



UNIVERSITI PUTRA MALAYSIA

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

NORULAIMAN BINTI YUSOFF

FBSB 2007 9



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

NORULAIMAN BINTI YUSOFF

**MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA**

2007



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

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**

By

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 its 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 dituliskan ini dianggarkan 33 kDa melalui kaedah gel filtrasi Sephadex G-100 dan SDS PAGE. Protease yang telah dituliskan 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-merkaptotanol.

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.



ACKNOWLEDGEMENTS

Alhamdulillah, praise to Allah on completion of my study. My special appreciation is to Umi and Abah, your sacrificing is invaluable, also for my lovely sisters Yuni, Awa and Roh, I love you all.

My sincere gratitude goes to Professor Dr. Raja Noor Zaliha Raja Abd. Rahman, Professor Dr. Abu Bakar Salleh, and Professor Dr. Mahiran Basri for the help, references, and advices. Especially, to Prof. Dr. Raja Noor Zaliha who always give me a hand when ever I needed helps. Last but not least, I would like to thank Dr. Basyaruddin Abdul Rahman and Dr. Azizah Shafie.

Also not forget to my labmates Leow, K. Ina, K. Ain, Abg. Rofandi, Bro. Mohammad, Fazlirahimi, Fairol, Azira, Syukuri, Chee Fah, K. Lia, Kok Whye, Ghani, Suriana, Syuhada, Tengku and Suhana thank for the help and guidance. My special thank goes to my housemate especially to Shalihah, thank for being my best friend.



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:

Tong Chow Chin, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Nor Aripin Shamaan, PhD

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Suhaimi Mustafa, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Rosli Md. Illias, PhD

Associate Professor
Faculty of Chemical Engineering and Natural Sources
Universiti Teknologi Malaysia
(External Examiner)

HASANAH MOHD. GHAZALI, PhD

Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

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:

Raja Noor Zaliha Raja Abd. Rahman, PhD

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Abu Bakar Salleh, PhD

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Mahiran Basri, PhD

Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD

Professor/ Dean
School of Graduate Studies
Universiti Putra Malaysia

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.

NORULAIMAN BINTI YUSOFF

Date: 9 August 2007



TABLE OF CONTENTS

	Page
ABSTRACT	
ABSTRAK	iii
ACKNOWLEDGEMENTS	vi
APPROVAL	vii
DECLARATION	ix
LIST OF TABLES	xiii
LIST OF FIGURES	xiv
LIST OF ABBREVIATIONS	xvi
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	4
2.1 Protease	4
2.1.1 Serine Proteinase	5
2.1.2 Cysteine Proteinase	8
2.1.3 Aspartic Proteinase	8
2.1.4 Metallo Proteinase	12
2.2 Sources of Proteases	14
2.2.1 Protease from Microorganism Sources	15
2.2.2 Protease from Plant and Animal Sources	16
2.3 <i>Pseudomonas aeruginosa</i>	16
2.4 <i>Pseudomonas aeruginosa</i> strain K	17
2.5 Classification of Protease from <i>Pseudomonas aeruginosa</i>	19
2.5.1 Elastase (Pseudolysin)	19
2.5.2 LasA (Staphylolysin)	21
2.5.3 Alkaline Protease (Aeruginolysin)	23
2.5.4 Protease IV (Lysine specific protease)	23
2.6 Organic Solvent	24
2.6.1 Organic Solvent Tolerant Microorganism	25
2.6.2 Organic Solvent Stable Enzyme	26
2.6.3 Application of Enzyme in Organic Solvent	30
2.7 16S rRNA Identification	31
2.8 Protease Purification	33
2.8.1 Ultrafiltration	33
2.8.2 Ion Exchange Chromatography	34
2.8.3 Hydrophobic Interaction Chromatography	35



