UNIVERSITI PUTRA MALAYSIA

PRODUCTION AND CHARACTERISATION OF THERMOSTABLE PROTEASE FROM *Bacillus stearothermophilus* STRAIN FI

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MASTER OF SCIENCE
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By

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LIST OF ABBREVIATIONS

APMSF	(4-aminophenyl)-methanesulfonyfluoride
E-64	Trans-Epoxysuccinyl-L-leucylamido (4-quanidino) butane
EDTA	Ethylenediaminetetraacetic acid
PMSF	Phenylmethylsulfonylflouride
TLCK	L-1-Chloro-3-(4-tosylamino)-7-amino-2 heptanone-hydrochloride
pCMB	p-Chloromercuricbenzoic Acid
ZPCK	benzyl oxycarbonyl-L-phenylalanine chloromethylketone
o-Phe	1,10-phenanthroline
phos	Phosphoramidon
BAPNA	N-α-Benzoyl-DL-arginine p-nitroanilide
DBAPNA	N-α-Benzoyl-D-arginine p-nitroanilide
BAME	N-α-Benzoyl-L-arginine methyl ester
BAEE	N-α-Benzoyl-L-arginine ethyl ester
ATEE	N-α-Acetyl-L-tyrosine ethyl ester
TAME	N-α-p-Tosyl-L-arginine methyl ester
BTEE	N-Benzoyl-L-tyrosine ethyl ester
B-Cys-4NA	S-Benzyl-L-cysteine-4-nitranilide
Chromozm t-PA	N-Methylsulfonyl-D-phenylalanine-glycine-arginine-4-nitranilide
Z-Phe-Leu-Glu-4NA	Carbobenzoxy-L-phenylalanyl-L-leucyl-L-α-glutamic acid-4-nitranilide
TEMED	N,N,N,N-Tetramethyllenediamide
SDS	Dodecylhydrogen Sulphate Sodium salt
TCA	Trichlororoacetic Acid
DMSO	Dimethylsulfoxide
CTAB	Cethyltrimethylammonium bromide
PEG	monomethoxypolyethylene glycol

Abstract of thesis submitted to the Senate of Universiti Pertanian Malaysia in fulfillment of the requirements for the degree of Master of Science.

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By

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MAY, 1994

Chairman : Associate Professor Che Nyonya Abdul Razak, Ph.D.

Faculty : Science and Environmental Studies.

Screening and isolation of thermophilic proteolytic bacteria were carried out from composting areas, high temperature ponding systems and areas surrounding palm oil mills. Twelve isolates were positive on Skim Milk Agar (10%) of which 11 produced protease in culture broth. A thermophilic and highly proteolytic isolate identified as *Bacillus stearothermophilus* strain F1 isolated from decomposed oil palm branch, was selected for further study. *B. stearothermophilus* strain F1 could grow up to 80°C within a broad pH ranges (pH 5 to 11) with an optimal growth temperature and pH at 70°C and 9.5, respectively. The doubling time of this bacteria at 60°C was 70 min.

Maximum protease production was achieved after 24 h cultivation when grown in 50 ml medium (pH 10.0) under shaking condition at 60°C. Static condition inhibited protease production but not growth. Cultures grown on peptone (iv) generated the highest amount of protease and lower production was observed when glucose or other rapidly metabolized carbon sources were added. Ammonium salt (0.5%)

and amino acids (0.5%) interfered with protease formation whenever they were added to the medium. Although the protease production was calcium independent, the presence of the ion at 4.5 mM enhanced the yield by two-fold. Protease production occurred during the mid-exponential growth phase of the bacterial growth and its onset coincided with sporulation.

