEKAARDAG  550
     ||||||||||||||| ||||||| |||||||||||||||||||||||||||
501  DWEVNEPERLAKVLSVYEDIQRELPKKVSIADLIVLGGSAAVEKAARDAG  550

551  FDVQTPFMPGRGDATEEQTDVESFSVLEPFADGFRNYEKKKYRVGPEELL  600
     |||  ||  ||||||| |||||||| |||||||||||| |  | | |||||
551  FDVKVPFFPGRGDATQEQTDVESFAVLEPFADGFRNYQKQEYSVPPEELL  600

601  VDKAQLLGLTAPEMTVLVGGLRVLGANYRDLPHGVFTDRIGVLTNDFFVH  650
     ||||||||||||||||||||||||||||||||||||||||||||||||||
601  VDKAQLLGLTAPEMTVLVGGLRVLGANYRDLPHGVFTDRIGVLTNDFFVN  650

651  LVDMNYEWVPRED-GLYDIRDRKTKAVRWTATRVDLIFGSNSILRSYAEF  700
     | ||||||||  | | | ||||||  ||||||||||||||||||||||
651  LLDMNYEWVPT-DSGIYEIRDRKTGEVRWTATRVDLIFGSNSILRSYAEF  700

701  YAQDDNKEKFVRDFIQAWVKVMNADRFDLRKKTKTSVTI*.........  750
     ||||||  ||||||||  ||||||||||||  ||   |||
701  YAQDDNQEKFVRDFINAWVKVMNADRFDLVKKARESVTA*.........  750
```

Fig.20 (continued)

Table 11 Expression of peroxidase gene for sequencing

Strains	Culture temp. (°C)	Specfic activity* (U/mg)	Ratio**
E.coli UM228	37	1.99	1
E.coli UM228 with pBPHS2.6	37	2.44	1.23
E.coli UM228 with pBPHS1.5	37	2.26	1.14
E.coli UM228 with pBPHS0.5	37	1.50	0.75

* The activity was assayed at 60 °C.

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

Chapter 4

Discussion

The peroxidases are widely distributed throughout plants, animals, and microorganisms. Plant peroxidases can be found in the cytoplasm, membrane, root cell culture, leaf and seed. The source of commercial peroxidase available is obtained from horseradish (*Armoracia rusticana*) : Horseradish peroxidase (HRP). They consist of more 40 components (Intrapruk, *et al.*, 1994). However, peroxidases are highly catalytic enzymes, they have very low specificity and exist in a multitude of isoenzymes, making it difficult to ascertain their actual functions (Kawaoka, *et al.*, 1994). Animal cells contain specific peroxidases such as myeloperoxidase, eosinophil peroxidases and platelet peroxidases. The most important problems both found in plants and animals enzyme production are related to the limited quantities, stability and high cost of purification. Therefore, peroxidases used in the presence are of microbial origin. It is cheaper and can be made in unlimited quantities (Loprasert, *et al.*, 1989). For this reason, the microbial peroxidase has attracted comparatively great attention for researchers. In contrast, thermostable peroxidase are of interest because of their potential industrial applications (Loprasert, *et al.*, 1988).

Most of the microorganisms possess bifunctional enzymes having peroxidase and catalase activity. These enzymes have been isolated from *Bacillus stearothermophilus* (Loprasert *et al.*, 1989), *E. coli* (Claiborne, *et al.*, 1979), *Rhodopseudomonas capsulata* (Hochman, *et al.*, 1987),

and *Comamonas compransoria* (Nies, *et al.*, 1982). Some of them (*E. coli* and *R. capsulata* enzymes) are tetrameric enzymes, and others (*B. stearothermophilus* and *C. compransoria*) are dimeric enzymes. Inspite of the differences in their subunit numbers, they have been found to contain one molecule of protoheme IX per two subunits. The gene of catalase-peroxidase HPI, from *E. coli* has been sequenced, No homology between HPI and other typical catalases which have no peroxidase activity has been found (Trigg-Raine, *et al.*, 1988).

