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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "Anti-cancer activity of microbial protease isolated from environmental sources" Submitted by Sri Nanda Singh who got his name registered on 13.09.2019 for the award of Ph. D. (Science) Degree of Jadavpur University, is absolutely based upon his own work under the supervision of Dr. Amit Pal and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

AP 2/5/23

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Anti-cancer activity of microbial protease isolated from environmental sources

Thesis submitted to Jadavpur University for the degree of
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By

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This thesis is dedicated to my mother and to my wife Sosmita

For their endless love, support and encouragement

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Nanda Singh

Table of Content:

Abstract

The purpose of this study was to identify novel proteases that can induce apoptosis on cancer cells. In this study we have purified secretory protease subtilisin from *Bacillus amyloliquefaciens*. The protease was purified from DHS-96 an environmental microbial strain. 16s rRNA sequencing confirmed that DHS 96 belongs to *Bacillus amyloliquefaciens*. Subtilisin induced apoptosis in colon (HT29) and breast (MCF7) cancer cells but showed no effect on mouse peritoneal macrophage and on normal breast cells (MCF10A). Western blot analysis showed that Bax, Bcl-2 level remained unchanged but tubulin level decreased significantly. Subtilisin does not induce the intrinsic pathway of apoptosis; rather it induced tubulin degradation in MCF-7 cells, whereas in normal cells (MCF-10A) tubulin degradation was not observed. In depth analysis showed subtilisin activates ubiquitination and proteasomal mediated tubulin degradation which was completely restored when proteasome inhibitor MG132 was used. We further observed PARKIN, one of the known E3-ligase is overexpressed and interacts with tubulin in subtilisin treated cells. PARKIN activation and tubulin degradation leads to ER-stress which in turn activates caspase-7 and PARP cleavage, thus guiding the subtilisin treated cells towards apoptosis. To our knowledge this is the first report of subtilisin induced apoptosis in cancer cells by proteasomal degradation of tubulin

CHAPTER 1

INTRODUCTION

Growth and reproduction are two fundamental characteristics of all living organisms. Eukaryotic organisms grow by a process called cell division, where a parent cell divides and produces two new daughter cells [1]. Cell division occurs through series of events called cell cycle. Cell cycle is a tightly regulated process and an abnormality in this process caused by genetic or environmental factors may lead to uncontrolled cell proliferation. The uncontrolled proliferation of cells further changes the molecular and genetic makeup of normal cells, as a result normal cell becomes cancerous cell [2]. There are different types of cancer that can be classified into five groups: Carcinomas, Sarcomas, Hematopoietic malignancies, Neuroectodermal malignancies and atypical tumors [3]. Among them carcinoma is the leading cause of death worldwide (Fig 1). The incident of cancer increases with age, as cellular repair machinery becomes less effective with age. Worldwide, cancer continues to be one of the leading causes of death each year [4]. In the year 2022, total 1,918,030 number of new cases and 609,360 number of deaths related with cancer have been reported in the USA [5]. In India, the adjusted mortality to incident ratio of cancer was 26.7 million in the year 2021 which may increase to 29.8 million by 2025 as predicted by Indian Council of Medical Research (ICMR) [6]. It is evident from these statistics that more research is required to develop better cancer treatments.

Prevention of cancer depends on various factors like life style changes, early detection of cancers and developing better cancer treatments that can reduce the risk of cancer progression in individuals [7,8]. A conventional approach to cancer treatment involves chemotherapy, radiation therapy, and surgery [9]. Treatments with these conventional approaches can prevent cancer and save lives but there is a high level of toxicity and disfigurement associated with these treatments. Surgery relies on removal of cancer containing mass of tissue and works well with solid tumor removal. Surgery may not be able to remove all cancerous tumors, as it may cause damage to normal tissue if the entire region is removed [10]. There is

also some risk associated with surgery, such as pain, infection, and recurrence of cancer after surgery [11]. Chemotherapy relies on the principle that a drug administered in the body

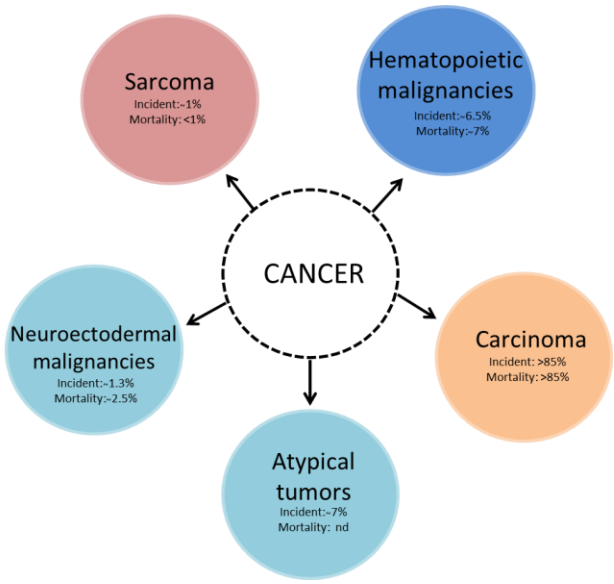


Figure 1.1: Cancer types and their incidence and mortality. (Link Wolfgang 2019)

can kill tumor cells more effectively than normal cells. Treatment for cancer remains largely based on chemotherapy. In some cases, chemotherapy can be effective, but sometimes its side effects can kill cancer patients. A major risk associated with chemotherapy is that along with the cancer cells, it kills the normal/healthy cells in the body, which can have serious consequences for the patient and may even change its genetic makeup in such a way that it becomes resistant to the treatment [12,13]. Similarly, radiation therapy reduces the size of tumors or kills cancer cells more effectively than normal cells when administered to a cancer affected area of the body. In radiation therapy different components of the cell can be targeted with ionizing radiation, including proteins, nucleic acids, and lipids, but the major target is the cellular DNA. Similar to chemotherapy radiation therapy also has major side effects. It is highly destructive against normal tissues and in addition it may induce new type of cancer in the body [14]. The fundamental problem of these conventional therapies is specificity against cancer cells. Due to the limitation of the conventional therapy, scientists

have discovered alternate approaches to treat cancer that target cancer cells more effectively without effecting normal cells. One of such alternate approaches is to find novel anti-cancer molecules/drug that can specifically kill cancer cells while minimizing the effect on healthy cells [15,16].

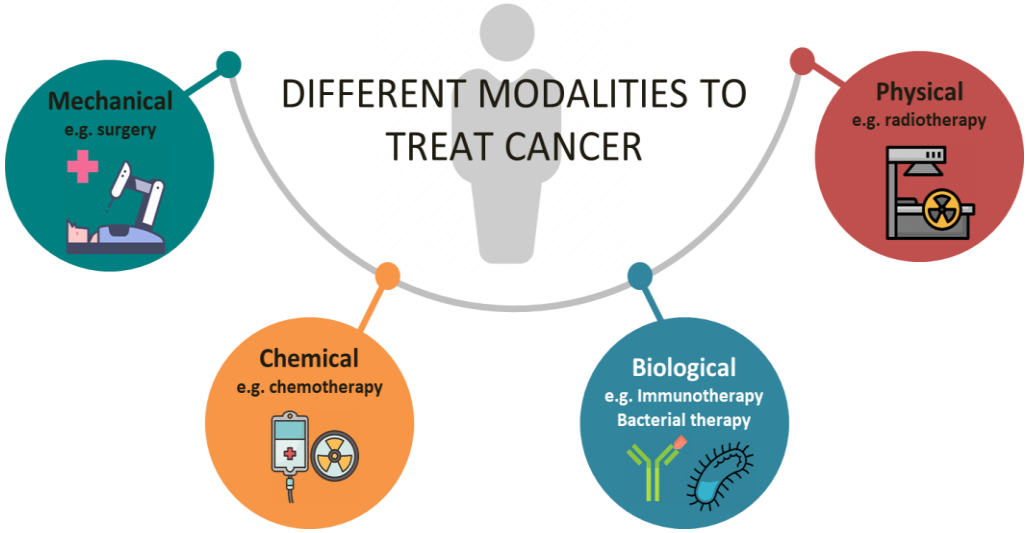


Figure 1.2: Different modalities of cancer treatment (Link Wolfgang 2019)

Anti-cancer molecules discovered from natural sources like microbes, plants and animals are more affordable therapy for cancer treatment. Beside the pharmaceuticals industry, research conducted by academic, government, and private laboratories has also contributed significantly to the discovery and development of anticancer agents isolated from natural sources [17]. As of now the anti-cancer research has led to discovery of large and diverse group of anti-cancer molecules which are not easy to categorize. There are many factors that can be considered when classifying anti-cancer molecules, from their chemical nature to their mechanisms of action. Traditionally they are classified as I) alkylating agent II) natural products III) antimetabolites IV) hormones and antagonist and V) miscellaneous. The miscellaneous group in recent years is growing with novel anti-cancer molecules, including

bacterial proteins, antibody and toxins [18]. In recent days, proteins and peptides from microorganisms have shown to be promising anti-cancer agents. Among them it has been shown that bacterial antibiotics, toxins, and proteases are effective against cancer cells. Some of these are already used in cancer treatment, and some are in experimental stage [19]. Advancement in biotechnology finds microbes to be a valuable source of bioactive molecules that can be used as therapeutics against various diseases such as cancer, cardiovascular disease and antimicrobial therapy and these molecules can be purified in a relatively cost-effective manner [20–23]. Microbes are found everywhere in nature, from desert to volcanic eruption and the number of microbial taxa we know is only 0.001% [24]. By exploring the environmental microbiome, we will be able to discover novel molecules that will provide us new information.

As early as the 1800s, molecules isolated from microbes were evaluated as anticancer agents. Bacteria and their by-products were first used in cancer treatment by physician William B. Coley and are referred to as ‘Coley's toxins’ [25]. The principle of Coley's toxin has been proven to be effective. A variety of anti-cancer agents derived from microbes are being evaluated for the treatment of cancer. In 1940 antibiotic actinomycin was isolated from *Streptomyces parvulus* and it was the first used antibiotic shown to have anti-cancer properties. Actinomycin-D was later approved by FDA and has been widely used as an anticancer molecule in the treatment of various types of tumors [26,27]. Microbial natural products such as bleomycin, epirubicin, doxorubicin and rapamycin were isolated from different microbial sources and were approved by the FDA for the treatment of cancer [28,29]. More than 60% of currently occurring anti-cancer molecules are from microorganisms. Besides wide use of microbial natural products, current research identifies the importance of microbial enzymes in disease therapeutics. Microbes are the source of many useful enzymes. Microbiological enzymes are widely used in textile, food, detergent,

leather, biofuels, and other industry [30]. The use of enzymes in biopharmaceutical industry has grown steadily in recent years [31]. Advantage of production of therapeutics enzymes from microbes is cost efficient and environmentally friendly. The concept of therapeutic enzymes is not new and from 1960s it has been used as a replacement therapy [32]. Among various enzymes, protease is the largest class of enzymes that hydrolyzes the peptide bond of proteins. Protease was the first therapeutic enzyme to be used. In 1978, US-FDA has approved 'Activase', a serine protease for the treatment of myocardial arrest and with this began the new era of enzyme therapeutics [33]. In recent year's proteases has been has been used as therapeutics candidate for different diseases, like treatment of sepsis, cardiovascular disease, digestive disorder, inflammation, cystic fibrosis, retinal disorder, cancer and other diseases. Proteolytic enzymes are established drug, prescribed to reduce symptom of irritable bowel syndrome (IBS), decrease inflammation and reduce muscle soreness for speedy recovery after surgery [34]. The U.S Food and Drug Administration (FDA) have approved variety of proteases for therapeutic applications (pancrelipase, Botulinum toxin B, Botulinum toxin A, Reteplase and Urokinase) and a few more are still in clinical trial [35].

Protease is an enzyme found in every living organism, including plants, animals and microbes. Protease play key role in processing, maturation and hydrolysis of other physiologically important proteins. In mammalian cells, proteases play vital role in tissue morphogenesis, differentiation, necrosis, apoptosis, autophagy, fertilization and wound-healing. Abnormality or any dysregulation of this proteolysis process leads to different pathological condition in our body, such as cancer, cardiovascular disease, inflammatory disorder and neurodegenerative disease. Targeting protease is believed to be effective in treating diseases [36,37]. Likewise in plants, protease play important role in plant development, seed germination, senescence, fruit ripening and nutrient redistribution. Plant proteases are commercially used in food processing industry for decades and the discovery of

new proteases from plants has great economic significance in many industries [38]. A protease mixture 'Bromelain' from *Ananas comosus* (Pineapple) plant is extensively used in anti-cancer activity and has been used as traditional anti-cancer medicine in South East Asia. Research has revealed that bromelain inhibits cancer by regulating NF-kB and Cox2 in cancer cells as an anti-inflammatory molecule [39]. Bacteria and others micro-organisms use proteases for their survival in response to different environmental conditions. Microbial proteases are essential to maintain their normal biochemical and physiological condition. Many pathogenic bacteria need proteases to infect their hosts [40,41]. Besides its wide use in industry, proteases isolated from microbial sources have shown promising results against various diseases including cancer. There are few reports where the anti-cancer properties of microbial proteases have been studied. Potential cytotoxic effect of microbial proteases induces apoptosis and can kill cancer cells. A protease isolated from *Serratia marcescens* showed anti-tumor activity when injected to a solid tumor mice model. This protease showed very promising results in regional treatments of tumors [42]. Similarly in our laboratory we have showed Hemagglutinin Protease (HAP) from *Vibreo cholerae* has anti-cancer properties. HAP induces apoptosis in breast and colon cancer cells. HAP increases cellular ROS levels, triggers the intrinsic apoptotic pathway through PAR-1 activation, and reduces tumor growth in solid tumor mouse model [43,44].

In search for similar microbial proteases from environmental sources which can induce cell death by apoptosis in cancer cells, we have identified and purified an extracellular protease subtilisin from an environmental isolate *Bacillus amyloliquefaciens*. Though commercial use of subtilisin has been extensively studied, its anti-cancer properties have not been addressed earlier. In the present study, we evaluated the anticancer properties of subtilisin and the mechanism of its action was assessed.

Subtilisin is an alkaline serine protease, belongs to ‘subtilase’ superfamily and can be found in wide range of organisms, such as bacteria, archaea and higher eukaryotic organisms. Historically, subtilisin has been a model for studying protein engineering because of its valuable biotechnological applications. Subtilisin is a well-studied commercially valuable enzyme extensively used in detergent, leather and textile industry. Subtilisin also been used in modern bio-waste management industry [45]. In addition to its wide use in detergents and waste management industry, there are a few reports suggesting its role in pharmaceuticals industry. Subtilisin in combination with antibiotics has been used to treat wound and burn injuries. It has been observed that subtilisin solubilizes necrotic cells from burns and wound injury without effecting normal tissue [46]. In a recent discovery engineered subtilisin has been used to target mutant RAS to treat cancer. Mutation of RAS can be observed in various cancers. Therefore, targeting RAS with modified subtilisin could be adapted to target other diseases-specific proteins [47]. In 1987 Maeda et al. from Japan reported that subtilisin treatment could reduce solid tumor sarcoma 180 in mice [42]. To the best of our knowledge this is the first report to identify the molecular mechanism through which subtilisin might prevent cancer.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Epidemiology:

Cancer is a group of disease that have potential to affect any part of the body. One of the unique characteristics of cancer cells is that they proliferate uncontrollably. The uncontrolled proliferation of the cells creates a mass of tissue called tumour which can then invade other normal parts of the body and induce them to convert into cancerous phenotype and this is called metastasis. Nearly 10 million peoples died in cancer in the year 2020 [48]. Among the various types of cancer, breast cancer is the highest reported (2.26 million cases) cancer in 2020 followed by lung cancer (2.21 million cases). According to World Health Organisation's (WHO) statistics, the highest causes of death in cancer is lung cancer (1.80 million deaths) followed by colon cancer (916,000 deaths) [49]. According to Indian Council of Medical Research (ICMR), 2.7 million people have cancer in the year 2020 and every year 13.9 lakhs new cancer patients are registered in India [50]. It is estimated that 8.5 lakh people die from cancer in India every year, and the cancer mortality ratio in the country will be 29.8 million by 2025 [51]. In India 1 in 20 urban males and 1 in 24 urban females have high risk of dying from cancer before 75 years of age [52]. Lip/oral cavity cancer, lung and stomach cancer are the most common causes (25% death) of deaths among males in India. Breast, uterine cervix and oral cancer are the most common cause (25%) of deaths among females in India (Fig 2.1). Burden of cancer is growing throughout the world. The distribution of cancer and the causes of death from cancer can be seen in different numbers in different countries. The socioeconomic development and government policies are also associated with the cancer burden. Most of the cancer type can be prevented by early diagnosis and treatment in early stages of the disease. It is therefore possible to significantly reduce the burden of cancer through public awareness and lifestyle changes.