The protease was purified to homogeneity by heat treatment, ultrafiltration and gel filtration chromatography with 128-fold increase in specific activity and 75% recovery. The protease is a serine-type enzyme, with a molecular weight of about 33,500 and 20,000 by SDS-PAGE and gel-filtration chromatography, respectively. It hydrolysed many soluble and insoluble protein substrates but exhibited no esterase, trypsin or chromotrypsin-like activities. Metal ions such as Mn^{2+} (5mM) and Ca^{2+} (5mM) stimulated the enzyme activity while Co^{2+} and Hg^{2+} markedly inhibited it. The protease was very thermostable retaining 100% activity for 9 h at 80°C. The stability at higher temperature was calcium dependent. The protease was very stable in denaturing agents, with minimal loss of activity (less than 10%) in the presence of 6 M urea, 1% SDS or 10% Triton-X for 48 h and 1 h at 4° and 70°C, respectively.

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PENGHASILAN DAN PENCIRIAN PROTEASE TERMOSTABIL DARIPADA *Bacillus stearothermophilus* STRAIN F1

Oleh

RAJA NOOR ZALIHA BT. RAJA ABD. RAHMAN

MEI, 1994

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Penyaringan dan pemencilan bakteria proteolitik termofilik telah dijalankan di kawasan membuat kompos, sistem kolam bersuhu tinggi dan kawasan persekitaran kilang memproses minyak kelapa sawit. Dua belas pencilan memberikan keputusan positif di atas Agar Susu Skim (10%) di mana 11 daripadanya berupaya menghasilkan protease dalam media kaldu. Satu pencilan yang termofilik dan penghasil protease tertinggi yang dikenalpasti sebagai *Bacillus stearothermophilus* strain F1, yang dipencil daripada dahan kelapa sawit yang terurai telah dipilih untuk kajian selanjutnya. *B. stearothermophilus* strain F1 ini berkeupayaan untuk hidup sehingga ke suhu 80°C dalam julat pH yang luas (pH 5 hingga 11), di mana suhu dan pH optimum pertumbuhannya masing-masing pada 70°C dan 9.5. Masa penggandaan bakteria ini pada suhu 60°C ialah 70 min.

Penghasilan protease pada aras maksimum diperolehi selepas 24 jam pengeraman, apabila dikultur dalam medium 50 ml (pH 10) dengan goncangan pada suhu 60°C. Keadaan statik merencat penghasilan

protease tetapi tidak mempengaruhi pertumbuhan bakteria. Penghasilan protease tertinggi diperolehi apabila bakteria dikultur dalam media yang mengandungi peptone (iv), manakala penghasilan yang rendah didapati apabila glukosa atau sumber karbon yang mempunyai kadar metabolisma yang cepat ditambah. Penambahan garam ammonium (0.5%) dan amino asid (0.5%) ke dalam medium mengganggu pembentukan protease. Walaupun pengeluaran protease bakteria ini tidak bergantung kepada ion kalsium, namun kehadiran ion tersebut pada tahap 4.5 mM meningkatkan penghasilan dua kali ganda. Penghasilan protease berlaku pada pertengahan fasa eksponen pertumbuhan bakteria dan ini bertepatan dengan sporulasi.

Protease dapat ditulenkan hingga homoginiti melalui pelakuan haba, turasan ultra dan penurasan gel kromatografi, dengan peningkatan aktiviti spesifik sehingga 128 kali ganda dan pulangan aktiviti sebanyak 75%. Protease ini adalah jenis serina dengan berat molekul sekitar 33,500 dan 20,000 masing-masing melalui kaedah SDS-PAGE dan penurasan gel. Enzim ini berkeupayaan menghidrolisiskan berbagai jenis protein larut dan tidak larut tetapi tidak menunjukkan aktiviti jenis esterase, tripsin atau kromotripsin. Ion logam seperti Ca^{2+} (5 mM) dan Mn^{2+} (5 mM) merangsang aktiviti enzim, manakala kehadiran Co^{2+} dan Hg^{2+} merencatkannya. Protease ini amat termostabil dengan pengekalan 100% keaktifan pada suhu 80°C selama 9 jam. Kestabilan pada suhu tinggi ini bergantung kepada kehadiran ion kalsium. Protease ini juga amat stabil dalam agen denaturasi, di mana pendedahan dalam 6 M urea, 1% (w/v) SDS atau 10%(v/v) Triton-X selama 48 jam dan 1 jam masing-masing pada suhu 4°C dan 70°C, mengakibatkan pengurangan aktiviti minimum (kurang dari 10%).

