UNIVERSITI PUTRA MALAYSIA

# PURIFICATION AND CHARACTERIZATION OF ORGANIC SOLVENT TOLERANT PROTEASE FROM *PSEUDOMONAS AERUGINOSA* STRAIN K

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**MASTER OF SCIENCE**
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**By**

**NORULAIMAN BINTI YUSOFF**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science**

**June 2007**

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

**PURIFICATION AND CHARACTERIZATION OF ORGANIC SOLVENT TOLERANT PROTEASE FROM *PSEUDOMONAS AERUGINOSA* STRAIN K**

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**NORULAIMAN BINTI YUSOFF**

**June 2007**

**Chairman: Professor Raja Noor Zaliha Raja Abd. Rahman, PhD**

**Faculty : Biotechnology and Biomolecular Sciences**

A bacterium known as Strain K was identified as *Pseudomonas aeruginosa* since it 16S rRNA sequence exhibited similarity of up to 99 % with *P. aeruginosa* from the NCBI database. Protease from the *P. aeruginosa* strain K was purified to homogeneity by 80.6 fold and 107 % recovery using a combination of ultrafiltration and ion exchange chromatography on Q-Sepharose. In second method, protease K-01 was purified to homogeneity by 116.2 fold purification and 199 % recovery, using a combination of ultrafiltration and hydrophobic interaction chromatography on Butyl-Sepharose. The purified protease was named as protease K-01.

The apparent molecular mass of the purified protease K-01 was estimated to be 33 kDa on gel filtration Sephadex G-100, and SDS PAGE. The purified protease hydrolyzed azocasein at optimum temperature of 55 °C. However, the enzyme lost its activity with a half life of more than 60 min at 55 and 60 °C. The optimum activity of the protease was observed at pH 8.0 and it was stable in the pH range of pH 6 to 13. The protease activity was completely inhibited by EDTA and 1,10-phenantroline, while 70 and 30 % reduction of protease activity was observed in the presence of DTT and 2-mercaptoethanol respectively.

Among the metal ions tested, $Mg^{2+}$ and $Ca^{2+}$ ions increased enzyme activity by 8 %. Protease activity was completely inhibited by $Fe^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Ag^{2+}$, $Zn^{2+}$, and $Hg^{2+}$ ions. $Fe^{3+}$ and $Co^{2+}$ ions were found to restore the activity of inactivated protease by 1mM EDTA. Protease K-01 was more stable in water miscible organic solvents (DMSO, methanol, ethanol, 2-propanol, n-butanol, and 1-decanol) than water immiscible organic solvent. For substrate specificity, protease K-01 was able to hydrolyze several native proteins such as casein, haemoglobin, albumin and gelatin.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**PENULENAN DAN PENCIRIAN PROTEASE YANG TOLERANT TERHADAP PELARUT ORGANIK DARIPADA *PSEUDOMONAS AERUGINOSA* STRAIN K**

Oleh

**NORULAIMAN BINTI YUSOFF**

**Jun 2007**

**Pengerusi: Profesor Raja Noor Zaliha Raja Abd. Rahman, PhD**

**Fakulti : Bioteknologi dan Sains Biomolekul**

Suatu bakteria yang dikenali sebagai Strain K telah dikenalpasti sebagai *Pseudomonas aeruginosa* apabila penjujukan 16S rRNA menunjukkan persamaan sehingga 99 % dengan *P. aeruginosa* dari pengkalan data NCBI. Protease daripada *P. aeruginosa* strain K telah ditulenkan sehingga homogeniti dengan 80.6 kali ganda ketulenan dan pulangan aktiviti sebanyak 107 %. Penulenan ini diperolehi dengan menggabungkan kaedah ultrafiltrasi dan kromatografi penukaran ion terhadap Q-Sepharose. Sementara dalam kaedah yang kedua, protease ini ditulenkan sehingga homogeniti 116.2 kali ganda ketulenan dengan pulangan aktiviti sebanyak 199 %. Penulenan ini diperolehi dengan menggabungkan kaedah ultrafiltrasi dan kromatografi interaksi hidrofobik iaitu Butil-Sepharose. Protease yang telah ditulenkan ini dinamakan sebagai protease K-01.

Berat molekul protease K-01 yang telah ditulenkan ini dianggarkan 33 kDa melalui kaedah gel filtrasi Sephadex G-100 dan SDS PAGE. Protease yang telah ditulenkan ini menghidrolisiskan azokasin pada suhu optimum 55 °C dan separuh hayat kehilangan aktiviti protease adalah lebih dari 60 min pada suhu 55 dan 60 °C. Aktiviti protease ini optimum pada pH 8.0 dan stabil pada julat pH dari pH 6.0 hingga 13.0. Aktiviti protease direncat keseluruhannya oleh EDTA dan 1,10-phenantrolin, sementara itu 70 dan 30 % penurunan aktiviti protease dapat diperhatikan oleh DTT dan 2-merkaptoetanol.