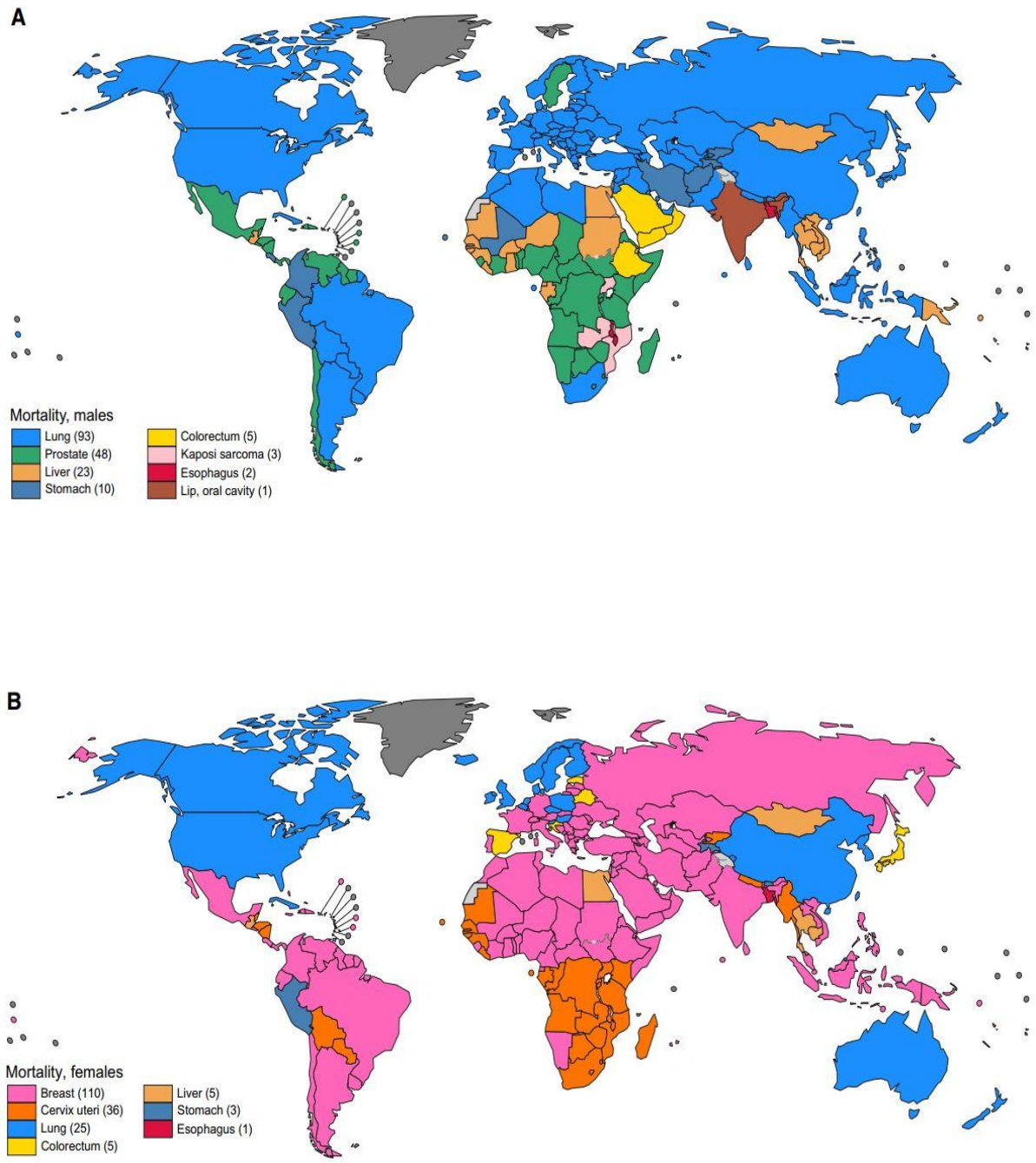


Fig 2.1: Types of cancer most prevalent among (A) men and (B) women in different countries in 2020. The number of country shown in the bracket**.**

(Sung H et. al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021 May)

2.2 Risk factors and causes:

To get rid of a problem we have to be familiar with the causes of that particular problem. What causes cancer is a fundamental question asked from decades. Advances in cancer epidemiology research helped us to understand the basis of cancer, it's diagnostics and how to prevent it. In recent years this has helped us to reduce cancer related mortality [53,54]. Numerous factors have been identified that significantly increase the risk of cancer development and the list continues to grow. It is difficult to categorise each factor. The major risk factors associated with cancer progression are discussed below.

2.2.1 Environmental exposure:

It is now known that lifestyle and environmental factors play a crucial role in causing cancer. It has also been established that some specific types of cancer can occur due to environmental exposer. Smoking is one of the main causes of lung cancer in the world. The environmental substances that induces the risk of developing cancer are called carcinogens. These carcinogens can be found everywhere in our environment. Chemicals present in tobacco, uv-radiation from sun, pollutants present in air and other carcinogenic compounds may cause cancer [55]. There are certain types of cancer that can be prevented if we avoid the exposure to such substances.

2.2.2 Radiation exposer:

Certain wavelength of radiation has power to damage the DNA or genetic components of organisms. This is called ionising radiation [56]. Majority of our radiation exposure comes from the sun. We could also get radiation exposure from other environmental pollutants like man-made nuclear hazards and equipment used in disease diagnosis. The role of radiation exposer to develop cancer was first observed during the incident of nuclear blast at

Hiroshima-Nagasaki. After the incident it was found that the children who had survived the incident had 2-3% increased risk of leukaemia and thyroid cancer [57]. Radiation used in diagnosis and screening could lead to increase in chances of developing cancer. It was reported that children who are exposed to X-ray could develop liver, bone and blood cancer. Pregnant women exposed to radiation due to their routine diagnosis had more chances of developing baby's childhood cancer [58]. CT scan, positron emission tomography (PET) scan, radiation therapy and X-ray can cause cellular damage and induce cancer progression. Those who undergo radiation therapy for certain childhood diseases are more likely to develop gastric cancer than those who do not receive radiation therapy [59].

Redon an environmental gas is another type of radiation that can cause cancer after prolonged exposure. Redon is produced by the breakdown of radioactive compound uranium and thorium. This gas is released from rock and soil after its formation [60,61]. Those living in a region with high levels of radon in their soil or environment have a high risk of lung cancer. Redon was identified as a cancer causing agent when researchers discovered that people who worked in uranium mines often died of lung cancer. A combination of this observation and laboratory experiments led to the discovery of Redon as a carcinogen [62–64].

Ultraviolet radiation or UV-ray from sunlight can cause cancer [65]. Wavelength between 10-400 nm is considered UV rays, and they are divided into several categories (UV A, B, C) depending on their frequency [66]. UV-rays act as a mutagenic substance and it can cause mutation in the genomic DNA of organisms that lead to DNA damage. Damaged DNA can induce the cells towards apoptosis or death. UV ray is very harmful to all organisms [67,68]. It is a well-established fact that UV exposer from sun can cause skin cancer. Excessive sun burns or prolonged exposure to sunlight could lead to melanoma and squamosal skin carcinoma [69,70].

2.2.3 Chemical exposer:

We live in a society where most materials we use are made up of some chemicals. Chemicals that we use have impacted our health. Some chemicals are also responsible for cancer development. Tobacco is one such chemical we are exposed to. Tobacco leaf used in various tobacco products is the leading cause of developing cancer [71]. It can cause various types of cancer including lung, oesophagus, lip, pharynx, bladder, kidney, colon and some form of leukaemia. Tobacco smoking produces highly carcinogenic compounds after burn, including nitrosamines [TSNA], N-nitrosonornicotine [NNN], 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [NNK]). These compounds can produce 16 types of cancer and thus tobacco smoking is considered to be most harmful form of chemical exposer [72]. Smokeless products of tobacco also have an impact on cancer development and as per United States (USA) National Cancer Institute, the major user of smokeless tobacco is in South East Asia where oral cancer is predominant [73].

Alcohol is another factor that could increase the risk of cancer. Consumption of alcohol increases the risk of developing mouth, throat, oesophagus, larynx, liver, and breast cancer [74,75]. Studies have shown that consumption of alcohol increase the risk of breast cancer by 11% and colon cancer by 8%. Excess consumption of alcohol could damage the tissues of liver and thus increase the chances of liver cancer [76]. It has been studied that consumption of alcohol increase the level of estrogen hormone that induces the risk of developing breast cancer in women [77,78]. Consumption of alcohol causes obesity in the individual and thus can increase the risk of developing various cancers [79]. According to American Cancer Society it is recommended not to drink alcohol, if anyone chose to drink he/she must take it in limited amount.

There are several other chemicals that are in use at home and our surroundings that increases the chance of getting cancer [80]. The risk of cancer occurs by exposer of such chemicals are provided in the table.

Table 2.1: Different chemicals associated with development of various forms of cancer

Cancer type	Chemical exposer
Lung	Arsenic, asbestos, cadmium, coke oven fumes, chromium compounds, coal gasification, nickel refining, foundry substances, radon, soot, tars, oils, silica
Bladder	Aluminum production, rubber industry, leather industry, 4-aminobiphenyl, benzidine
Nasal cavity and sinuses	Formaldehyde, isopropyl alcohol manufacture, mustard gas, nickel refining, leather dust, wood dust
Larynx	Asbestos, isopropyl alcohol, mustard gas
Pharynx	Formaldehyde, mustard gas
Mesothelioma	Asbestos
Lymphatic and hematopoietic	Benzene, ethylene oxide, herbicides, x-radiation system
Skin	Arsenic, coal tars, mineral oils, sunlight
Soft-tissue sarcoma	Chlorophenols, chlorophenoxyl herbicides
Liver	Arsenic, vinyl chloride
Lip	Sunlight

(Sources: American Cancer Society Fact Sheet “Occupation and Cancer”; International Agency for Research on Cancer.)

2.2.4 Age:

Age is one of the reasons of increasing risk of cancer. Cancer can occur at any age in an individual but with age the efficiency of cellular protection machinery or immunity against cancer gets decreased. As a result, if any abnormality or fault occurs within the cells then the chance of recovery is less efficient in an aged person than a younger person [81]. According to USA National Cancer Institute, the median age of cancer patients is 66 years [82]. The cancer incident rate under the age of 20 is about less than 25 cases in 1,00,000 people but the incident rate is more than 500 cases in 1,00,000 people when the age is 50 (Fig 2.2).

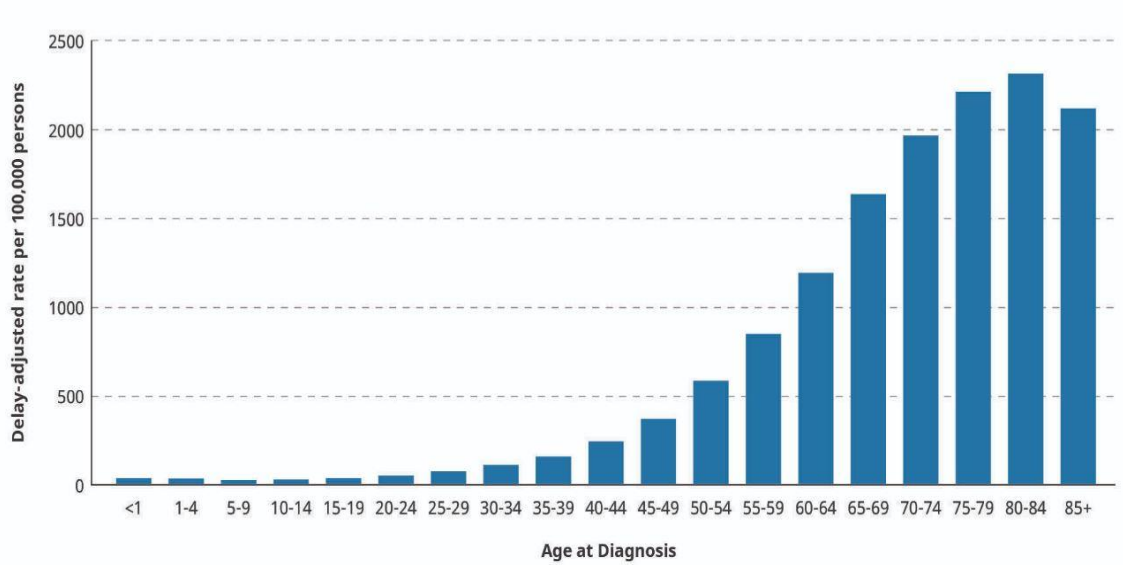


Fig 2.2: Cancer incident rate with age. (Source: The Surveillance, Epidemiology and End Results (SEER)'21 2013–2017, National cancer Institute, USA)

2.2.5 Infections:

Exposer of certain type of infectious agent could also increase the risk of cancer. This occurs because certain types of infections alter cellular signalling, causing normal cells to turn cancerous. It is possible for certain types of infections to inactivate or suppress our immune system, preventing our bodies from fighting off cellular changes that lead to cancer[83–86].

Some viral infections such as Hepatitis-C virus causes hepatocellular carcinoma, T-cell leukemia virus causes leukemia and papilloma virus causes cervical cancer [87,88]. There are also some bacterial infections that could increase the risk of cancer. *Helicobacter pylori* infections could increase the chance of gastric cancer [89]. *Chlamydia trachomatis* infections can be found in sexually active women and studies have shown that infections of this bacteria can increase the risk of cervical cancer among female [90,91]. It has been reported earlier that flatworms have also been found to causes cancer in human. *Schistosoma hematobium* flatworm can induce bladder cancer and *Opisthorchis viverrine* infection can cause cancer in bile duct [92–95].

2.2.6 Genetics:

Though several environmental factors contribute to the development of cancer, ultimately it is the dysregulation of genes inside our cells that causes cancer. Two types of genes that are mainly related with cellular growth, have directly or indirectly contributed to the development of cancer, one type is proto-oncogene and another one is tumor-suppressor gene [96,97]. Overactivation of proto-oncogene and inactivation of tumor-suppressor gene leads to cancer progression. There are several reports to show that these genes with mutations or changes can be passed from the parents to child. This means that if a family history of cancer exists, the disease can be passed on from one generation to the next generation [98–100]. BRCA1 and BRCA2 are two genes if mutated have high chance of breast cancer (45%-80%) or any other type of cancer. The chance of getting breast cancer is high if a child inherits a mutated BRCA1 or BRCA2 gene from their parents [101,102]. Genetic test can be done to check the inherited genetic profile for cancer risk.