CHAPTER 1
INTRODUCTION

Proteases classified as hydrolases (EC 3.4), are enzyme that play a central role in the degradation of protein by hydrolysing peptides bonds. Under modern unified scheme, proteases are divided into two classes namely, endopeptidases (EC 3.4.21-99) and exopeptidases (EC 3.4.11-19) (Barrett and McDonald, 1986). Endopeptidases are proteases that initiate degradation of proteins by cleaving internal peptide bonds, whereas exopeptidases degrade peptide chains from their ends, removing one, two or three amino acids at a time. In general, proteases are classified by their origin, behaviour against inhibitors, or pH optimum. Based on their pH optimum, they are normally categorised into three groups, namely acid (pH 2-4), neutral (pH 7-9) and alkaline (pH 9-11) proteases. In view of the potential biotechnological applications, the exploitation of these proteases will lead to various products since most of the bioconversion processes are pH dependent.

Although proteases can be obtained from animal, plant and microbial sources, due to various technological and economical reasons, microbial proteases are superseding the other sources. Among the outstanding attributes that contributed to their achievement are, firstly, the microbes have shorter generation time, thus they can be grown vigorously and rapidly in large quantities by established fermentation techniques. Secondly, they can be easily manipulated to improve the productivity by genetic engineering. Thirdly, their secretion mechanisms can be further controlled by various factors governing their growth

and enzyme production. In addition, the purification of microbial proteases to homogeneity is possible with the recent technical advances through chromatographic and electrophoretical methods.

Microbial proteases are probably the most widely and commercially used enzymes with 48 percentage of sales on the world market (Kula *et al.*, 1987). They are becoming increasingly important in medicine, food processing, timber preservation, leather and detergent industries. Detergent proteases account for approximately 25% of total worldwide enzyme production which exemplify a successful commercial product.

Microbial proteases have been studied in a wide variety of microorganisms which include bacteria, yeast and fungus. Within the bacteria, protease production in various species have been investigated, this include *Bacillus* spp. (Shimogaki *et al.*, 1991; Takami *et al.*, 1989; Manachini *et al.*, 1988), *Vibrio* sp. (Fukasawa *et al.*, 1988a), *Chromobacterium* sp. (Dainty *et al.*, 1978), *Aeromonas* sp. (Schalk *et al.*, 1992), *Pseudomonas* sp. (Azcona *et al.*, 1989), *Lactococcus* spp. (Tan *et al.*, 1991; Bosman *et al.*, 1990), *Lactobacillus* sp. (Miyakawa *et al.*, 1991), *Clostridiun* spp. (Croux *et al.*, 1990: Park and Labbe, 1990), *Thermus* sp RT 41A (Peek *et al.*, 1992), and *Desulfurococcus* (Cowan *et al.*, 1987). Many species of yeast and fungus such as *Candida albicans* (Homma *et al.*, 1993), *Candida humicola* (Ray *et al.*, 1992), *Agricus biosporus* (Burton *et al.*, 1993) *Aspergillus oryzae* (Fukushima *et al.*, 1991), *Fusarium oxysprum* (Castro *et al.*, 1991), *Aureobasidium pollulans* (Donaghy and McKay, 1993) and some other yeast and mold have also been studied for their protease production.

Proteases which are known to be active and stable in highly alkaline conditions are secreted by both neutrophilic and alkalophilic bacilli. They are of interest since they represent a major source of commercially produced proteolytic enzymes. These proteases are shown to be very stable, albeit to differing extent, in the presence of a variety of detergents. They have been used as laundry additives for several decades to provide cleaning improvements on a variety of proteinaceous soil types. Their ability to enhance the detergent action in the washing of soiled clothes have been investigated in detail. Two such commercially available enzymes are Alcalase^{R} and Esperase^{R}, both being produced by bacilli (Dambmann and Aunstrup, 1980). The commercial success of the above detergent proteases resulted in a search for a new and novel enzymes with better stain-removing properties and stabilities in the washing suds. Enzyme-assisted de-hairing of animal hide is another industrial process which is currently in focus. This process is made possible with proteolytic enzymes that are stable and active under alkaline conditions (pH 12) (Kroll, 1990).