Currently, *Bacillus* sp PHS155 has been isolated from hot pond in the south of Thailand. Thongma (1997) reported that the peroxidase of *Bacillus* sp. PHS155 was a functional enzyme having peroxidase without catalase activity and can tolerate high temperature (60°C). A peroxidase was purified from the crude extract of *Bacillus* sp. PHS155 by ion exchange chromatography (DEAE-Sephacel), gel filtration on Sephadex G-200, G 50-150 and Sephacryl S-300. The enzyme was purified about 8.76 times with a recovery of 0.05%. The specific activity of enzyme was 3.33 U/mg. It shown optimum pH 5-7 at 60 °C.

Ms. Kintkand Na pathalung (1996) cloned a 9.0 kb chromosomal DNA fragment containg peroxidase gene from *Bacillus* sp. PHS155 into λ-GEM-11. In this thesis, The DNA sequence of the peroxidase gene was confirmed. The gene consists of 2,187 basepairs with the deduced amino acid of 82,321.89 daltons. The amino acid alignment between the catalase I of *B. stearothermophilus* and peroxidase of *Bacillus* sp. PHS155 was shown in Fig. 15. The catalase I from *B. stearothermophilus* consists of 731 amino acid residues, which is very close to the 728 amino acid residues of 82,321.89 daltons of peroxidase from

Bacillus sp. PHS155. While at this molecular weight did not close to the 67 kDa of the peroxidase, which was purified by using ion exchange chromatography on DEAE-Sephacel, gel filtration on Sephadex G-200, G 50-150 and Sephacryl S-300 and determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Thongma, 1997). These observations suggest that the determination of the peroxidase by using gel filtration might be in error. The amino acid Met-Gul-Asn-Lys-Cys-Pro of catalase I from *B. stearothermophilus*, which are not found in mature proteins, might be presumably removed posttranslationally (Loprasert *et al.*, 1989). Sequence comparisons of this peroxidase gene of available animal and plant peroxidases showed no signs of similarity (Welinder, 1991). In contrast, the catalase I (hydroperoxidase from *B. stearothermophilus*) showed homology with *E. coli* catalase (hydroperoxidase I, HPI), *Salmonella trphimurium* catalase HPI and yeast CCP of 63%, 62% and 40% respectively (Trakunaleamsai *et al.*, 1992). On the other hand, sequence comparisons of catalase I from *B. stearothermophilus* showed homology with peroxidase from *Bacillus* sp. PHS155 85.3%. No obvious homology is observed between *B. stearothermophilus* peroxidase and horseradish enzyme (Fujiyama, *et al.*, 1988). These observations might suggest that the group of enzymes having peroxidase without catalase activity is different from typical catalase and typical peroxidase. It seems that the peroxidases and these bacterial catalases might be evolutionarily related (Trakunaleamsai *et al.*, 1992).

Fujiyama and colleague (1990) compared the peroxidase gene from tobacco, potato, *E. coli*, *B. stearothermophilus* and cytochrome c

peroxidase (CCP) gene. The results revealed that amino acid sequences of plant peroxidase were 36-50% identical, whereas these 5 horseradish peroxidases (HRPs) show lower homology with microbial peroxidase sequences.

The hydroprofiles of catalase I from *B. stearothermophilus* was compared with peroxidase from *Bacillus* sp. PHS155, it was found that the two profiles were very similar (85.3% homology). In addition, Dr. Savitr Trakunaleamsai and colleagues (1995) reported a comparison of the hydroprofiles of catalase I from *B. stearothermophilus* and cytochrome c peroxidase. The two profiles are also very similar (40% homology). This high similarity suggests similar protein folds in the matched regions of these enzyme. Therefore, it seems possible to predict the tertiary structure of the N-terminal half of two bacterial catalases and peroxidase of *Bacillus* sp. PHS155 using the known structure of the cytochrome c peroxidase (Finzel, *et al.*,1984) and the aligment shown in Fig. 19.

The expression of peroxidase gene was determined by assaying peroxidase activity of the crude extracts at 60°C. The pBPHS5.5 produced the enzyme more than the host *E. coli* UM228 but less than *Bacillus* sp. PHS155 (Table 7). These results prove that the *perA* of *Bacillus* sp. PHS155 as expressed in *E. coli* UM228 is similar to the expressed product of peroxidase gene from *B. stearothermophilus* in *E. coli* UM228 (Loprasert, *et al.*, 1990). However, the level of acivity was lower than wild type. In addition, studies on the expression of peroxidase gene from pBPHS2.6, pBPHS1.5 and pBPHS0.5 in *E. coli* UM228 (Table 11). The pBPHS2.6, and pBPHS1.5 showed peroxidase

activity about one time higher than pBPHS0.5 and more than *E. coli* UM228. These results suggests that expression vector may require 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 " enhancers " as well as " minicitron " in translationally coupled systems. (Makrides, 1996). The expressed product had enzyme activity, suggesting that protoheme was incoporated correctly in the expressed product. The enzyme also exhibited themostability, which is a remarkable property of *Bacillus* sp. PHS155 peroxidase .