2.2.7 Other causes of cancer:

Many other risk factors that could cause cancer like daily diet, obesity or overweight and sedentary lifestyle could induce risk of cancer [103,104]. There are certain drugs that are used for treatment of diseases and can be carcinogenic and can increase the risk of cancer in an individual [105,106]. Different pollutant in air, water and soil causes the risk of developing cancer [107–109]. Unsafe sex or sexual lifestyles also have impact on developing cancer of an individual [110,111]. There is no specific reason for developing cancer but exposer to various agents can induce the risk of developing cancer in an individual.

2.3 Diagnosis:

Combination of test and physical examination is essential to find and diagnose cancer. Several tests are available to diagnose cancer and the strategy of diagnosis is very important for early detection of cancer.

2.3.1 Laboratory test:

Many types of lab test are there for detection of cancer, such as blood test, urine test and cancer bio-marker test. Blood test and urine test can help to identify the abnormality related with cancer but these are not confirmatory test for cancer diagnosis [112–114]. Cancer marker test is very helpful to detect any specific type of cancer. Presence of elevated level of marker in the blood could be very helpful to detect the cancer [115,116]. This result in combination with other test can diagnose cancer at early stage.

2.3.2 Genetic screening:

Genetic screening of cancer cells is very important to identify the mutated genes or variations in DNA of an individual. Scientific advancement in genome sequencing makes cancer cell genetic screening easy and cost effective [117–119]. Persons with family history of cancer should go for genetic screening for better understanding of the hereditary profile of any particular cancer [120]. Genetic screening helps to determine any cancer susceptibility within an individual and this is important for detecting cancer at early stage. Sometimes clinicians do genetic screening before chemotherapy or surgery to understand the outcome of the treatment. For example, a female individual who has family history of cancer can go through early genetic screening and if BRCA positive mutation is found in that patient, then the clinician can suggest her to go with Risk-Reducing Prophylactic Mastectomy [121]. Personalized medicine or precision medicine is becoming popular nowadays and with the help of genetic screening, clinicians can plan therapeutic options for an individual cancer patient with personalized medicine [122,123].

2.3.3 Imaging:

The use of new imaging tools and techniques helps clinicians diagnose cancer more accurately. In this process imaging of bodies internal structure is captured by radiologist and then analysed by expert clinicians [124]. Depending upon how the image is taken, there are three types of imaging available. Imaging by transmission, imaging by emission and imaging by reflection. Example of transmission imaging is Computed transmission scan (CT scan), Positron emission tomography scan (PET scan) and X-ray. Ultrasound imaging (USG) uses reflection property to generate image and Magnetic resonance imaging (MRI) uses emission imaging [125]. Imaging data help clinicians to find how cancer spreads into the body, what stages it is, how well patient responses against the treatment. Recent advancement in machine

learning and artificial intelligence (AI) uses the data generated from the imaging and applies it for better understanding of cancer phenotyping [126].

2.3.4 Biopsies:

Biopsy is a process where a small piece of tissue is removed surgically from patient body and observed under microscope to check if any cancer cell is present [127,128]. Depending upon the position of tumour, different procedures are there to take biopsy sample. Tumor biopsy is done by removing small portion of tissue or by removing the entire organ. In liquid biopsy sample is taken through needle aspiration [129,130]. Biopsy is often done to check if the tumor is malignant.

2.3.5 Endoscopy:

Endoscopy is a technique to investigate sonographically inside our body using endoscopic scanning probe. With this technique gastrointestinal (GI) tract can be visualized clearly and the organ proximal to GI tract could also be effectively visualized [131]. This technique helps to identify any abnormality or malignancy in esophagus, intestine and colon. Endoscopy accurately determines the different stages of gastric cancer and has been effectively used to diagnose gastric cancer [132–134].

2.3.6 Other tests:

Several other methods have been developed for cancer diagnosis, such as, cancer specific antigen test, stool DNA, blood test and nanotechnology based diagnostic test [135]. For accurate diagnosis of cancer, clinicians need to rely on multiple test results.

2.4 Treatment:

Several approaches are there to treat cancer. Chemotherapy, surgery and radiation therapy are the conventional approaches to treat cancer [9]. Recent advancement in cancer research brings new approaches to treat cancer such as, immunotherapy, hormonal therapy and targeted therapy [136,137]. Since the last 15-20 years new anti-cancer therapeutics strategies have been developed and new anti-cancer drug from various sources have been identified. The treatment options of cancer are very wide in today's world and depending upon the type and stages of cancer, clinicians can chose better options to treat cancer.

2.4.1 Surgery:

Surgery is one of the best options to cure cancer if it is diagnosed at early stage. Surgery relies on principle to remove tumor from the body and is useful to cure the local solid tumor but not for malignant tumors [10]. Sometimes surgery is done to prevent the risk of cancer in an individual. Mastectomy is done to reduce the risk of cancer in women with family history of breast cancer [138]. There are various limitations of surgery, sometimes cancer can relapse from the area where surgery has been done [139]. Surgery can also damage the normal tissue of surrounding tumor and this can cause other health issues to the patient. Surgery may not be possible in patients who are physically unfit or who cannot tolerate surgery, like in aged patients [11].

2.4.2 Radiation therapy:

In the treatment of cancer, radiation has been in use for a very long time. Ionizing radiation (X-rays, gamma rays) have the potential to invade cells or tissue of our body and can damage the DNA which in-turn indues cell death [140]. Radiation is given to the cancer patients from the external sources or by injecting radioactive drugs near the effected place to treat cancer

[141]. Though radiation therapy has been proven to have the potential to cure cancer, there are certain limitations of radiation therapy. Radiation therapy can kill cancer cells but it can also damage normal cells and in some cases serious post treatment side effects occur in the patient. Radiation itself can damage the DNA of normal cells and could induce the normal cells to become cancerous cells [142].

2.4.3 Chemotherapy:

Chemotherapy uses chemicals to treat cancer and it was first used in 1940 when 'mastered gas' was discovered as an anti-cancer agent. Since then, numerous agents have been discovered to treat cancer. There are various types of molecules discovered as anti-cancer agents, such as, DNA binding agents, microtubule targeting molecules, cell surface receptor targeting agents, cytotoxic molecules etc [143,144]. The conventional chemotherapy has potential to cure cancer but it has various side effects and limitations. The main limitation is specificity against cancer. Chemotherapeutic treatment can kill non-specifically normal cells and thus causes various side effects [145,146].

2.4.4 Targeted therapy and alternative sources of ant-cancer drugs:

In conventional cancer therapies, specificity against cancer is a major problem. To overcome the limitations of conventional cancer treatment, scientists have developed alternative treatment approaches that target cancer cells more effectively without harming normal cells. Targeted therapy is one such approach [147]. In targeted therapy, advances in cancer research help to identify the cancer specific targeted molecule for therapeutics specificity. Identification and discovery of drugs against targeted molecule reduce the burden of toxicity and side effects found in conventional therapy.

In anti-cancer research, one of the objectives is to discover novel molecules/drugs that can specifically inhibit cancer. Molecules discovered from natural resources are the major sources for drug development in today's world. Over the years, research has been done to isolate molecules from plants, animals and microbes and study their anti-cancer effect. Around 25% of currently approved anti-cancer drugs are discovered from these sources [148–150]. Microorganisms remain great sources of molecules that are useful to mankind. One of the main reasons is because microbes have great diversity in nature and isolation of compounds from microbes is relatively cost effective [151–153]. The use of microorganisms as source of anti-cancer drugs is therefore very promising and includes the use of microbial metabolites, proteins, peptides and toxins [154].

2.5 Microbial enzymes in cancer therapy:

Microbes are a source of different enzymes that are used in different sectors, from industry to health. Currently, microbial enzymes have a market worth 9.9 billion dollars (USD) in 2019, and this growing demand encourages researchers to screen for more of these enzymes [155,156]. Over the past few years, microbial enzymes have been increasingly used in biopharmaceutical industry. The production of therapeutic enzymes from microbes is environment friendly and cost-effective [31]. Microbial enzymes have also been screened for cancer therapeutics in recent years. For example, cytosine deaminase (CD) from bacteria and fungus has been used for prodrug activation in cancer gene therapy. CD enzyme converts 5-flurocytosine to 5-flurouracil and has anti-cancer property [157,158]. L-asparaginase from microbial origin is already FDA approved enzyme used in acute lymphoblastic leukemia. L-asparaginase causes depletion of L-asparagine amino acid, which is a non-essential amino acid and can take part in cell metabolism, glycoprotein bio-synthesis and epithelilal-mesenchymal Transition (EMT) of cancer. Depletion of the amino acid by L-asparaginase

helps in inhibiting cancer cell growth [159,160]. Microbial enzyme heparinase was first isolated from bacteria *Pedobacter heparinus* and showed anti-tumor activity [161,162]. There are several other microbial enzymes to have anti-cancer properties.

2.5.1 Microbial proteases in cancer therapeutics:

Among various types of enzymes, proteases are the most abundant enzymes that hydrolyze peptide bonds in proteins. Proteases act as an important enzyme in every organism. It plays many roles, including maturation of intracellular proteins, digestion of nutrients, and taking part in immune cascades etc [35]. Due to their unique function proteases have been

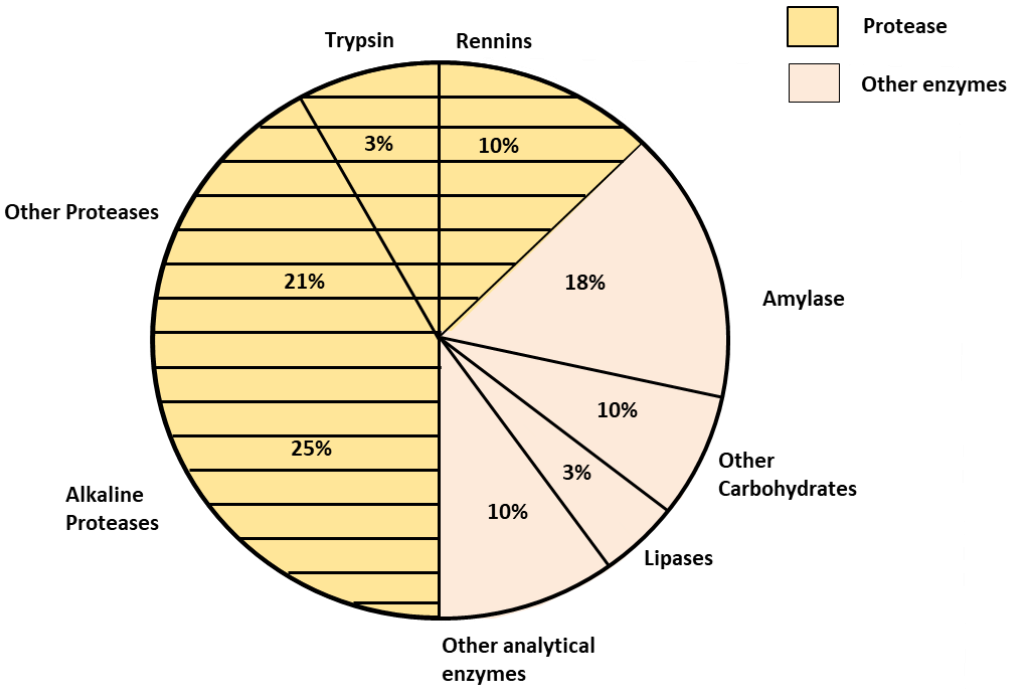


Fig 2.3: Distribution of commercial enzymes market (Rao B. Mala et al: Molecular and Biotechnological Aspects of Microbial Proteases)

used in commercial industry, such as, detergent industry, leather industry and food-processing industry. Out of other industrial enzymes market, proteases serve 60% of total worldwide sale of enzymes (Fig 2.3) and a significant part of this protease market comes from microbial proteases, which represent 40% of the total [163].

Protease is a group of enzymes that hydrolysis protein polypeptide chain into smaller peptide. Proteases are classified into two groups, endopeptidase and exopeptidase. Exopeptidase cleave proteins at their C or N terminal end while endopeptidase cleave the proteins at anywhere in between the two ends of the polypeptide chain. When the structure and mechanism of protease catalysis were discovered, they were classified into six groups: metalloproteases, serine proteases, cysteine proteases, aspartic proteases, glutamic proteases, and threonine proteases [164]. The name of each protease type is related to the interacting amino acid present at the active site with the substrate. Protease can be found in variety of organisms from single cell to multicellular organisms. Among these, proteases from microorganisms got popular, because of its easy availability, genetic manipulation and cost-effective purification. Microbial proteases are classified into alkaline, acidic, and neutral proteases depending on their activity at different pH levels. The activity of the alkaline protease is optimum at an alkaline environment, similarly, the activity of neutral protease is optimum at neutral pH and the activity of acidic protease is optimum at acidic pH [165].

Microbial proteases have been used in industry for years but use of proteases in biopharmaceuticals industry has been growing in recent years. A significant step toward the use of proteases from industry to therapeutics was made when the FDA approved the enzyme ‘Activase’ for the treatment of myocardial damage in 1978 [34]. Following that, identification of proteases from diverse origins and study of their therapeutic effects on different diseases brought the protease industry into a new direction. Later, FDA has approved various other proteases for therapeutic use and many are in clinical trial [35]. The role of microbial proteases in anti-cancer research has been reported in few studies and it has been found that it induces apoptosis, inhibits cell proliferation, and induces cytotoxicity in cancer cells. Further research is needed to identify novel bacterial proteases against cancer. The use of bacterial proteases in anti-cancer research is discussed below.

2.5.1.1 Bacterial serratia proteases an anti-cancer agent:

In 1987 Maeda et al. from Japan isolated protease from *Serratia marcescensand* and showed that it has a potent anti-tumor activity when injected into radiation induced syngenic mouse tumor [42]. It has been shown that serratia protease in low dose can completely eradicate tumour locally, thus for regional tumour treatment this protease can be a good option. In high dose this protease is toxic to animals and the anti-tumour activity is completely inhibited when incubated with ovomacroglobulin (inhibitor of serratia protease). Serratia protease is inhibited *in vivo* by alpha-2 macroglobulin but after 5-6 hrs, protease activity is regained. It has been found that serratia protease form complex with alpha-2 macroglobulin (E/I complex) after getting internalized via alpha-2 macroglobulin receptor. Following internalization cellular integrity is lost and eventually the cell died [166].

2.5.1.2 Serralysin- extracellular protease from *Serratia marcescens* showed Cytotoxic Effects on Colorectal Cell:

Another extracellular metalloprotease serralysin from *Serratia marcescens* was isolated and purified which showed cytotoxic effect on Caco-2 cell line [167]. MTT assay showed 24 hrs exposer of 24.8 μg/ml serralysin to Caco-2 cell line can kill 50% of the cell population. Inhibition of the protease by EDTA showed no cytotoxic effects on cancer cells. However, the subcellular target and mode of action of this protease has not been explored yet.