In industries most enzymatic processes are being carried out at an elevated temperature. Not only it accelerated the processes but also increased the substrate solubilities, accelerated the rate of diffusion, decreased the viscosity and reduced the risks of contaminations (Edwards, 1990; Doig, 1973). In addition, operating at high temperature provided a means of distilling volatile chemical, thus allowing cheap and rapid purification of the product (Edwards, 1990). Washing with proteases containing detergents is usually done at 20-50°C or above (Aaslyng *et al.*, 1991). Thus there is a great necessity to use enzymes which are heat

stable. Thermostable enzymes are now gaining their importance in a wide range of biotechnological applications. Their inherent stability at elevated temperatures, in organic solvents and denaturing agents enable their use in processes where conventional enzymes are restricted.

Even though thermostable enzymes may be obtained from mesophilic microorganisms, chances are usually greater for them to be produced by thermophiles. Similarly, most alkaline proteases were obtained from alkalophilic microorganisms. These alkalophilic micro-organisms may provide new proteases with useful characteristics suitable for industrial applications. Therefore, the search for new microorganisms producing new and novel proteases for industrial purposes should be continuously pursued. Thus this research was undertaken with the following objectives:

a) to isolate a thermophilic bacteria producing thermostable proteases.
b) to identify the bacteria.
c) to optimise the protease production.
d) to purify the enzyme to homogeneity.
e) to characterise the purified enzyme.

CHAPTER 2
LITERATURE REVIEW

The Classes of Proteases

In general, protease is synonymous with peptide hydrolases and can be applied to both exopeptidases and endopeptidases. Proteases differ from almost all other enzymes, in that their substrate specificities are extremely difficult to define (Barrett, 1986). Hartley (1960) observed that the protease seemed to act through four distinct catalytic mechanisms, and therefore could be refered as 'serine', 'thiol', 'acid' or 'metal' proteases. With recent knowledge on the chemistry of the catalytic sites, the concept of distinguishing these groups of enzymes remains completely valid, although three of the four names have been amended, and now called 'serine', 'cysteine', 'aspartic' and 'metalo-' proteases (Barrett, 1986). In the enzyme nomenclature scheme, these forms sub-sub-classes 21, 22, 23, and 24 respectively, of the peptide bond hydrolyses (sub-class 3.4) (IUB Nomenclature Committee, 1984).

Serine Proteases (EC 3.4.21)

The enzymes which belong to this first group are most numerous, extremely widespread and diverse. They can be divided into two superfamilies, namely the *chymotrysin* superfamily and the *subtilisin* superfamily (James, 1976; Hartely 1960). Enzymes related to subtilisin have been found only in bacteria, whereas the chymotrypsin-related proteases are found in both prokaryotic and eukaryotic microorganisms, plant and both invertebrate and vertebrate animals.

When classified according to their substrate specificity, there are three major types of serine proteases (Polgar, 1987). They are trypsin-like enzymes which cleave substrates with positively charged amino acids residue (lysine and arginine), chymotrypsin-like enzymes which prefer substrate with aromatic or large aliphatic, and elastase-like enzyme which prefers substrate of small aliphatic side chains. *Staphylococcus aureus* protease Q-8 was a special case as it cleaves only glutamate and aspartate residues (Drapeau, 1976).

The serine proteases normally have a molecular weight of 15,000 to 30,000 (Morihara, 1974). Their alternative name, alkaline protease, reflects their high pH optima of 9 to 13. There are no absolute activator requirements, but Ca^{2+} ions are required in the activation reaction of some of the proenzymes as well as for stabilisation (Barret, 1986).