2.8.4	Affinity Chromatography	37
2.8.5	Gel Filtration Chromatography	38
2.9	Application of Proteases	39
2.9.1	Detergent	40
2.9.2	Food Industry	42
2.9.3	Pharmaceutical Industry	43
2.9.4	Leather Industry	44
2.9.5	Other Applications	45
3	METHODOLOGY	46
3.1	Source of Microorganism	46
3.2	Extraction of Genomic DNA	46
3.3	Quantification and Purity of Genomic DNA	47
3.4	Agarose Gel Electrophoresis	48
3.5	Polymerase chain reaction (PCR)	49
3.6	Purification of the PCR Product	49
3.7	Competent Cell of <i>Escherichia coli</i>	50
3.8	Bacterial Identification using 16S rRNA	51
3.8.1	Gene Amplification of 16S rRNA	51
3.8.2	Cloning and Transformation of 16S rRNA	52
3.8.3	Screening for the Positive Colonies	53
3.8.4	Sequencing and Analysis of 16S rRNA	54
3.9	Optimum Protease Production	55
3.10	Preparation of Inoculums	55
3.11	Protease Assay	56
3.12	Protein Assay	56
3.13	PAGE Electrophoresis	58
3.13.1	Native PAGE Electrophoresis	59
3.13.2	SDS PAGE Electrophoresis	59
3.14	Purification of Protease	60
3.14.1	Culture Preparation for Purification	60
3.14.2	Ultrafiltration	61
3.14.3	Buffer Exchange	61
3.14.4	Ion Exchange Chromatography (IEC)	62
3.14.5	Hydrophobic Interaction Chromatography (HIC)	63
3.15	Characterization of Protease	64
3.15.1	Molecular Weight Determination	64
3.15.2	Effect of Temperature on Protease Activity and Stability	65
3.15.3	Effect of pH on Protease Activity and Stability	66
3.15.4	Effect of Inhibitors on Protease Activity	67
3.15.5	Effect of Metal Ions on Protease Activity	67
3.15.6	Effect of Organic Solvents on Protease Activity	68



3.15.7	Substrate Specificity	68
4	RESULTS AND DISCUSSION	70
4.1	<i>Pseudomonas aeruginosa</i> strain K	70
4.2	16S rRNA Identification	72
4.2.1	Extraction of Genomic DNA	72
4.2.2	Quantification and Purity of Genomic DNA	74
4.2.3	Amplification and Sequencing of 16S rRNA gene	75
4.2.4	16S rRNA Sequence Analysis	79
4.2.5	Phylogenetic Tree of 16S rRNA	86
4.3	Optimum Protease Production	90
4.4	Purification of Organic Solvent Tolerant Protease K-01	90
4.4.1	Ion Exchange Chromatography	91
4.4.2	Hydrophobic Interaction Chromatography	97
4.5	Characterization of Protease K-01	104
4.5.1	Determination of Molecular Weight	104
4.5.2	Effect of Temperature on Protease Activity and Stability	108
4.5.3	Effect of pH on Protease Activity and Stability	115
4.5.4	Effect of Inhibitors on Protease Activity	120
4.5.5	Effect of Metal Ions on Protease Activity	123
4.5.6	Effect of Organic Solvents on Protease Activity	128
4.5.7	Substrate Specificity of Protease K-01	134
5	CONCLUSION	137
5.1	Conclusion	137
5.2	Recommendation	139
	REFERENCES	140
	APPENDICES	151
	BIODATA OF THE AUTHOR	160



LIST OF TABLES

Table		Page
2.1	Organic solvent stability of <i>P. aeruginosa</i> strain K proteases (Rahman <i>et al.</i> , 2006)	18
2.2	Properties of <i>Pseudomonas aeruginosa</i> Proteases (Caballero <i>et al.</i> , 2001)	20
2.3	Some reported strains of organic solvent tolerant bacteria and their characteristics (Sardessai and Bhosle, 2002)	27
2.4	Organic solvent tolerance mechanisms of bacteria (Sardessai and Bhosle, 2002)	28
2.5	Potential advantages of enzymatic processes in organic media (Sheldon, 1996)	29
2.6	Industrial enzyme market (Sheldon, 1996)	41
4.1	Concentration and purity of extracted genomic DNA	75
4.2	Anion exchange chromatography purification table of protease K-01	94
4.3	Hydrophobic interaction chromatography purification table of protease K-01	100