2.5.1.3 Hemagglutinin protease from *Vibrio cholerae* is a potent anti-cancer molecule:

In our laboratory, we have shown that *Vibrio cholerae* hemagglutinin protease (HAP) is effective against various cancer cells. It showed significant cytotoxic effects on colon cancer (HT-29) and breast cancer (MCF-7) cell line. Solid tumor developed in syngenic mouse model was completely abolished when treated with HAP [43]. Importantly, HAP can induce

cytotoxic effects on cancer cells but not on normal cells (MCF-10A, normal breast epithelial cell). Molecular mechanism revealed that HAP acts on PAR-1 receptor on the cell surface and activates it. Activated PAR-1 receptor further activates its downstream signalling NF-κB and MAPK [44]. This signalling pathway enhanced ROS generation in cancer cells and induced the intrinsic pathway of apoptosis. Detailed analysis showed that *V. cholerae* hemagglutinin protease (HAP) cleaved PAR1 at a site that is different from its conventional thrombin mediated cleavage site. The N-terminal site of cleaved PAR1 interacts with one of its transmembrane domains and activates its downstream signaling pathways. HAP exposed a new N-terminal sequence "PFISED" which by itself could be used as an activating peptide for PAR-1 [168].

2.6 Apoptosis is the key target for anti-cancer molecules:

Apoptosis or programmed cell death is the cellular physiological process by which cells activate its own death program [169]. Series of pro-apoptotic or anti-apoptotic proteins can be regulated in this process that leads to ultimate death of the cells [170]. Apoptosis occurs for the elimination of unwanted or abnormal cells of an organism. Thus, this process is important for maintaining cellular homeostasis [171]. Apoptosis in the cells can be activated through two major pathways, intrinsic or mitochondrial pathway and extrinsic or death receptor pathway. Intracellular signal such as DNA damage, cytokine deprivation and unfolded protein response can activate the intrinsic pathway of apoptosis and extracellular signal such as cellular damage, pathogen infections can induce the extrinsic pathway of apoptosis [172–174].

In the intrinsic pathway, pro-apoptotic proteins induces the damage of mitochondria or disrupt the mitochondrial membrane potential, as a result cytochrome-c is released from mitochondria to the cytoplasm that in-turn activates apoptotic protease activating factor 1

(APAF1) and form apoptosome with pro-caspase-9. This apoptosome complex helps to cleave caspase-9 and activate it. This activated caspase-9 then activates executioner caspases, caspase-3/7/6 that leads to execution of the apoptosis in the cell [175,176]. Some of the pro-apoptotic proteins Bax, Bad, Bim, Bid etc and p53 play important role in the regulation of these proteins [177,178].

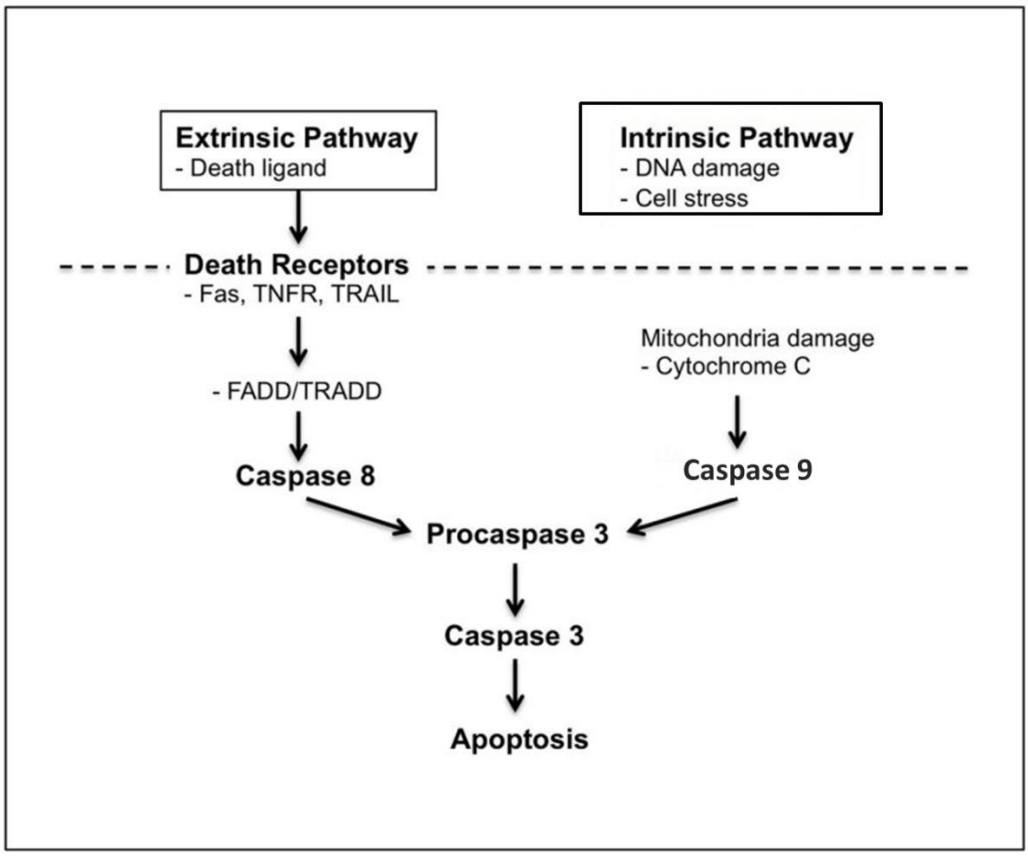


Fig 2.4: Key steps of intrinsic and extrinsic pathway of apoptosis schematically represent. (Cheng N *et. al* APP Overexpression Causes Aβ-Independent Neuronal Death through Intrinsic Apoptosis Pathway. eNeuro. 2016)

In the extrinsic pathway of apoptosis, extracellular signal induces apoptosis through death receptors such as, Fas receptors, tumor necrosis factor receptors (TNF) and TNF-related apoptosis-inducing ligand (TRAIL) receptors [179]. The death domain of these receptors then

recruits pro-apoptotic proteins which in-turn activates initiator Caspase-8. Caspase-8 activates its downstream executioner caspase, pro-caspase-3, resulting in the execution of apoptosis [180,181]. So in the execution phase of apoptosis there is crosstalk between intrinsic pathway and extrinsic pathway of apoptosis occurs [182].

One of the major characteristics of cancer cells is uncontrolled proliferation of the cells and for this, inhibition of apoptosis is necessary. Inhibition or deregulation of apoptosis pathway leads to the uncontrolled proliferation of cancer cells. In cancer cell, apoptosis can be deregulated by different mechanisms. Over-expression of anti-apoptotic proteins and down regulation of pro-apoptotic proteins is common in most of the cancer types. For example, anti-apoptotic protein bcl-2 is up-regulated and loss of bax or mutation in Bax gene is reported in various cancers [183–185].

In anti-cancer research different strategies are employed to target cancer cells and targeting apoptosis is one of the common approaches [186]. Many anti-cancer molecules have been shown to induce apoptosis in cancer cells. There are various anti-cancer molecules reported which can target intrinsic and extrinsic pathway of apoptosis. Curcumin found in turmeric is reported to have anti-cancer activity through inhibiting bcl-2 and x-linked inhibitor of apoptosis protein (XIAP) [187,188]. Quercetin, founds in apple and onion activate executioner caspases and induces apoptosis [189]. Cytotoxic necrotizing factor Y (CNFy) from *Yersinia pseudotuberculosis* induces PARP cleavage and activates caspase-3 mediated apoptosis in cancer cells [190]. Several others example are there where anti-cancer molecules isolated from microbes, plants and other sources have potential to induce apoptosis in cancer cells.

Table 2.2: Anti-cancer molecules isolated from plants and their mechanism of inducing apoptosis

Compound name	Origin	Mechanisms
Aloe-emodin	*Rheum palmatum*	Induces cytochrome c release
Black cohosh	*Actaea racemosa*	Activates caspases
Curcumin	Tumeric	Inhibits BCL-2 and XIAP
Graviola	*Annona muricata*	Inhibits BCL-2 and activates BAX
Epigallocatechin-3-gallate	Green tea component	Activates cell death receptors
Genistein	Soybeans	Cell cycle arrest activation
Juglone	*Juglans mandshurica*	Increase caspase 9 cleavage

(Source: Pfeffer CM et. al. Apoptosis: A Target for Anticancer Therapy, Int J Mol Sci. 2018)

2.7 Microtubule targeting agents (MTAs) in anti-cancer research:

Microtubules are dynamic structure of the cells cytoskeleton, made up of actin and tubulin heterodimer [191]. Microtubules are dynamic polymer and require high level of regulation to maintain its dynamic nature. It is an important component of a cell, helps in maintaining shape of cells, transport of proteins, vesicle and others organelle of the cells. It also plays an important role in cell division during mitotic chromosome alignment and separations [192,193]. Targeting microtubule is one of the effective components for anti-cancer drugs. In cancer therapy most of the MTAs inhibit cancer cell growth by cell cycle arrest [194]. MTAs are mainly of two types, microtubule stabilizing agents (MSAs) and microtubule de-stabilizing agents (MDAs). MSAs inhibit depolymerization of microtubules and as a result inhibit the mitotic spindle separation, thus block the cell cycle progression [195,196].

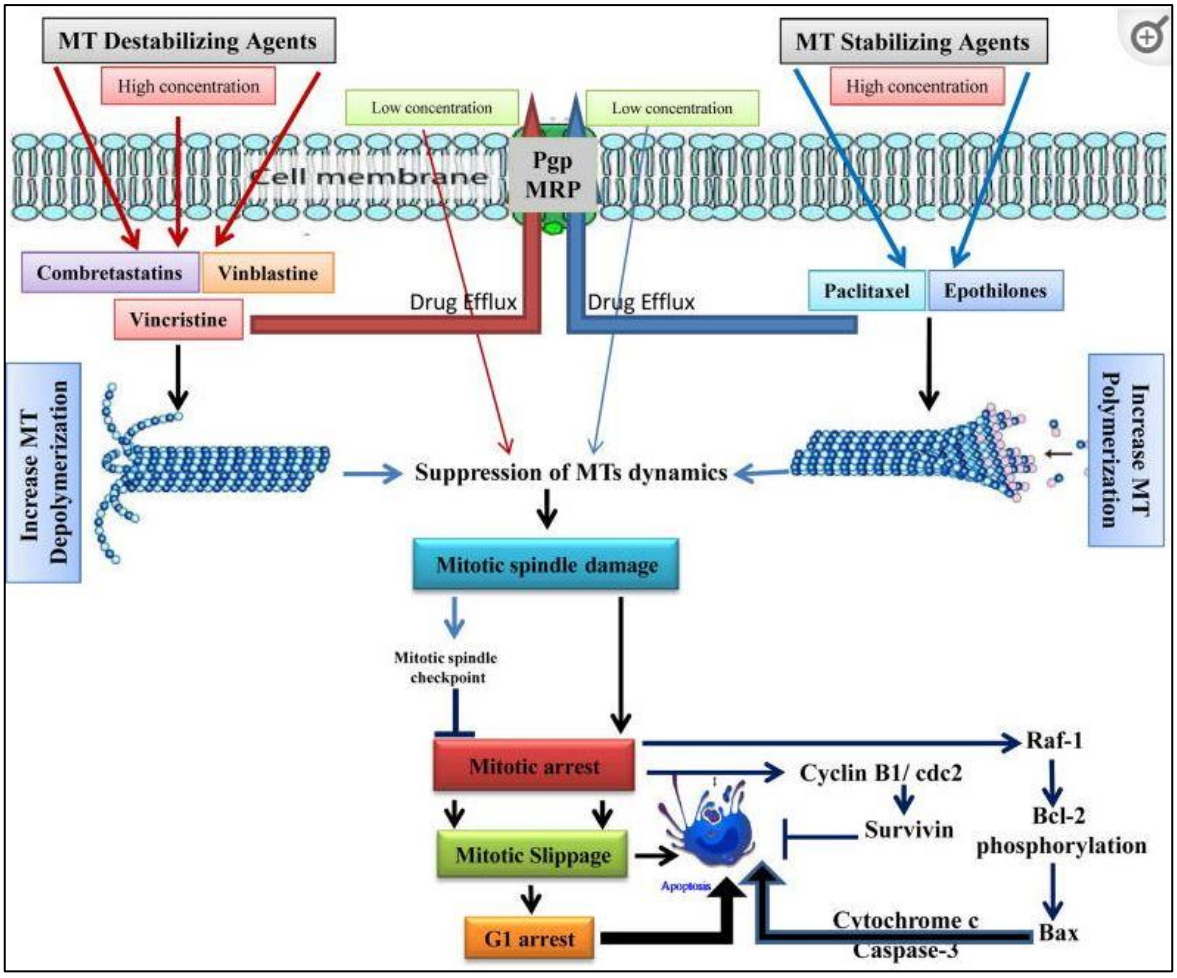


Fig 2.5: A schematic representation of the mechanism by which MTAs inhibits the growth of cancer cells. (Mukhtar E. *et al.* Targeting microtubules by natural agents for cancer therapy. Mol Cancer Ther. 2014)

Paclitaxel, docetaxel are class of MSAs that are used in cancer chemotherapeutics to inhibit cancer cell division, resulting in apoptosis in the cells [197,198]. MDAs destabilize the microtubule and induce its depolymerization which ultimately causes cell cycle arrest. Some of the examples of MDAs are vincristine, vinblastine and combretastatins [199,200]. There are some other classes of MTAs which can specifically inhibit tubulin by its degradation. These types of molecules can target tubulin in the cells, inducing its degradation through the ubiquitin-proteasome system that causes cell cycle arrest or stress in the cells that leads to apoptosis or cell death. Withaferin-A, a natural product could destabilize tubulin and induces

its ubiquitin-proteasome mediated degradation. Withaferin-A isolated from *Withania somnifera*, is a traditional anti-tumor, anti-angiogenic medicine used in India [201,202]. Another example is small molecule T0070907 which can induce tubulin degradation through proteasome mediated pathway [203]. These type of tubulin targeting molecules are novel agents for cancer therapeutics.

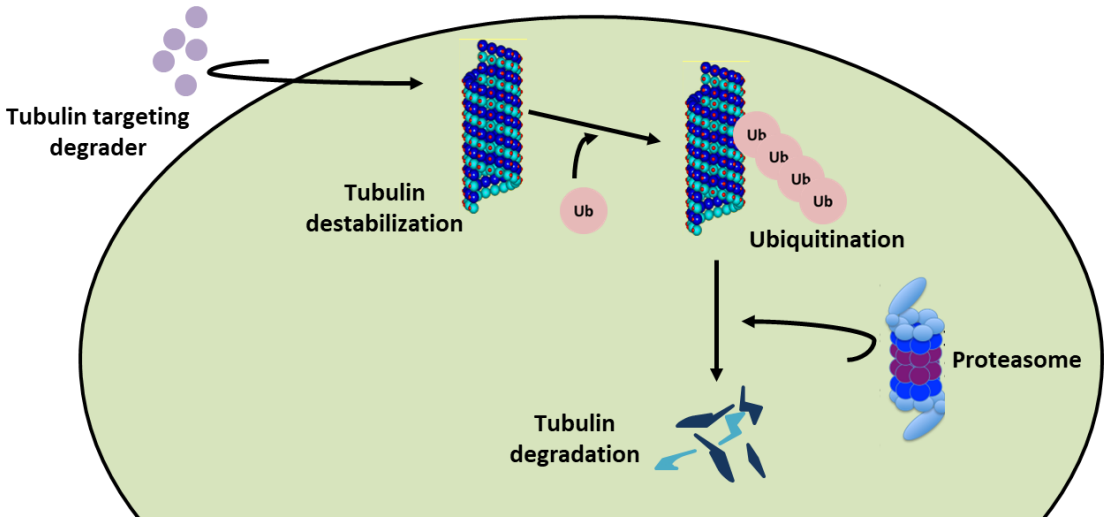


Fig 2.6: Schematic representation of tubulin degradation by microtubule targeting agents.