Phenylmethanesulphonyl fluoride (PMSF) was first shown to stoichiometrically inactivate trypsin and chymotrypsin in 1963 (Gold and Fahrney, 1964; Fahrney and Gold , 1963). Since then sulphonyl fluorides have been used widely as serine protease inhibitors. In addition, several substituted sulphonyl fluorides have also been reported as reactive and specific inhibitors of trypsin-like enzymes (Tanaka *et al.* 1983 ; Laura *et al.* 1980). However, the most potent inhibitor for trypsin-like enzyme, is 4-amidinophenylmethane sulfonyl fluoride (APMSF) (Laura *et al.* 1980). Unlike PMSF, APMSF does not inhibit chymotrypsin.

Several peptide aldehydes of microbial origin, have been reported as inhibitors of serine proteases. The first aldehyde, chymostatin was reported to inhibit chymotrypsin-like enzymes (Umezawa *et al.* 1970) but

did not inhibit trypsin-like enzymes. Elastinal is a good and specific inhibitor of elastase (Okura *et al*. 1975; Umezawa *et al*. 1970) and does not inhibit chymotrypsin or trypsin. Two peptide aldehydes, leupeptin and antipain have been reported as inhibitors of trypsin-like enzymes (Umezawa and Aoyagi, 1977; Umezawa, 1976) and are widely used in the characterisation of a new trypsin-like proteases.

More than 40% of the microbial proteases so far reported belong to the serine proteases (Tsuru and Yoshimoto, 1987). Recently a number of serine proteases produced by bacteria (Peek *et al*. 1992), yeast (Donaghy and McKay, 1993) and fungi (Burton *et al*., 1993) have been reported.

Cysteine Proteases (EC 3.4.22)

The cysteine proteases, which were known previously as thiol proteases, constitute the group of endopeptidases whose member rely for catalytic activity in the presence of a thiol group of cysteine residue in the enzyme molecule. Cysteine proteases have been isolated from a large number of biological sources which encompass plants (papain, bromelain, ficin) (Glzer and Smith, 1971), animal (cathepsin B) (Barret and McDonald, 1980), bacteria (*Streptococcus* protease, clostripain) (Mohihara, 1974) and eukaryotic microorganisms (North, 1982).

Most cysteine proteases are small protein with molecular weight in the range of 20,000-35,000 (Brocklehurst *et al*., 1987) and are most active at around neutral pH. The isoelectric point of the various cysteine proteases cover a wide range from about 3 to 11.7 (McDowall, 1970).

Cysteine proteases contain a catalytically active cysteine sulphydryl groups (Cys-25) and a histidine imidozole group (His-159) within the active site of the enzymes (Giles and Keil, 1984; Polgar and Halasz, 1982). Reagents, such as N-ethylmaleimide, iodo- and bromo acetates, p-chloromercuric benzoate or pyridyldisulphides (Brocklehurst and Little, 1973) resulted in alkylation of cysteine sulphydryl group which render the enzyme catalytically inactive. The inhibitor Tos-phe-Ch_2Cl (TPCK) inactivated cysteine protease by alkylation of thiol group on Cys-25. E-64 ([N-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amino (4-quanido)butane) an irreversible inhibitor of cysteine proteases was discovered by Hanada *et al.* (1978). The most recent report showed that sulphonium methyl ketones as the most potent class of calpain inhibitor reported to date (Pliura *et al.*, 1992).

Aspartic Proteases (EC 3.4.23)

The aspartic proteases may be one of the younger classes of proteases which appear to be confined to eukaryotes. Fungal aspartic proteases, generally have a molecular weight values in the range 30,000-45,000 and isoelectric points below pH 5.1 (North, 1982). Well-characterised microbial acid proteases have been isolated and in some cases crystallised, from strain of *Candida albicans* (Ruchel, 1981), *Monascus kaoling* (Hwang *et al.*, 1980) and *Fusarium moniliforme* (Kaloczewski *et al.*, 1983).

Typical pH optima for aspartic proteases are in the range of pH 3.5 to 5.5. In general, aspartic proteases act best on peptide bonds between bulky hydrophobic amino acid residues. Thus Leu-Try, Try-Leu, Phe-