LIST OF FIGURES

Figure		Page
2.1	The subdivided class of proteases under group of exopeptidase and endopeptidase (Rao <i>et al.</i> , 1998).	5
2.2	Catalytic mechanism of serine protease (Dunn, 1989).	7
2.3	Catalytic mechanism of cysteine protease (Dunn, 1989; Rao <i>et al.</i> , 1998).	9
2.4	Catalytic mechanism of aspartic protease (Dunn, 1989; Rao <i>et al.</i> , 1998).	11
2.5	Catalytic mechanism of metallo protease (Dunn, 1989).	13
4.1	Single colony, pure culture and gram staining of <i>P. aeruginosa</i> strain K.	71
4.2	Genomic DNA extraction from <i>P. aeruginosa</i> strain K.	73
4.3	Amplified of 16S rRNA PCR product.	76
4.4	16S rRNA nucleotide sequence of <i>Pseudomonas aeruginosa</i> strain K.	77
4.5	Nucleotide sequence alignment (CLUSTAL W) of <i>Pseudomonas aeruginosa</i> strain K (DQ889450).	80
4.6	Phylogenetic relationship between 16S rRNA sequences of <i>Pseudomonas aeruginosa</i> strain K and selected thirty other <i>Pseudomonas</i> species.	87
4.7	Purification profile of protease K-01 on Q-Sepharose chromatography.	93
4.8	SDS-PAGE of purified protease K-01 by Q-Sepharose chromatography.	95
4.9	Purification profile of protease K-01 on Butyl-Sepharose chromatography.	99



4.10	SDS-PAGE of purified protease K-01 by Butyl-Sepharose chromatography.	102
4.11	Molecular weight determination of protease K-01 by Gel filtration (Sephadex G-100).	105
4.12	Molecular weight determination of protease K by SDS-PAGE.	106
4.13	Molecular weight determination of protease K by Native-PAGE.	107
4.14	Effect of temperature on protease activity.	110
4.15	Thermal stability of protease K at various temperatures.	112
4.16	Thermal stability of protease at 55 °C and 60 °C.	113
4.17	Effects of pH on protease activity.	116
4.18	Effects of pH on protease stability.	118
4.19	Effect of inhibitors on protease activity.	121
4.20	Effect of metal ion on protease activity.	124
4.21	The effect of metal ion on inactivated protease activity by EDTA.	127
4.22	Effect of various organic solvent on protease activity	129
4.23	Substrate specificity of protease K-01.	135



LIST OF ABBREVIATIONS

bp	Base pair
cm/h	Centimeter per hour
°C	Degree centigrade
dH ₂ O	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).

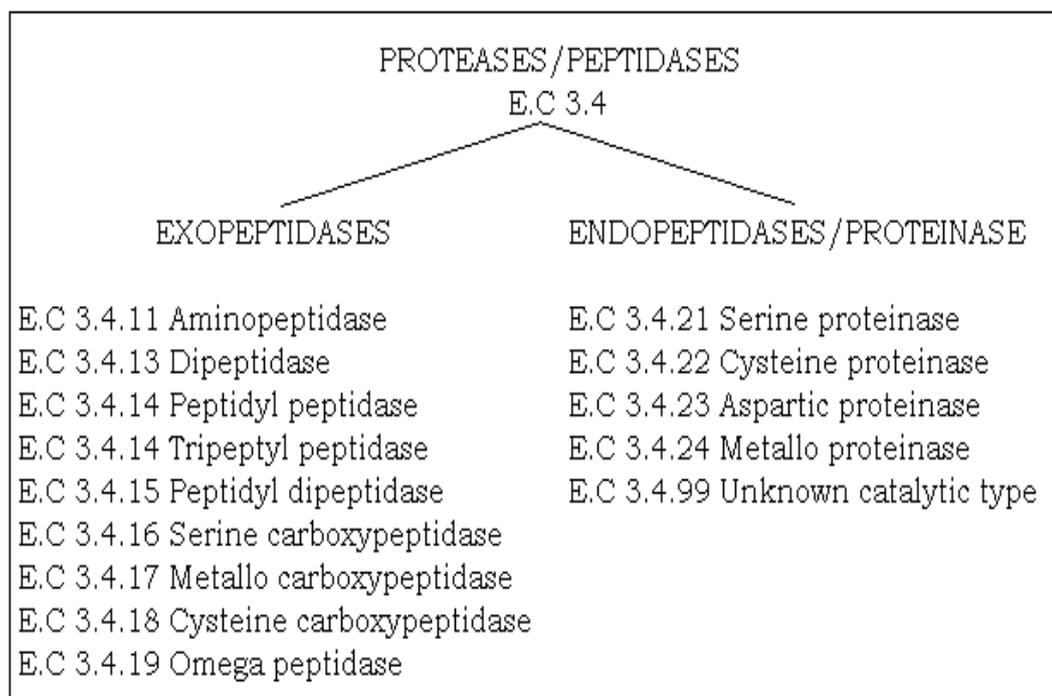


Figure 2.1: The subdivided class of proteases under group of exopeptidase and endopeptidase (Rao *et al.*, 1998).

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).