CHAPTER 3

OBJECTIVES

Conventional cancer therapies often fail to prevent cancer in patients, and most of the time it causes major side effects. There is need for new approaches to treat cancer. Microbial based therapy is promising alternative approach to treat cancer. Earlier studies showed that microbial proteases can induce apoptosis in cancer cell line [42,44,167]. We have shown in our earlier studies that HAP (Hemagglutinin protease from *V. cholerae*), a metalloprotease can induce apoptosis in cancer cell and regress tumor in mice model [43,168]. The aim of this work is to purify proteases from environmental microbial isolates with potential anticancer properties that could be used to kill cancer cells.

The objectives of my study are-

1. Screening of environmental bacterial strains for protease secretion.
2. Test the strains showing protease activity for apoptosis or cell necrosis on cancer cells.
3. Purification of extra cellular proteases showing apoptosis or cell necrosis on cancer cells.
4. Study the anticancer activity of bacterial proteases in-vitro, on cancer cell line and in-vivo, on cancer mice model.
5. Investigate the molecular mechanism of anti-cancer activity caused by microbial proteases in cancer cell.

CHAPTER 4

MATERIALS AND METHODS

4.1 Reagents:

Bacteriological media Nutrient broth (NB), Luria broth (LB), Tryptic Soy broth (TSB) and Bacto-agar were from Difco-BD (USA). Azocasein, ammonium sulphate, Tri-chloro acetic acid (TCA), diethyl pyrocarbonate (DEPC), phenylmethylsulphonyl fluoride (PMSF), ethylene diamine tetra-acetic acid (EDTA), 1, 10-phenanthroline, sodium dodecyl sulphate (SDS), phosphate buffer saline (PBS) tablet, sodium chloride (NaCl), Tris, Glycine, Ethidium bromide (ETBr) and other general chemicals were from Sigma-Aldrich (USA). Bovine serum albumin (BSA), Nitrocelluslose membrane, Tween-20 and Bradford reagent were from Bio-Rad USA. Skim milk and coomassie brilliant blue were from HIMEDIA, India. Methanol, Sodim chloride (NaOH), β-marcaptoethanol, Glacial acetic acid, isopropanol, chloroform, phenol and Glycerol were from SRL, India. Ethanol, polyvinylidene fluoride (PVDF) membrane, Triton-X-100, N,N,N',N'-Tetramethyl ethylene diamine (TMED) from E, Merek Limited. Penicillin-streptomycin, fetal bovine serum (FBS), minimum essential medium (MEM), trypsin, dimethyl sulphoxide (DMSO), trypan blue and Dulbecco's modified Eagle's medium (DMEM) were from Gibco (Rockville, MD). Mammary epithelial cell growth basal medium (MEBM) and mammary epithelial cell growth medium (MEGM) were from Lonza (Swizerland). DNA ladder, BCA assay kit, HRP-substrate developer solution and DreamTaq green mastermix from Thermo-Fisher (USA), protein ladder from Genetix (India). Genomic DNA isolation kit, TRIZOL reagent, cDNA synthesis kit were from Qiagen (Germany). SYBER-Green mastermix was from Applied Biosystem (ABS). Annexin V-PI staining kit was from BD-Bioscience (USA), PARKIN si-RNA was from Santa Cruz Biotechnology and proteasome activity assay kit was from Abcam (UK). All the primary antibodies, protein A/G agarose beads and prolong anti-fade DAPI were from Cell signalling technology (USA). Anti-tubulin antibody and all the secondary antibodies were from Santa-Cruz Biotechnology (USA). Primers used here were purchased from IDT (USA).

4.2 Bacterial strains and culture condition:

A total of more than 140 bacterial isolates were collected from salt farm of the Council of Scientific and Industrial Research- Central Salt and Marine Chemical Research Institute (CSIR-CSMCRI), Bhavnagar, Gujrat, India (Lat. 21°47´51´´N and Long. 72°7´38´´E). Sterile cotton swab was used to collect the bacteria from the surface of the different part of the farm. Swab was then strike directly on nutrient agar plate. Plates were kept at 37ºC and single colony with different morphology was restrike onto sterile stab culture for long term storage. During experiment, the bacterial isolates from the stab culture were revived on nutrient media and were stored at -80ºC in 25% glycerol. To screen for protease activity all the bacterial isolates were revived in nutrient broth (NB) (containing 2% NaCl, pH-8) and incubated at 37 ºC with shaking at 18o rpm till the OD reached 0.6. The pre-culture was then inoculated in final culture of nutrient broth at a ratio of 1:100 and incubated overnight. The overnight grown culture was centrifuged at 10,000 rpm for 5 mins and the collected supernatant was used for protease screening by azocasein assay.

4.3 Azocasein assay:

Azocasein assay is a protease screening assay which helps to measure protease activity of the culture supernatant of bacterial isolates and for the proteins eluted during purification as described in our earlier study [204]. We used this assay in our experiment to screen various bacterial culture supernatant for protease secretion. Azocasein is a nonspecific protease substrate, where Azo dye is tagged with casein protein. Incubating azocasein solution with samples containing protease will result in hydrolysis of Casein. The Hydrolysis of the casein releases the Azo dye into the solution where it is detected by absorbance at 440 nm in presence of 500 mM NaOH solution.

4.3.1 Preparation of Reagents:

0.7% Azocasein in 100 mM Tris-HCL (pH 8)

10% TCA

500 mM NaOH

4.3.1 Procedure:

Overnight grown bacterial culture supernatant and purified proteins (5 µg) were incubated with 100 µl 1% azocasein solution at 37°C for 1 hr. After 1 hr incubation, 200µl 10% TCA was added to stop the reaction and incubated for 30 min at 4ºC. The precipitation was centrifuged at 10,000 rpm for 10 minutes. Supernatant was collected and mixed with 0.5 M NaOH at a ratio of 1:1. The absorbance was measured at 440nm. NB was used as a negative control and purified haemagglutinin protease (HAP) from *Vibrio cholerae* was used as a positive control.

4.4 Ammonium sulphate precipitation and Dialysis:

Protein present in the bacterial culture supernatant was precipitated by adding solid ammonium sulphate to the solution. Protein solubility is affected by ions. At low ion concentrations (<0.5M), protein solubility increases along with ionic strength. Ions in the solution shield protein molecules from the charge of other protein molecules which is known as ‘salting-in’. At a very high ionic strength, protein solubility decreases as ionic strength increases in the process known as ‘salting-out’. Thus, salting out can be used to separate proteins based on their solubility in the presence of a high concentration of salt. Few proteins are soluble only in water and most require at least a small concentration of salt to remain folded and stable. Often, proteins that contain positively and negatively charged regions self-

aggregate under very low salt conditions. When salt is present, however, the anions and cations neutralize charges on the protein surface, preventing aggregation. As the salt concentration is increased even further, the surface of the protein will become so charged that once again, the protein molecules will aggregate [205,206]. The salting-out ability of multiple charged anions such as sulphate follows the Hofmeister series. Hofmeister series for anions: $PO_4$3-> SO_4^{2-}> CH3COO^-> Cl^-> B^r-> ClO_4-> I-> SCN-Hofmeister series for cations: NH_4^+> Rb^+> K^+> Na^+> Li^+> Mg^{2+}> Ca^{2+}> Ba^{2+} Ammonium sulfate, $(NH_4)_2SO_4$, is often used for salting out because of its high solubility, which allows for solutions of very high ionic strength, low price, and availability of pure material. Additionally, NH4+ and SO_4^{2-} are at the ends of their respective Hofmeister series and have been shown to stabilize protein structure. Some proteins follow a reversal of the Hofmeister effect when pH< pI (e.g., lysozyme and crystallins) [207].

Salting out removes proteins that are easily aggregated from those that are very soluble making it a good initial purification step for small soluble proteins [208]. It is one of the few protein purification methods that does not require the presence of a purification tag on the protein thus, if the protein is naturally expressed in the host cell, cloned in the absence of tags, or has an unexposed tag, salting out is an ideal choice for purification. This method may also be applied to purify a protein of unknown sequence. Since the sample is in a high concentration of $(NH_4)_2SO_4$ at the end of the experiment, hydrophobic interaction chromatography (HIC) may be used immediately to further purify the sample. The primary shortcoming of using salting out to purify proteins, however, is that contaminants often precipitate with the protein of interest. To obtain a pure protein sample, further purification steps such as ion exchange chromatography and gel filtration chromatography are needed. Also, the protein is in a high concentration of salt at the end of the experiment. Dialysis is generally the best method to remove $(NH_4)_2SO_4$ from a sample.

TABLE 4.1: Ammonium Sulfate Precipitation Table

Starting Concentration	Final Concentration									
	55%	**60%**	**65%**	**70%**	**75%**	**80%**	**85%**	**90%**	**95%**	**100%**
0%	351	390	430	472	516	561	608	657	708	761
5%	319	357	397	439	481	526	572	621	671	723
10%	287	325	364	405	447	491	537	584	634	685
15%	255	292	331	371	413	456	501	548	596	647
20%	223	260	298	337	378	421	465	511	559	609
25%	191	227	265	304	344	386	429	475	522	571
30%	160	195	232	270	309	351	393	438	485	533
35%	128	163	199	236	275	316	358	402	447	495
40%	96	130	166	202	241	281	322	365	410	457
45%	64	97	132	169	206	245	286	329	373	419
50%	32	65	99	135	172	210	250	292	335	381
55%	0	33	66	101	138	175	215	256	298	343
60%		0	33	67	103	140	179	219	261	305
65%			0	34	69	105	143	183	224	266
70%				0	34	70	107	146	186	228
75%					0	35	72	110	149	190
80%						0	36	73	112	152
85%							0	37	75	114
90%								0	37	76
95%									0	38
100%										0

Grams of Ammonium Sulfate add to 1000 ml of solution

(Source: Protein Purification: Principles and Practice. 1982. R.K. Scopes. 282 pages)

Dialysis is the movement of molecules by diffusion from high concentration to low concentration through a semi-permeable membrane. Only those molecules that are small enough to fit through the membrane pores are able move through the membrane and reach equilibrium with the entire volume of solution in the system. Once equilibrium is reached, there is no further net movement of the substance because molecules will be moving through the pores into and out of the dialysis unit at the same rate. By contrast, large molecules that cannot pass through the membrane pores will remain on the same side of the membrane as they were when dialysis was initiated.

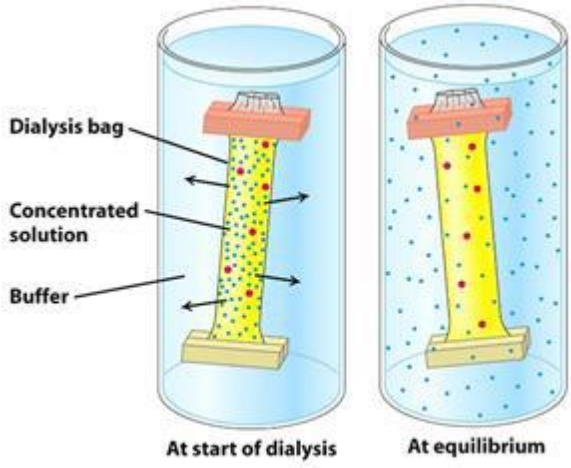


Fig 4.1: Demo picture showing the process of Dialysis (Image taken from https://comis.med.uvm.edu/)

4.4.1 Preparation of Reagents:

25mM Tris-HCL (pH 8) buffer

4.4.2 Procedure:

600 ml of the cell- free supernatant was collected in a 1 litre glass beaker. Gently added ammonium sulphate and dissolved continuously with the help of magnetic stirrer. Addition of

ammonium sulphate $(NH_4)_2SO_4$ should be very slow since frothing is undesired. After complete addition of 80% ammonium sulphate $(NH_4)_2SO_4$ it is allowed to stirrer for 30 minutes. Then the beaker was kept overnight at 4ºC.

After ammonium sulphate precipitation, the solution was centrifuged at 10,000 rpm for 10 min at 4ºC and the precipitated ware collected. The precipitated was dissolved in 1 ml of 25 mM Tris-HCl buffer (pH 8.8). Before the Suspended protein loaded on a dialysis membrane, the membranes were cut into appropriate size and boiled with the 25mM Tris-HCl (pH 8.8) for 10 minutes. The membrane boiling is necessary to wash the cellulose present on the membrane that block the pore. After boiling the membranes were washed with distilled water. The bottom of the dialysis bag was clipped with the membrane clip to seal well. Add the dissolved protein and closed it with the clip. The dialysis bag is suspended in 25mM Tris-HCl buffer (pH 8.8). Put a magnetic stirrer and allow rotating overnight on a low rpm. Next day, the buffer was changed so as to allow proper dialysis and put it on 4ºC for the rest of the day. The diluted protease was collected on a 15 ml Falcon Tube and the used buffer was discarded.

4.5 Ion exchange chromatography:

Ion exchange chromatography is one of the most efficient methods for the separation of charged particles. Ion exchange chromatography is most often performed in the form of column chromatography. However, there are also thin-layer chromatographic methods that work basically based on the principle of ion exchange. Column materials used for ion exchange chromatography contain charged groups covalently linked to the surface of an insoluble matrix. When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite charge. In this “ion cloud”, ions can be reversibly exchanged without changing the nature and the properties of the matrix. The charged groups

of the matrix can be positively or negatively charged. A positively charged matrix will bind negatively charged ions from the solution. Therefore, it is called an anion exchanger. Cation exchanger matrices have negative charges. Based on the structure of the ion exchange matrix, we distinguish ion exchangers with hydrophobic and hydrophilic matrices. Ion exchangers with a hydrophobic matrix are most often highly substituted polystyrene resins. These are suitable for the binding of inorganic ions, e.g. in water softening applications.

The first stage is equilibration in which the ion exchanger is brought to a starting state, in terms of pH and ionic strength, which allows the binding of the desired solute molecules. The exchanger groups are associated at this time with exchangeable counter-ions (usually simple anions or cations, such as chloride or sodium).

The second stage is sample application and adsorption, in which solute molecules carrying the appropriate charge displace counter-ions and bind reversibly to the gel. Unbound substances can be washed out from the exchanger bed using starting buffer.

In the third stage, substances are removed from the column by changing to elution conditions unfavourable for ionic bonding of the solute molecules. This normally involves increasing the ionic strength of the eluting buffer or changing its pH. desorption is achieved by the introduction of an increasing salt concentration gradient and solute molecules are released from the column in the order of their strengths of binding, the weakest bound substances being eluted first.

The fourth and fifth stages are the removal from the column of substances not eluted under the previous experimental conditions and re-equilibration at the starting conditions for the next purification.

Separation is obtained since different substances have different degrees of interaction with the ion exchanger due to differences in their charges, charge densities and distribution of charge on their surfaces. These interactions can be controlled by varying conditions such as ionic strength and pH. The differences in charge properties of biological compounds are often considerable, and since ion exchange chromatography is capable of separating species with very minor differences in properties, e.g. two proteins differing by only one charged amino acid, it is a very powerful separation technique.

In ion exchange chromatography one can choose whether to bind the substances of interest and allow the contaminants to pass through the column, or to bind the contaminants and allow the substance of interest to pass through. Generally, the first method is more useful since it allows a greater degree of fractionation and concentrates the substances of interest. Ion exchange separations may be carried out in a column, by a batch procedure or by expanded bed adsorption.