Therefore, the sequencing of a peroxidase encoding gene from *Bacillus* sp. PHS155 was studied to understand the structure, properties, and sequences of peroxidase. In addition, these clones (pBPHS5.5, pBPHS2.6) in the thesis might be useful in the production of peroxidases for the development of biotechnological processes, for clinical diagnosis which is refered to as enzymes-linked immunosorbent assay (ELIAS), including the treatment of wastewater containing phenolic compounds.

Chapter 5

Summary

1. A 15 kb of chromosomal DNA of *Bacillus* sp. PHS155 which contained the peroxidase gene (*perA*) was cloned into *Bam*HI site of λ-GEM-11. After that, 5.5 kb *Cla*I fragment was subcloned into pBluescript II SK+. A *perA* gene was detected with Southern-hybridization by using pOD68 probe. The resulting 2.7 kb *Eco*RI containing *perA* gene was sequenced.

2. The peroxidase-encoding gene (*perA*) of *Bacillus* sp. PHS155 was subcloned in *E coli*. UM228. The gene consisted of 2,187 base pairs (inclued stop codon), by open reading frame encoding a peroxidase of 728 amino acid residues, and encodes a protein of M_r 82,321.84. The G+C content of the peroxidase gene was 48.46% and isoelectric point was 5.31 indicating that it was acidic peroxidase .

3. The comparison of the deduced amino acid sequence peroxidase of *Bacillus* sp. PHS 155 showed 85.3% homology which was most closely related to the amino acid sequence of the calalase I (hydroperoxidase) from *Bacillus stearothermophillus* IAM 11001.

Table 12 The comparison a peroxidase gene (*perA*) of *Bacillus* sp. PHS155 with a catalase I of *B. stearothermophilus* IAM 11001

	Bacillus sp. PHS 155	*B. stearothermophilus* IAM 11001
Nucleotide sequence	2.7 kb *EcoR*I	3.1 kb *EcoR*I
Amino acid	728	731
Molecular weight (daltons)	82,321.84	82,963
G+C content (%)	48.46	55
pI	5.31	4.92
Expression	*E.coli* UM228	*E.coli* UM228

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Appendix

1. LB (Luria-Bertaini) Medium (100 ml)

Bacto-yeast Extract	0.5 g
Bacto tryptone	1.0 g
NaCl	0.5 g

Adjust pH to 7.5 with NaOH and autoclave when the solution has cooled add 1 ml of 1 M $MgSO_4.7H_2O$ and 1 ml of 20% maltose.

2. SM Buffer (per liter)

50 mM Tris-HCl ; pH 7.5

100 mM NaCl

8.0 mM $MgSO_4$

0.01% Gelatin

3. LB Top Agar (100 ml)

Bacto-yeast Extract	0.5 g
Bacto tryptone	1.0 g
NaCl	0.5 g
Agarose	0.6 g

Adjust pH to 7.5 with NaOH and autoclave when the solution has cooled add 1 ml of 1 M $MgSO_4.7H_2O$ and 1 ml of 20% maltose.

4. Phage Buffer (100 ml)

20 mM Tris-HCl ; pH 7.5

100 mM NaCl and 8.0 mM $MgSO_4.7H_2O$

5. S Buffer (100 ml)

Phage Buffer + 2% (w/v) gelatin

6. Phage Precipitation Solution (100ml)

20% (w/v) Polyethylene glycol (MW 8,000)

2M NaCl

7. 1×TAE Buffer (100 ml)

0.04 M Tris-HCl

2 mM EDTA

8. TE Buffer (100 ml)

10mM Tris-HCl ; pH 8.0

1 mM EDTA

9. 10×TBE Stock Solution Buffer (pH 8.3)

Tris base	107.8 g
Boric acid	55.0 g
Na_2EDTA	8.2 g
dH_2O add to	1.0 L

Filter using Whatman # 1 filter paper and store at 4°C up to two weeks.