Diantara ion-ion logam yang diuji, ion $Mg^{2+}$ dan $Ca^{2+}$ meningkatkan aktiviti enzim sehingga 8%. Aktiviti protease direncat sepenuhnya oleh ion $Fe^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Ag^{2+}$, $Zn^{2+}$, dan $Hg^{2+}$. Ion $Fe^{3+}$ and $Co^{2+}$ didapati mengembalikan aktiviti protease yang telah dinyahaktifkan dengan 1 mM EDTA. Protease K-01 lebih stabil di dalam pelarut organik yang larut air (DMSO, methanol, ethanol, 2-propanol, n-butanol, and 1-decanol) berbanding pelarut organik yang tidak larut air. Protease K-01 berupaya menghidrolisiskan beberapa protein semulajadi seperti kasin, hemoglobin, albumin dan gelatin.

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I certify that an Examination Committee has met on 11th June 2007 to conduct the final examination of Norulaiman Binti Yusoff on her Master Science thesis entitled "Purification and Characterization of Organic Solvent Tolerant Protease from *Pseudomonas aeruginosa* strain K" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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Date: 16 August 2007

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date: 13 September 2007

## DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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**NORULAIMAN BINTI YUSOFF**

Date: 9 August 2007

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| | |
|---|---|
| bp | Base pair |
| cm/h | Centimeter per hour |
| °C | Degree centigrade |
| $dH_2O$ | Distilled water |
| g | Gram |
| g/L | Gram per liter |
| Xg | Times gravity |
| h | Hour |
| kDa | Kilo dalton |
| L | Liter |
| M | Molar |
| ml | Milliliter |
| min | Minute |
| nm | Nanometer |
| mw | Molecular weight |
| mM | Millimolar |
| M | Molar |
| ml/min | Milliliter per minute |
| mwco | Molecular weight cut off |
| mg/ml | Milligram per milliliter |



| | |
|---|---|
| $ms^{-1}$ | Meter per second |
| mA | Milliamphere |
| PAGE | Polyacrylamide gel electrophoresis |
| rpm | Revolutions per minute |
| SDS | Sodium dedocyl sulfate |
| % | Percentage |
| µg | Microgram |
| µl | Microliter |
| µm | Micrometer |
| V | Volt |
| $V_e$ | Elution volume |
| $V_o$ | Void volume |
| $V_t$ | Total column volume |
| v/v | Volume per volume |
| w/v | Weight per volume |

# CHAPTER 1

## INTRODUCTION

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteases execute a large variety of functions, extending from the cellular level to the organ and organisms level, to produce cascade systems such as homeostasis and inflammation. Their involvement in the life cycle of disease causing organisms has led them to become potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS (Rao *et al.*, 1998).

The current estimated value of the worldwide sales of industrial enzymes is USD 1 billion. Seventy five percent of the industrial enzymes are hydrolytic. Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes. Proteases have long history of application in the food and detergent industries. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance (Rao *et al.*, 1998).

A major driving force in the application of enzymatic process in the chemical process industries is environmental concern and regulation. There is a rapidly growing demand for cleaner, alternative technologies that produce less waste, for example high-atom utilization and low salt processes which are less energy intensive and avoid the use of toxic reagents and solvents. High selectivity under mild reaction conditions, without the need for protection and deprotection of functional groups are the characteristic features of enzymatic processes which make them highly attractive from an environmental viewpoint (Sheldon, 1996).

Similarly, there is a growing demand for products that are more targeted in their action and more environmentally benign. This manifests itself, for example, in the market trend towards the use of enantiomerically pure pharmaceuticals and agrochemicals and polymers that are biodegradable. Many biologically active substances such as pharmaceuticals, herbicides, fungicides, insecticides, flavors and fragrances, are chiral molecules. The high stereo selectivity inherent in many enzymatic transformations makes them eminently suitable for the synthesis of pure enantiomers (Sheldon, 1996).

*Pseudomonas aeruginosa* is a gram negative, aerobic rod belonging to the bacterial family Pseudomonadaceae. These bacteria are common

inhabitants of soil and water. They occur regularly on the surfaces of plants and occasionally on the surfaces of animals. Pseudomonas *aeruginosa* reported to secrete several types of protease; elastase, alkaline protease, Las A and protease IV. Proteases are assumed to play a major role during acute *P. aeruginosa* infection (Madigan *et al.*, 1997; Lyczak, *et al.*, 2000).

*P. aeruginosa* strain K was reported to produce three types of proteases (Rahman *et al.*, 2006). This bacterium was isolated from contaminated soil of a wood factory in Selangor, Malaysia. Nutritional and physical factor on protease production of *P. aeruginosa* strain K has been reported by Rahman *et al*, (2005a); Rahman *et al*, (2005b). One of the protease has been classified as alkaline protease. The protease has been purified and characterized (Rahman *et al.*, 2006). The other two proteases are remained to be disclosed. The solvent tolerant protease from *P. aeruginosa* strain K has been choosing for further studies. This research was undertaken with the following objectives:

- To identify the bacteria by 16S rRNA Identification
- To purify the organic solvent tolerant protease
- To characterize the purified protease

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Protease

Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all living organisms (Rao *et al.*, 1998). The nomenclature for enzyme, Enzyme Commission (EC) is used to describe each enzyme and broadly classified the enzyme based on its mechanisms. EC 3 refers to hydrolases, which catalyze the hydrolysis of various bonds. EC 3.4 is the nomenclature for the protease which is responsible for cleavage of peptide bonds in peptides and protein (Dunn, 1989).