4.5.1 Preparation of Reagents:

25mM Tris-HCl (pH-8.8) buffer

0.1N, 0.2N, 0.3N, 0.5N NaCl solution

4.5.2 Procedure:

Pre-swollen DE-52 (DEAE cellulose 52) was kept in 25mM Tris-HCl (pH-8.8) buffer. Then the beads were packed in a 20 x 2.5 cm column and thereafter washed vigorously with 25mM Tris-HCl (pH-8.8) buffer to equilibrate the column. Then, the column was connected with the BioLogic Duo Flowcytometry system (Bio-Rad, USA) which gives the absorbance at 280 nm as a peak when the protein elution occurs. Next, the protein sample was loaded onto the column. When the sample is completely adsorbed in the cellulose beads, the flow through fraction was collected by

using 25mM Tris-HCl (pH-8.8) buffer. The bound proteins were eluted from the resin by using increasing salt concentration of 0.1N, 0.2N, 0.3N, 0.5N NaCl. Each fraction was collected and azocasein assay was done to measure the presence of protease.

4.6 Gel filtration chromatography:

Gel filtration chromatography or size-exclusion chromatography separate molecules based on the size of the molecules. The columns made of porous beads with specific diameters that allow smaller size molecules to travel more paths to elute than larger size molecules when they pass through beads. Most commonly used gel filtration chromatographic beads are Sephadex, Superdex, these are based on agarose linked dextran. They are stable even after autoclave. Gel filtration chromatography allows the separated molecules to remain at their native state without losing their functional properties. Because of this properties gel filtration chromatography widely used for separation of proteins, peptides and other biological molecules.

4.6.1 Preparation of Reagents:

25 mM Tris-HCl (pH-8.8) buffer

4.6.2 Procedure:

The DE-52 eluted protease positive fraction was pooled, dialysed and concentrated. The DE-52 eluted protein was then further purified by Sephadex G-75 gel filtration chromatographic column. The Sephadex G-75 beads were swollen in 25 mM Tris-HCl (pH 8) and packed in 1.5×30 cm column equilibrated with the same buffer. DE-52 eluted protein was loaded onto the column and the fraction was eluted by adding 25 mM Tris-HCl (pH 8). Different fractions were pooled and screened for protease activity by azocasein assay.

4.7 Identification of protease by mass spectrometry:

Mass spectrometry is a technique which is used in proteomics to generate high quality data to precisely know the proteins sequence and its modifications. The following steps were followed in Mass spectrometry for proteins identification. Proteins are partially digested with trypsin and then peptide generated from the digestion analysed in the mass spec according to their m/z values, which is called peptide-MS analysis. As trypsin digest peptide bond at C-terminus of Lys and Arg, digestion pattern of proteins will be unique for each protein. As a result, each protein will generate a different peptide mass fingerprint (PMF) from peptide-MS analysis. PMF data search in the database is to identify the best match protein. Amino acid sequence details of the peptide fragment were further analysed by MS/MS. In this way MS and MS/MS data were combined to get the final protein sequence.

4.7.1 Preparation of Reagents:

Digestion buffer [50 mM Ammonium bicarbonate (NH_4HCO_3)]

Destaining buffer [25 mM NH_4HCO_3, 50% Ethanol]

Reduction buffer [10 mM Dithiothreitol (DTT), 50 mM NH_4HCO_3]

Alkylation buffer [55 mM Iodoacetamide, 50 mM NH_4HCO_3]

Trypsin solution [used at a concentration of 12.5 ng/µl]

Protein extraction solution [3% Tri fluoro acetic acid (TFA), 30% Acetonitrile]

4.7.2 Procedure:

Protease active fractions from G-75 column were eluted, concentrated, and analysed by NATIVE and SDS-PAGE. The major bands from SDS and NATIVE-PAGE were excised from Coomassie stained gel and destained with destaining buffer until all the stain removed. After that the gels were dehydrated with 100% acetonitrile at room temperature for 10-15 min. Then the cut gels were rehydrated with reduction buffer at 56ºC for 1 hr. In the next step alkylation of the gel was done by adding alkylation buffer to the gel in dark for 1 hr. After

that gels were washed in digestion buffer and dehydrated in 100% acetonitrile repeatedly. After repeated washing and dehydration, gels were dried completely in vacuum. Then the dried gels were incubated with trypsin solution at 37ºC for overnight. The digested peptide from the gel was extracted by incubating gel with extracted buffer at a ratio of 1:1 for 15 min. The gel was then again dehydrated twice with acetonitrile for 10 min to remove any traces of peptides if present. The extracted peptide now dried under vacuum to remove acetonitrile. Finally the peptide sample dissolved in 0.1% TFA and 50% acetonitrile and analysed in MALDI-ToF instrument at National Centre for Biological Science in Bangalore, India. The peptide-MS and MS/MS data generated from the analysis combined and used for peptide sequence generation. Peptide sequence was search in NCBI and UniProt database to find the homology and protein identification. The fragmented peptide sequence was rearranged in-silico with the homolog full length protein sequence and the sequence coverage was quantified.

4.8 Inhibition of protease activity:

To study the inhibitory effect of purified protease, inhibitor like PMSF (10 mM) and EDTA (10 mM) were used. 100 mM stock of PMSF and 500 mM stock of EDTA were prepared in sterile water. Protease inhibition was done using 5 µg of purified protease incubated with each of the inhibitor at 37ºC for 1 hr and after incubation with inhibitor, azocasein assay was used to measure the protease activity as described earlier.

4.9 Determination of optimum pH for protease activity:

Activity of enzymes can vary at different pH and every enzyme has optimum pH at which it has highest activity. In our study to measure the optimum pH of the protease for its activity, purified protease was dialysed against a buffer with a pH range of 2 to 12 and the activity of the protease was determined using azocasein assay. For pH 2-5, we have used 25 mM acetate

buffer, similarly for pH 6-7, we have used 25 mM PBS buffer and for pH 8 12 we have used 25 mM Tris-HCl buffer.

4.10 Determination of optimum temperature for protease activity:

Similar to pH, every enzyme has an optimum temperature, where it exhibits its greatest activity. Different enzyme can be applied to different functions based on its optimum temperature. In this study subtilisin was purified as described earlier and to find out the optimum temperature for its protease activity, 5 µg of subtilisin was incubated at different temperatures: 25 ◦C, 40 ◦C, 50 ◦C, 60 ◦C, 70 ◦C, 80 ◦C and 100 ◦C for 60 min and after incubation protease activity was performed by azocasein assay as described previously.

4.11 Genomic DNA isolation:

Genomic DNA from DHS-96 strain was isolated using Qiagen genomic DNA isolation kit. 1ml overnight grown bacterial culture was taken in a 1.5 ml microcentrifuge tube. Culture was spin down at 14,000 × g for 2 minutes. The bacterial cells were then resuspended in 480 µl of 50 mM EDTA. 120 µl of 10 mg/ml lysozyme was added to the culture suspension and mix gently by pipetting to hydrolyse the polysaccharide present in the bacterial outer layer. The mixture was then incubated at 37 ºC for 60 minutes and inverting up-down 10 min interval for bacterial membrane disruption. Following incubation, the mixture was centrifuge at 14,000 × g for 2 min and the pellet was collected. 600 µl of nuclei lysis solution was added to the supernatant and mix gently by pipetting up and down. The solution was then incubated at 80 ºC for 5 min to complete the lysis. 3 µl of RNase solution was added to the mixture and incubated at 37 ºC for 30 min. After incubation 200 µl of protein precipitation solution was added to the cell lysate and vortex vigorously at high speed for 20 sec to mix the protein precipitation solution to the cell lysate. The mixture incubated for 5 min at room temperature and following incubation centrifuged at 14,000 × g for 10 min. Now, carefully transfer the supernatant to a clean 1.5 ml microcentrifuge tube. 600 µl of isopropanol added to the

supernatant and mix by inverting until a thread like structure appear. Now, the mixture was centrifuged at 14,000 × g for 5 min. Supernatant was carefully pour off and drain the tube to a clean absorbent paper. 70% ethanol was added to the tube and gently inverted the tube for several times to wash DNA pellet. After washing, the tube was centrifuged at 14,000 × g for 2 min and ethanol was carefully aspirated. The tube was again drain to the clean absorbent paper and air dry for 15 min. 100 µl of DNA rehydration solution was added and incubated 65 ºC for 1 hr and the DNA solution was stored at -20 ºC until use.

4.12 Quantification of DNA and analysis of purity:

DNA purification can be analysed by different methods, such as spectroscopy method, agarose gel electrophoresis method and fluorescence method. The most common method used in the laboratory is the spectroscopy method. In spectroscopy method, the DNA analysis was done by taking the absorbance of the DNA by UV spectroscopy. DNA absorbance was taken at 260 nm of wavelength and as the main contaminant during DNA purification is protein, the ratio of DNA absorbance (at 260 nm) and protein absorbance (280 nm) was taken to check the purity of the DNA solution. In fluorescence method, DNA was quantified by using DNA binding fluorescence dye and the fluorometric absorbance was taken from emission-excitation value. In agarose gel method, DNA gives a specific band on agarose gel and if any contaminant like RNA is present it can be identified by visualizing the band, since RNA will give different size band. DNA concentration in agarose gel was estimated by measuring DNA intensity and compares it with the DNA intensity of known concentration of DNA.

In our experiment we have quantify the DNA by UV-spectroscopy. 5 µl of DNA was diluted in 595 µl of nuclease free water and the OD at 260 nm was measured. From the OD value DNA concentration was measured from the following equation:

DNA concentration (µg/µl) = **OD at 260 x 120** (dilution factor) **x 50 / 1000** (1OD of ds DNA = 50 µg/ml)

The purity of the DNA was measured by taking OD_{260}/OD_{280} absorbance ratio. OD_{260}/OD_{280} of the pure DNA is close to 1.8.

4.13 Oligonucleotides:

Following oligonucleotides listed in the table were used in this study.

Table 4.2: List of primers used in this study for PCR

Gene	Primer sequence (5´-3´)	Annealing (°C)	Product size (bp)
16s-rRNA	GGATGAGCCCGCGGCCTA CGGTGTGTACAAGGCCCGGGAACG	50	~1500
Subtilisin (B. subtilis)	AGCGGGATCCATGAGAAGCAAAAAATTGT GGATC AATACTCGAGCGTTGTGCAGCTGCTTGTACGTT	52	~1100
Subtilisin (B. amylol-quefaciens)	AGCGGGATCCATGAGAGGCAAAAGTATGATCA GT AATACTCGAGCGCTGAGCTGCCGCCTGTACGT	52	~1100
Tubulin (Human)	CGGGCAGTGTTTGTAGACTTGG CTCCTTGCCAATGGTGTAGTGC	60	~150
Actin (Human)	GGACTTCGAGCAAGAGATGG AGCACTGTGTTGGCGTACAG	60	~150

4.14 Polymerase chain reaction:

The polymerase chain reaction (PCR) is a laboratory technique for DNA replication that allows a "target" DNA sequence to be selectively amplified. PCR can use the smallest sample of the DNA to be cloned and amplify it to millions of copies in just a few hours. Discovered in 1985 by Kary Mullis, PCR has become both and essential and routine tool in most biological laboratories. As the name implies, it is a chain reaction, a small fragment of the DNA section of interest needs to be identified which serves as the template for producing the

primers that initiate the reaction. One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building block, i.e., the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates. It is then possible to clone DNA whose sequence is unknown. This is one of the method's major advantages. Genes are commonly flanked by similar stretches of nucleic acid. Once identified, these patterns can be used to clone unknown genes - a method that has supplanted the technique of molecular cloning in which DNA fragments are tediously copied in bacteria or other host organisms. With the PCR method this goal can be achieved faster, more easily and above all in vitro, i.e., in the test-tube. Moreover, known sections of long DNA molecules, e.g. of chromosomes, can be used in PCR to scout further into unknown areas. PCR components such as dNTPs, DNA polymerase, Mg^{2+}, primers, ATPs, appropriate buffers, template DNA were mixed together and are taken through series of 3 major cyclic reactions conducted in an automated, self-contained thermocycler machine. Denaturation, annealing and elongation are the three-cycle involved in a PCR reaction.

Denaturation: This step involves heating the reaction mixture to 94°C for 15-30 seconds. During this, the double stranded DNA is denatured to single strands due to breakage in weak hydrogen bonds.

Annealing: The reaction temperature is rapidly lowered to 54-60°C for 20-40 seconds. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

Elongation: Also known at extension, this step usually occurs at 72-80°C (most commonly 72°C). In this step, the polymerase enzyme sequentially adds bases to the 3′ each primer, extending the DNA sequence in the 5′ to 3′ direction. Under optimal conditions, DNA polymerase will add about 1,000 bp/minute.

4.14.1 PCR for 16s-rRNA sequencing:

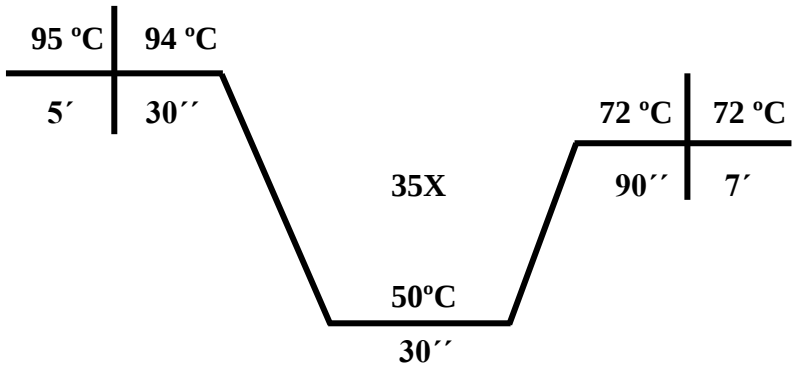
Identification of the bacterial isolate DHS-96 was done by 16s-rDNA sequencing. First, genomic DNA was purified from overnight grown bacterial culture and 16s-rDNA was amplified from template gDNA by using 16s-rRNA gene specific forward and reverse primer. Amplified product was then extracted from agarose gel and purification was done. Purified PCR product were then sequenced bi-directionally.

4.14.1.1 Preparation of reagents:

DNA-	1 µl (50/100 ng)
16s Forward Primer-	10 µM
16s Reverse Primer-	10 µM
dNTPs (2.5mM each)-	4 µl
10X Taq DNA polymerase Assay Buffer-	10 µl
Taq DNA polymerase enzyme (3U/µl)-	1 µl
Nuclease free water-	adjusted to 100 µl
Total Reaction Volume-	100 µl

4.14.1.2 PCR amplification conditions:

Following PCR cycle conditions were followed:



4.14.2 PCR for subtilisin gene amplification:

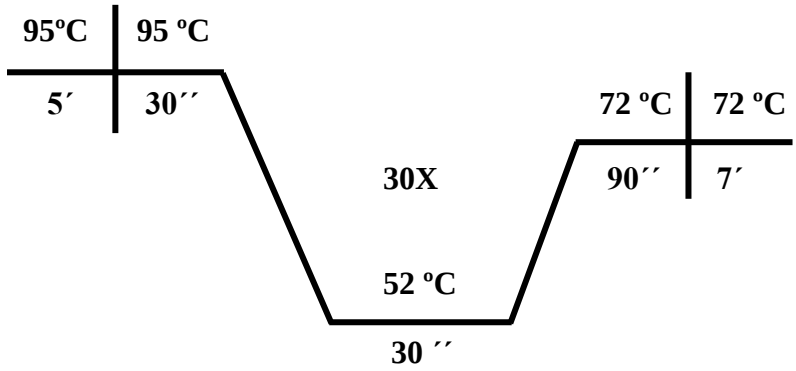
To confirm the species of the bacterial isolate DHS-96, subtilisin gene amplificaion was done. Genomic DNA from the overnight grown bacterial culture was taken as template of the PCR reaction and species specific primers were used to amplify subtilisin gene. The primers were came as a lyophilized powder from the IDT and was dissolved in nuclease free water to make a stock of 100 µM.