10. 1×TBE Buffer (1500 ml)

10×TBE Stock Solution 150 ml

dH_2O 1350 ml ; Stir for 5 min. Make fresh daily.

11. 40% Acrylamide Stock Solution

19:1(w/w) Acrylamide/Bis-acrylamide powder (Bio-Rad)

dH_2O 48.0 ml

Stir for 30 min and store at 4°C up to a month.

12. 10% Ammonium Persulfate (w/v)

Ammonium Persulfate	0.2 g
dH_2O	2.0 ml

Stir until dissolved and this solution can be store at 4°C for 5 days.

13. 2% x-Gal (5-Bromo-4-chloro-3-indoyl-β-D-galactoside) (w/v)

x-Gal	0.2 g
Dimethylformamide (DMT)	100 ml

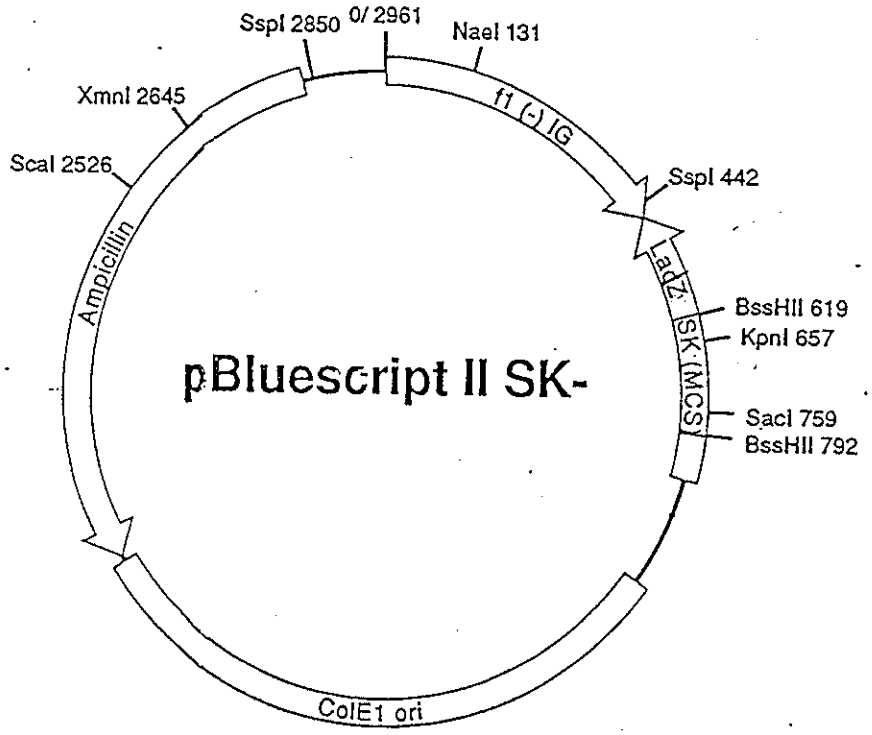
Stir until dissolved and this solution can be store at 4°C.