Protease is synonymous with peptidase. Peptidases comprise two groups of enzymes; exopeptidase and endopeptidase (Figure 2.1). The exopeptidases are enzymes that cleave peptide bonds at amino or carboxy-terminus while the endopeptidases cleave peptide bonds internally in the polypeptide chain. Endopeptidase is also known as proteinase. Proteinases are classified according to their catalytic mechanisms. Four mechanistic classes have been recognized by the

International Union of Biochemistry and Molecular Biology (IUBMB) (Dunn, 1989).

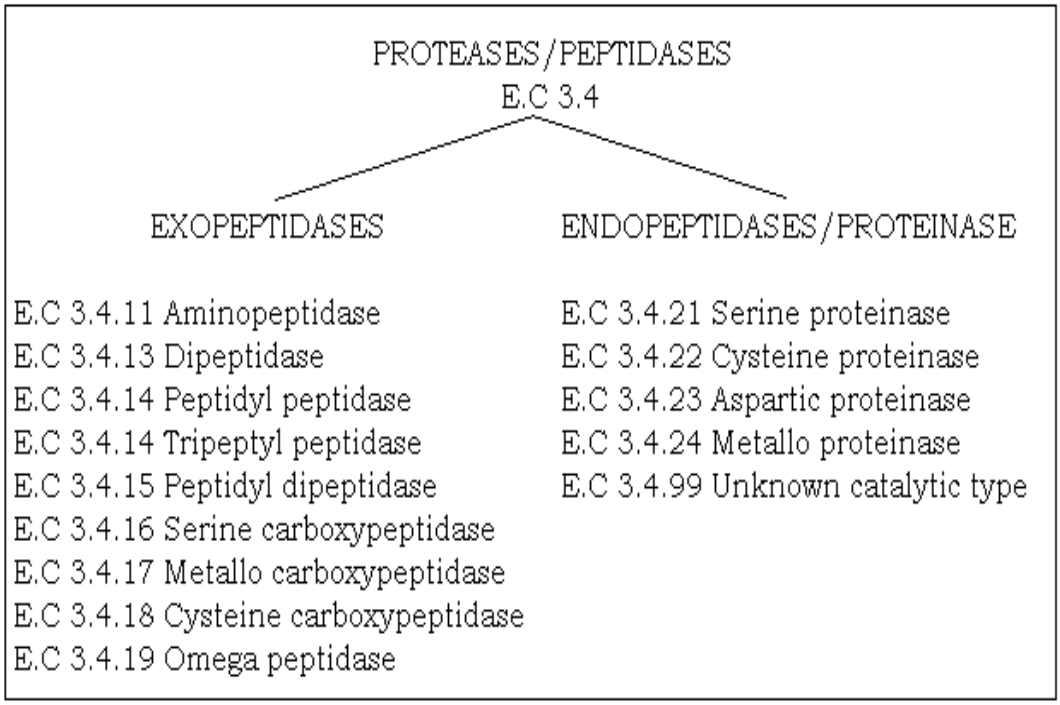


**Figure 2.1: The subdivided class of proteases under group of exopeptidase and endopeptidase (Rao *et al.*, 1998).**

### 2.1.1 Serine Proteinase (E.C 3.4.21)

Serine proteinases are characterized by the presence of a serine group in the active site. Their molecular masses range between 18 and 35 kDa. Generally, serine proteases are inhibited by phenylmethanesulfonyl fluoride (PMSF), di-isopropylflourophosphate (DFP) and tosyl lysyl chloromethyl ketone (TLCK). Serine proteases are generally active at neutral and alkaline pH, with an optimum pH of between pH 7 and pH 11. The isoelectric points are generally

between pH 4 and pH 6. Serine proteinases exhibit different substrate specificities which are related to amino acid substitution in various enzyme subsites interacting with the substrate residues (Rao *et al.*, 1998).

Three residues which form the catalytic triad are essential in the catalytic process; histidine, aspartic, and serine. The first step in catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential serine. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate and then the peptide bond is cleaved (Figure 2.2).

During the second step or deacylation, the acyl-enzyme intermediate is hydrolyzed by water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme. The deacylation which also involves the formation of tetrahedral transition state intermediate proceeds through the reverse reaction pathway of acylation. A water molecule is the attacking nucleophile instead of the Ser residue. The histidine residue provides a general base and accepts the OH group of the reactive Ser (Dunn, 1989; Rao *et al.*, 1998).