4.14.2.1 Preparation of reagents:

DNA-	1 µl (50/100 ng)
subtilisin Forward Primer-	10 µM
subtilisin Reverse Primer-	10 µM
2X DreamTaq Green Master Mix-	12.5 µl
Nuclease free water-	Adjusted to 25 µl
Total reaction volume-	25 µl

4.14.2.2 PCR amplification conditions:

The PCR was done using following condition:



4.15 Agarose gel electrophoresis:

Agarose gel electrophoresis has been widely used to separate DNA. Both polyacrylamide gel and agarose gel can be used to separate DNA, but agarose gel is used to separate large range of DNA (50bp to several megabase). Separation of DNA by using electrophoresis technique was first used by virologist Vin Thorne [209,210]. Agarose made from red seaweed extraction of two species *Gelidium* and *Gracilaria*. Agarose gel is formed due to hydrogen bond formation and hydrophobic interaction between agarose molecules and for this reason when DNA molecule moves through this gel, no interaction or alteration observed in DNA, thus making it suitable medium for electrophoretic separation. The resolution of the separation of DNA in agarose gel depends on the pore size of the gel and the pore size is proportional to the concentration of agarose used in the formation of gel. In general, 0.5-2% agarose is used for DNA analysis, depending on its size. Agarose gel electrophoresis runs in different types of electrophoresis buffer. Two most common buffer used in agarose gel electrophoresis is Tris acetate EDTA (TAE) and Tris borate EDTA (TBE). To visualize the resolved DNA in the gel, intercalating dye Ethidium Bromide (EtBr) widely been used [211].

4.15.1 Preparation of reagents:

50 X TAE buffer [2M Tris 1M Glacial acetic acid, 0.5 M EDTA]

10X Sample loading buffer [0.21% Bromophenol blue, 0.21% Xylene Cyanol, 50% Glycerol, 0.2M EDTA]

0.8% agarose gel [mix 0.8 gm of agarose in 100 ml 1X TAE buffer and micro wave for 3-5 minutes]

4.15.2 Procedure:

Depending on the PCR product size 0.8- 1.2 % of agarose gel was prepared by weighing appropriate agarose and mixed in 1X TAE buffer. Then the mixture boiled in microwave for 3-5 minutes until dissolved completely. 0.5 µg/ml final concentration of EtBr mixed with the agarose solution and subsequently poured in the gel casting tray for solidify. 10-20 µl of PCR reaction mixed with the 1X loading dye before loading into the gel well with appropriate DNA ladder and run at 60-100 Volte. Gels were observed under UV light or on Bio-Rad Gel Doc machine when the loading dye front reached 60% of the gel.

4.16 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE is an electrophoresis technique used to separate protein molecule in the laboratory. Prior to electrophoresis protein molecules denature under reducing condition and negative charge to the protein applied by using SDS. In the electric field, all negatively charged protein molecules migrate from negative to positive and small size molecule migrate faster than larger molecule due to less resistance in the electric field. In this way proteins separated on the basis of molecular size in SDS-PAGE.

4.16.1 Preparation of reagents:

Solution A (30% Acrylamide stock solution) [29.2 g Acrylamide, 0.8 g N, N'-ethylene bis acrylamide]

Solution B [1.5 M Tris-HCl buffer (pH 8.8), 0.4% SDS]

Solution C [0.5 M Tris-HCl buffer (pH 6.8), 0.4% SDS]

10% Ammonium persulphate solution (APS) [1g of APS in 10 ml dH_2O]

N,N,N′,N′-Tetramethylethylenediamine (TEMED)

4.16.2 Preparation of resolving and stacking gel:

We have use 1.0 mm spacer of Bio-RAD Mini-PROTEAN gel-casting system. To fill the 1.0 mm gel cassette 5.6 ml of resolving gel and 3 ml of stacking gel is required. We used different percentages of resolving gel, such as 10%, 12.5%, and 7.5%, depending on the

Table 4.3: Stacking and resolving gel recipe

	Resolving gel				Stacking gel
Reagents	15%	12.5%	10%	7.5%	4.5%
Solution A	4.5 ml	3.75 ml	3 ml	2.25 ml	1.35 ml
Solution B	2.25 ml	2.25 ml	2.25 ml	2.25 ml	
Solution C	-	-	-	-	0.45 ml
10% APS	0.063 ml	0.063 ml	0.063 ml	0.063 ml	0.01 ml
TEMED	0.01 ml	0.01 ml	0.01 ml	0.01 ml	0.01 ml
Water	2.25 ml	3 ml	3.75 ml	4.5 ml	1.2 ml
Total Volume	9 ml	9 ml	9 ml	9 ml	3 ml

experimental conditions, while 4.5% resolving gel was used for all experiments. Resolving and stacking gel was prepared using different reagents with the concentration mention in the following table.

4.16.3 Electrophoresis buffer:

Electrophoresis buffer or SDS-PAGE running buffer was prepared by dissolving 14.4 gm of glycine, 3 gm of Tris and 1 gm SDS in 1 litre dH_2O.

4.16.4 Preparation of sample loading buffer:

5X sample loading buffer stock was prepared by mixing following reagents: 150 mM Tris-HCl (pH 6.8), 5% SDS, 12.5% beta-marcaptoethanol 50% glycerol and 0.1% bromophenol blue. This sample buffer is also named as Laemmli buffer.

4.16.5 Sample preparation for SDS-PAGE:

Protein samples which were needed to be separated by SDS-PAGE, mixed with 1X sample loading buffer and boiled for 10 min in water bath. After boiling samples were keep at room temperature to cold. Afterwards, samples were short spin and loaded onto gels according to their concentrations.

4.16.6 Electrophoresis:

SDS-PAGE was done on the Bio-Rad vertical electrophoresis system. In SDS-PAGE samples are stacked first in the stacking buffer and then resolved in the resolving buffer. For stacking, the gel was run at 70 volts, while for resolving, 110 volts was used at room temperature.

4.16.7 Staining and destaining of the gel:

When dye front of the sample completely run out from the gel, gel was removed from the electrophoresis system and washed in dH_2O. The gel was stained in 0.1% coomassie brilliant blue staining solution. (Staining solution preparation: 0.1% coomassie brilliant blue was prepared by dissolved 0.1 gm of coomassie powder in 45 ml methanol and 10 ml glacial acetic acid. Stir continuously until dissolved, then filter with whatman filter paper and water is added to adjust the volume to 100 ml). The gel was stained for 30 min with shaking at room temperature. Afterwards the stained gel was destained with destaining solution at room temperature until the gel destain completely and protein band can be visualised. (Destaining solution preparation: 100 ml methanol, 100 ml glacial acetic acid mix with 800 ml dH_2O)

4.17 NATIVE-PAGE:

Proteins can be separated on their native state by using non-reducing polyacrylamide gel electrophoresis, also known as NATIVE-PAGE. A protein sample does not denature in this electrophoresis process, and native proteins are separated according to their structure and charge. To keep the proteins in their native state, in this electrophoresis process performed without detergent (SDS) and reducing agent (beta marcaptoethanol). Apart from the following exception, all reagent preparations for NATIVE-PAGE are identical to those for SDS-PAGE. NATIVE-PAGE electrophoresis buffer devoid of SDS and the sample buffer devoid of SDS and beta marcaptoethanol.

4.18 Cell culture and treatment:

Human breast cancer cell line MCF-7 was cultured and maintained in MEM, human colon cancer cell line HT-29 and mouse peritoneal macrophages cells were cultured in DMEM supplemented with 10% FBS, 100 µg/ml Streptomycin sulphate and 100 U/ml penicillin-G at

37°C in presence of 5% CO_2. MCF-10A, a non-tumorigenic breast epithelial cell line, was grown in MEBM along with MEGM-bullet kit (containing BPE, hEGF, Insulin, hydrocortisone) and 100 ng/ml cholera toxin. Mouse peritoneal macrophages were isolated from BALB/c mice using Phosphate Buffer Saline (PBS) and cultured in DMEM, with 10% FBS, 100 µg/ml Streptomycin and 100 U/ml penicillin-G. MCF 10A cell was purchased from American Type Culture Collection (ATCC). MCF-7 and HT-29 cells were provided by Chittaranjan National Cancer Institute (CNCI), Kolkata. Growth of the cells were observed in Olympus phase contrast microscope.

For treatment, six-well tissue culture plates were seeded with 1 X 10^6 numbers of HT-29 and MCF-7 cells. After 70% confluency, cells were washed with 1X PBS twice and starved for 6 hrs in serum-free medium. HT-29 cells were treated with filter sterilized bacterial culture supernatant and MCF-7 cells were treated with different doses of subtilisin (0, 3, 5 µg/ml) for 24 hrs for flow cytometric analysis. MCF-7 cells were treated with 5 µg/ml of subtilisin for immunoblot, immunoprecipitation, immunofluorescence, Real-Time PCR and proteasome activity measurement. For treatment with proteasome inhibitor MG-132, MCF-cells were pre-incubated with MG-132 for 1 hr prior to treatment with subtilisin.

4.19 WESTERN BLOT:

Western blotting uses specific antibodies to identify proteins that have been separated based on size by gel electrophoresis. The immunoassay uses a membrane made of nitrocellulose or PVDF (polyvinylidene fluoride). The gel is placed next to the membrane and application of an electrical current induces the proteins to migrate from the gel to the membrane. The membrane can then be further processed with antibodies specific for the target of interest, and visualized using secondary antibodies and detection reagents.

4.19.1 Preparation of reagents:

4.19.1.1 Buffer preparation:

Transfer buffer [25 mM Tris, 192 mM glycine, 20% methanol]

Tris buffer saline (TBS) [20 mM Tris, 500 mM NaCl]

TBS-Tween 20 (TBST) [TBS + 0.1% Tween 20]

5% skim milk (5 gm of skim milk dissolved in 100 ml TBS)

4.19.1.2 Protein lysate preparation from cell culture

Place the cell culture dish on ice and wash the cells with ice-cold PBS. Aspirate the PBS, then add ice-cold lysis buffer (1 mL per 10^7 cells/100 mm dish/150 cm^2 flask; 0.5 mL per $5x10^6$ cells/60 mm dish/75 cm^2 flasks). Scrape adherent cells of the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled microcentrifuge tube. Alternatively cells can be trypsinized and washed with PBS prior to resuspension in lysis buffer in a microcentrifuge tube. Maintain constant agitation for 30 min at 4°C. Following incubation centrifuge the microcentrifuge tube at 4°C. The centrifugation speed and time may vary depending on the cell type. In our experiment we centrifuge for 10 min at 12,000 rpm. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice, and discard the pellet.

4.19.1.3 Quantification of protein:

Protein concentration should be determined before loading onto the gel for western blot as loading equal amount of protein for each cell lysate is important to compare the protein expression level. To determine protein concentration, Bradford assay and Bicinchoninic assay (BCA) was performed depending on the nature of the sample. Different sample components used in different experimental setup will not be compatible with any one quantification assay.

It should therefore be decided whether to use BSA or Bradford depending on the experimental setup.

4.19.1.3.1 Bradford assay:

Bradford assay is one of the most common protein quantification assay used in the laboratory. The Bradford assay is a colorimetric assay and it uses Coomassie brilliant blue G-250 as a protein binding dye. Upon binding with protein, it gives colour which can be absorbed at 595 nm. The concentration of unknown protein was measured from the standard curve made by known protein concentration. The standard curve for Bradford assay was prepared by plotting OD_{595} value in the Y-axis against protein concentration in the X-axis. Bradford reagent is not compatible with ionic detergent and for that adding SDS turn Bradford blue. For this reason Bradford assay is not suitable when RIPA buffer is used for cell lysis.

Procedure:

Different concentration of BSA prepared from diluting 10 mg/ml stock BSA in the dH_2O in the following manner. 50 µl of different concentration of BSA were transferred to a 96 well plate and 150 µl of Bradford solution was added to each standard. The plate was incubated for 5 min at room temperature and the absorbance was measured at 595 nm. Standard curve was prepared by plotting OD value at Y axis and the BSA concentration on X- axis.

Table 4.4: BSA dilution method for standard curve preparation

Reagent	Dilution 1 (0.0 mg/ml)	Dilution 2 (0.062 mg/ml)	Dilution 3 (0.125 mg/ml)	Dilution 4 (0.25 mg/ml)	Dilution 6 (0.5 mg/ml)	Dilution 7 (1.0 mg/ml)
H_2O	100 µl	99.38 µl	98.75 µl	97.5 µl	95 µl	90 µl
BSA	0 µl	0.62 µl	1.25 µl	2.5 µl	5 µl	10 µl

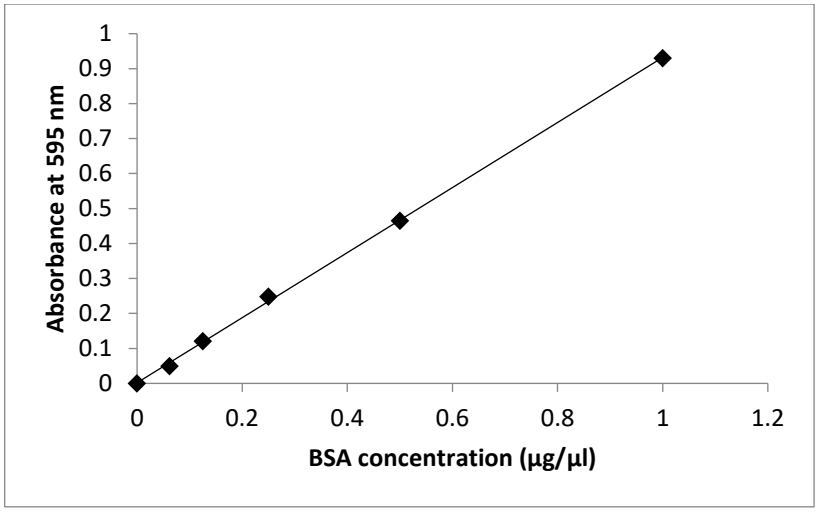


Fig 4.2: Bradford standard curve

4.19.1.3.2 BCA assay:

Bicinchoninic acid (BCA) protein assay is another highly sensitive colorimetric method for detection of proteins or peptides in the solution. The assay is based on biuret reaction, where in an alkaline solution in presence of two or more peptides, blue Cu^{2+} ions convert into purple Cu^{+} ion. As a result, reaction solution produces purple colour in presence of proteins. Protein concentration is correlated with the intensity of the colour and more intensity of the colour means more peptide bonds are present. In the BCA assay similar principle has been used. In an alkaline environment in presence of protein, cupric cation of copper reduced to cuprous cation. In the next step, Cu^{+} ions chelate with two molecules of BCA. This BCA-copper complex produces an intense purple colour which can be detected at 562 nm. Similar to Bradford assay, BCA assay requires known protein standard concentrations to quantify unknown protein concentrations. BCA assay is not suitable in presence of some chemical like tris, EDTA, EGTA etc, so avoid using this assay if the protein sample is containing these chemicals. In our experiment BCA assay was performed by using 'Pierce BCA assay kit'

Procedure:

Different concentrations of BSA were prepared by diluting 10 mg/ml stock BSA in the dH_2O in the following manner. The assay kit contains BCA Reagent A and B, which were

Table 4.5: BSA dilution method for standard curve preparation

Reagent	Dilution							
	1 (0.0 mg/ml)	**2 (0.025 mg/ml)**	**3 (0.250 mg/ml)**	**4 (0.500 mg/ml)**	**6 (0.750 mg/ml)**	**7 (1.0 mg/ml)**	**8 (1.5 mg/ml)**	**9 (2.0 mg/ml)**
H_2O	100 µl	99.75 µl	97.5 µl	95 µl	92.5 µl	90 µl	85 µl	80 µl
BSA	0 µl	0.25 µl	2.5 µl	5.0 µl	7.5 µl	10 µl	15 µl	20 µl

mixed in a 50:1 ratio and mixed by vortexing. To prepare the standard curve, 390 µl of BCA reagent was mixed with 10 µl of protein solution and the mixture was kept at 37 °C for 30 min. Following incubation, the absorbance of the solution was measured at 562 nm. Standard curve was prepared by plotting OD value at Y axis and the BSA concentration on X- axis.