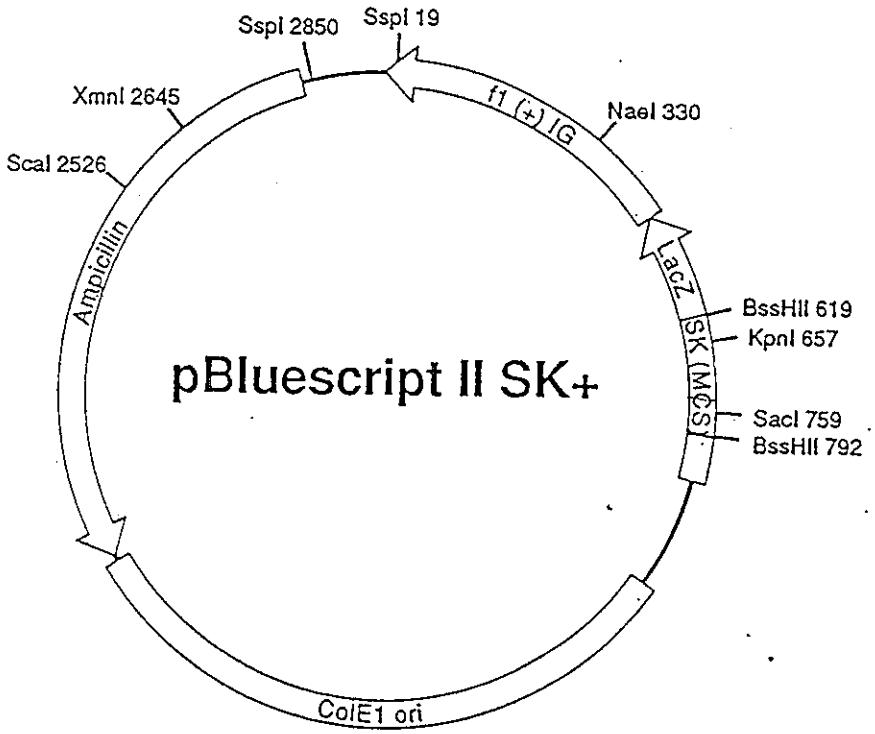
14. Genotype of DH5α

F^-, ϕ80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17. (r^-_k, m^+_k), *sup*E44, *rel*A1, *deo*R, Δ (*lac*ZYA-*arg*F) U169.

15. Genotype of LE392

F^-, *had*R574, (r^-_k, m^+_k), *sup*E44, *sup*F58, *lac*Y1 or Δ (*lac*IZY)b, *gal*K2, *gal*T22, *met*B1, *trp*R55.





17. pBluescript II SK+ vector

Reverse Primer
5' AACAGCTATGACCATG 3'
MET

T3 Primer
5' ATTAACCCTCACTAAAG 3'
BssH II T3 Primer +1→

SK Primer
5' TCTAGAACTAGTGGATC 3'
SalI Sac II Bst X I Eag I Not I Xba I Spe I BamH I Sma I Pst I Eco RI

5' GGAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG

3' CCTTTGTCGATACTGGGTACTAATGCGGTTCGCGCGTTAATTGGGAGTGATTTCCCTTGTTTTCGACCTCGAGGTGGCGCCACCGCCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAA

Beta-Galactosidase

Eco RV Hind III Cla I Acc I Sal I Hinc II Xho I Dra I Apa I Kpn I BssH II Rescued Strand

AATTCGATATCAAGCTTATCGTACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGGAGTCGTATTACGCGCGCTCACTGG CCGTCGTTTTACAA 3'

GCTATAGTTCGAATAGCATGGCAGCTGGAGCTCCCCCCCGGGCCATGGGTTAAGCGGGATATCACCTCAGCATAATGCGCGCGAGTGACCGGCAGCAAAATGTT 5'

←-1 T7 Primer

3' GCATGGCAGCTGGAGC 5'
KS Primer

3' GATATCACCTCAGCATAA 5'
T7 Primer

3' GACCGGCAGCAAAATG 5'
M13 - 20 Primer

18. Flurescence-base automated DNA sequencing

Automated-sequencing designed using fluorescent dye lable terminators. The principle of automated-sequencing method is based on Sanger's enzymatic sequencing method employing chain-terminating dideoxynucleotides (Sanger's *et al.*, 1977). Four primers were used, each labeled with a different fluorescent dye. Each primer is used in a separate reaction which includes one of the four dideoxynucleotides. Because each of the four dyes fluoresces at different wavelength, It is possible to perform the electrohoresis on a single lane of a polyacrylamide gel. During gel migration, the fluorescently labeled DNA fragments are excited by an argon ion laser at a fixed position. Detectors register the fluorescent-dye-specific signal and give the user the analyzed sequencing data in the form of chromatograms.

19. Terminator Premix

A-Dye Terminator, C-Dye Terminator, G-Dye Terminator, T-Dye Terminator, dITP, dATP, dCTP, dGTP, dTTP, Tris-HCL (pH 9.0), $MgCl_2$, thermal stable pyrophosphatase, and AmpliTaq DNA Polymerase

Vitae

Name : Miss Supreya Pongprerachok

Birth Date : 21 July 1964

Education Attainment :

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biology)	Prince of Songkla University	1986

Scholarship Awards during Environment

National Science and Technology Development Agency (NSTDA).