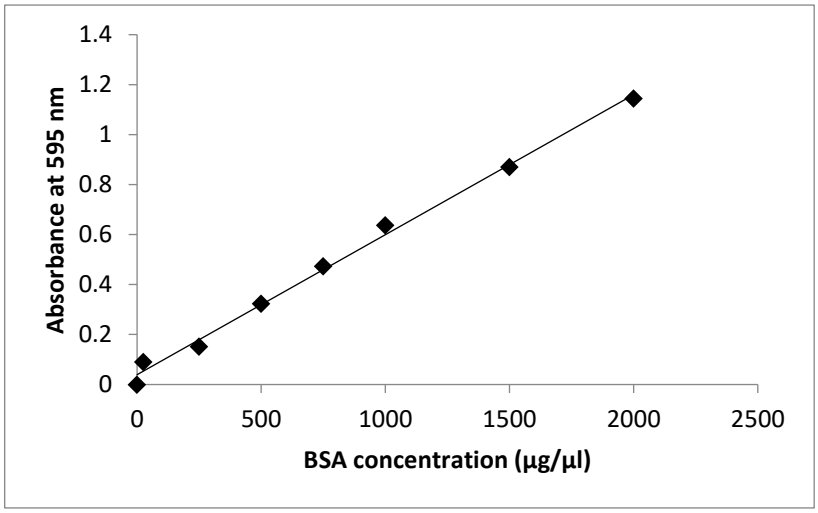


Fig 4.3: BCA standard curve

4.19.1.2 Protein sample preparation:

For sample preparation equal concentration of protein samples were taken and mixed with 1X Laemmli buffer. The mixture was boiled at 100°C for 5 min. Lysates can be aliquoted and stored at -20°C for future use. Samples were kept at room temperature to cold. Afterwards, samples were short spin and loaded onto gels according to their concentrations.

4.19.2 Procedure:

Equal quantity of proteins was loaded into the gel and run at 100 V until dye front is completely run out from the gel. After electrophoresis, the proteins are transferred from the gel to the membrane. The membrane can be either nitrocellulose or PVDF. If PVDF is used, the membrane should be activated with methanol for 1 min and should be rinsed with transfer buffer before preparing the stack. The time and voltage of transfer may require some optimization. We did the transfer for 2 hrs at constant voltage of 100 V. 'Ponceau S' staining can be used before blocking, to check the transfer of proteins in the membrane. Membrane was then blocked with blocking buffer (5% milk or BSA) for 1 h at room temperature or overnight at 4°C in gentle shaking. Membrane was then washed with TBS and incubated with appropriate dilutions of primary antibody in blocking buffer in gentle shaking at 4 °C for overnight. Following incubation, the membrane was washed thrice with TBST for 7 min each. Thereafter, the membrane was incubated with the recommended dilution of HRP-conjugated secondary antibody in blocking buffer at room temperature for 1 h. Wash the membrane thrice with TBST for 5 min each. Finally, the membrane was developed with HRP substrate. The image was acquired in chemi-doc.

4.20 Immunoprecipitation:

Immunoprecipitation is a technique to isolate a protein from a mixture of proteins using specific antibody against a specific protein which needs to be purified. In our study to

observe the interacting partner of tubulin and to study the ubiquitination of tubulin, immunoprecipitation technique was used. Immunoprecipitation was done as described earlier [212].

4.20.1 Preparation reagent:

NP-40 lysis buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1 mM sodium fluoride, 0.5% Nonidet P-40 (NP-40), 50 mM β-glycerophosphate, 10% glycerol]

5X sample loading buffer [150 mM Tris-HCl (pH 6.8), 5% SDS, 12.5% beta-marcaptoethanol 50% glycerol and 0.1% bromophenol blue.]

4.20.2 Procedure:

Subtilisin (5 µg/ml) and/or MG-132 (10 µM) treated or untreated cells were lysed by NP-40 lysis buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1 mM sodium fluoride, 0.5% Nonidet P-40 (NP-40), 50 mM β-glycerophosphate, 10% glycerol] on ice. The proteins in the lysates were estimated and 500 µg of proteins from each lysate was incubated overnight with α and β-tubulin specific antibody (1:50) at 4 °C in an end-to-end rotor with mild rocking. Tubulin antibody incubated lysate was incubated with Protein A/G agarose beads for 4 hrs with gentle rocking followed by centrifugation at 3,824 RCF at 4 °C for 3 min. A/G beads with immune-complexes were washed with NP-40 lysis buffer to remove any non-specific bindings. Beads were re-suspended in 2X-SDS-PAGE loading buffer and boiled for 10 minutes before loading to SDS-PAGE. Immunoblotting was done using anti-ubiquitin (1:500) antibody to study tubulin ubiquitination and anti-PARKIN (1:500) antibody, to observe tubulin-PARKIN interaction.

4.21 MTT assay:

MTT assay was performed for the assessment of cytotoxicity of subtilisin against cancer cells. The full name of MTT is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. It is a yellow-coloured tetrazolium salt and in presence of metabolically active cells this yellow-coloured salt transformed into a purple colour insoluble formazan crystal by mitochondrial NAD(P)H-dependent oxidoreductase enzymes. This crystal can be solubilized in organic solvent like DMSO and absorbed at 500-600 nm range. Higher the absorbance greater the viable and metabolically active cells.

4.21.1 Procedure:

$1X10^4$ number of cells were seeded into 96 well plate and grown overnight. Grown cells were treated with different concentration of subtilisin (0.0 µg/ml – 10 µg/ml) for 6 hrs. Each concentration was taken in triplicate and following treatment cell culture medium was aspirated gently. 0.5 µg/ml final concentration of MTT was added to the each well and keep the culture plate at 37 ºC for 3-4 hrs until formazan crystal visible under microscope. Following incubation MTT solution aspirated carefully from the well and solubilise solution (10 % SDS and 1 % acetic acid in DMSO solution) was added to solubilize the crystal. Pipetting up and down several time and kept for 10 min at room temperature. Measured the OD at 590 nm. Plot the OD in Y-axis against protease concentration in X-axis. OD value from the positive control experiment was considered as 100% viable cells.

4.22 Apoptosis assessment by flow cytometric analysis:

Flow cytometry is a popular cell biology technique that utilizes laser-based technology to count, sort, and profile cells in a heterogeneous fluid mixture. Using a flow cytometer machine, cells or other particles suspended in a liquid stream are passed through a laser light

beam in single file fashion, and interaction with the light is measured by an electronic detection apparatus as light scatter and fluorescence intensity. If a fluorescent label, or fluorochrome, is specifically and stoichiometrically bound to a cellular component, the fluorescence intensity will ideally represent the amount of that particular cell component. Flow cytometry is a powerful tool because it allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second. This makes it a rapid and quantitative method for analysis and purification of cells in suspension. Using flow, we can determine the phenotype and function and even sort live cells.

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Here we have used Annexin-V-PI staining kit from BD to observe apoptosis. Annexin V is a 35–36 kDa Ca2+-dependent phospholipid-binding protein with high affinity for PS and binds to exposed apoptotic cell surface PS. Annexin V can be conjugated to fluorochromes while retaining its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells undergoing apoptosis. Staining with Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) for identification of early and late apoptotic cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Therefore, cells that are considered viable are both Annexin V and PI negative, while cells that are in early apoptosis are Annexin V positive and PI negative, and cells that are in late apoptosis or already dead are both Annexin V and PI positive.

4.22.1 Procedure:

A quantitative assessment of apoptosis was done using an apoptosis detection kit (BD-bioscience, USA), where cells were stained with FITC conjugated annexinV/propidine iodide

(PI) and analysed in flow cytometer. Six-well tissue culture plates (Corning, USA) were seeded with 1×10^6 MCF-7 and HT-29 cells. Cells with 70 % confluency were washed once with 1× PBS and starved for 6 h in serum-free medium. Cells were treated with flter sterilized bacterial culture supernatant and with subtilisin in different doses (0, 3, 5 μg/ml) for 24 h. To observe apoptosis in presence of proteasome inhibitor, MCF-7 cells were pre-incubated with different doses of MG-132 (1, 3, 10, 25 μM). After treatment, cells were pelleted down by 1000 rpm for 10 min and washed with PBS. After washing cells were dissolved in 500 μl 1X binding buffer provided by the kit manufacturer. Annexin V (2 μl) and PI (1 μl) were added to each tube. Following incubation for 5 min at room temperature in dark, samples were analysed in FACS Aria II using 'Cell Quest' software.

4.23 Immunofluorescence:

Immunofluorescence is a form of fluorescent antibody technique utilizing a fluorochrome conjugated to primary antibody or secondary antibody, which is added directly to a tissue or cell suspension for the detection of a specific antigen. Using immunofluorescence, position and localisation of cellular component inside or outside of the cell can be easily visible. It is an important technique widely been used in molecular and cell biology experiment. Immunofluorescence staining of MCF-7 cells was carried out to study tubulin degradation after subtilisin treatment.

4.23.1 Procedure:

Preparation of cover slip:

Before sterilizing, the cover slip is soaked with the mixture of nitric acid and hydrochloric acid (mixed at 2:1 ratio) for 2 hrs. Then washed with clean dH_2O for several times and waited for dry. Then the coverslip was autoclaved for 15 min at 121ºC. Autoclaved coverslip was placed into the well of cell culture plate during plating of the cultured cells.

Immunostaining of the cells:

Desired number of cells were seeded onto sterile coverslip and when the cells reached at 70 % confluence cells were treated with subtilisin in presence of different doses of proteasome inhibitor MG-132 (1 µM, 3 µM, 10 µM and 25 µM). Following treatment cells were fixed with acetone/ethanol (1:1) for 5 min at room temperature. Cells were then rinsed with PBS and were blocked with 5% FBS prepared in PBS for 1 hr at room temperature. Thereafter 150 µl of Anti-tubulin antibody (1:500 in 5% FBS; Santa-cruz Biotechnology) was placed on a parafilm and the cells containing coverslips were placed invertedly onto the drop of antibody. The setup was incubated at 4ºC for overnight. Next day, coverslips were placed again into the well of the tissue culture plate and washed four times with PBS for 5 min each. Afterward, 200 µl of FITC-conjugated secondary antibody (1:1000 in 5% FBS; Santa-cruz Biotechnology) was placed dropwise in a clean parafilm and carefully the coverslip was placed invertedly on the drop of secondary antibody solution. The setup was incubated for 1.5 hrs at room temperature. Following incubation, coverslips were placed into the well of the tissue culture plate and washed three times with PBS for 5 min each.

Coverslip mounting and imaging of the cells:

Microscope slides were washed with 70% ethanol and soaked with tissue paper to dry completely. Small drop of mounting media (with DAPI-ProLong gold antifade reagent) put on the slides and then carefully by forceps immunostaining cells containing coverslips were placed invertedly onto the slides. The mounting media was allowed to harden for a few minutes, and then the coverslips were sealed with white nail polish. Now, samples are being imaged in the ICMR-NICED central instrumentation facility and observed using confocal microscopes (Zeiss-LSM510s). Images were acquired using 405 nm blue channel (for nucleus), 4 nm green channel (for tubulin) and 40X objective lens.

4.24 RNA extraction:

Total RNA from subtilisin treated MCF-7 cells was isolated using TRIzol reagent. It is a mixture solution of guanidine isothiocyanate and phenol. When cells are mixed with trizol reagent, it dissolves cellular component DNA, RNA and proteins. Then by mixing with chloroform different cellular components can be purified from separated aqueous and organics phase.

4.24.1 Procedure:

The experimental cells were grown on 6-well cell culture plate. Following treatment of the cells media from the well was removed and washed with PBS. After washing 300 µl of TRizol (for 10^7 number of cells) was added to the well and cells were collected by scrapping. The suspension was collected in microcentrifuge tube and mixed by pipetting several times. Following 5 min incubation chloroform was added at a ratio of 1:5 to the suspension and vortex vigorously. After 2 min the mixture was centrifuged at 12,000 rpm for 15 min at 4º C. After centrifugation lower organic layer (containing protein), upper aqueous layer (containing RNA) and middle interphase (containing DNA) could be seen clearly. The aqueous phase was collected carefully in a microcentrifuge tube and Molecular grade isopropanol was added twice the volume of the aqueous phase to precipitate the RNA. The mixture was then incubated for 10 min at 4º C and centrifuged at 12,000 rpm for 10 min. Supernatant was discarded and the RNA pellet was dissolved in 70 % ethanol to wash. Solution was centrifuged at 12,000 rpm at 4º C for 5 min and the supernatant was discarded. Pellet was air dried for 5-10 min and finally dissolved in DEPC treated water at 65º C for 30 min. The isolated RNA was stored at -70º C until further use.

4.25 RNA quantification and purity analysis:

RNA concentration was measured after extraction using UV-Vis spectrophotometer at 260 nm. Purity of the RNA was assessed by taking the ratio of OD_{260}/OD_{280}. 5 µl of RNA solution was mixed with 595 µl of DEPC treated water and the absorbance was measured at 260 nm. The concentration of the RNA was measured by following formula

RNA concentration (µg/µl) = [OD_{260} X 120 (Dilution factor) X 40] / 1000

(For 1 cm pathlength, 1 OD_{260} = 40 µg/µl RNA)

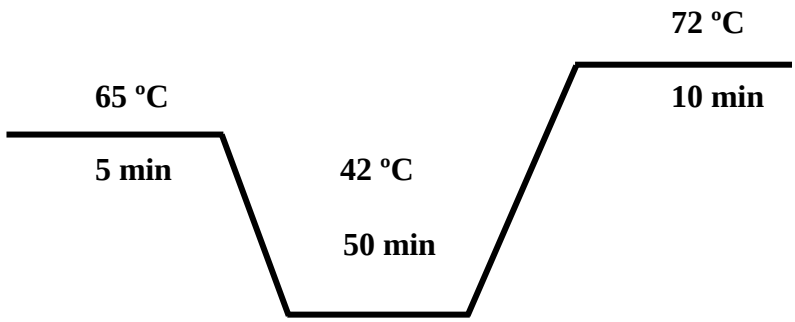
4.26 cDNA synthesis:

cDNA or complementary DNA synthesis is a process by which DNA is synthesized from RNA template using RNA dependent DNA polymerase called reverse transcriptase (RT) enzyme. In our study cDNA was synthesized using Qiagen cDNA synthesis kit. The reagents were- reverse transcriptase enzyme (100 units/µl), 10x RT buffer, random hexamer, dNTPs (2.5 mM) and RNase inhibitor (10 units/µl). All the components were thawed on ice and then the reaction was set as follows:

Total RNA-	1 µg
Random hexamer-	2 µl
10X RT buffer-	2 µl
RNase inhibitor-	1 µl
RT enzyme-	1 µl
Nuclease free water-	adjusted to 20 µl
Total volume-	20 µl

PCR amplification conditions:

The PCR was done using following condition:



RNA/random hexamer mixed well and incubated at 65 ºC for 5 min. After incubation placed on ice and rest of the reagents were added. Then the reaction was done at 42 ºC for 50 min and 72 ºC for 10 min. After the final step, RNase H was added to the tube and incubate at 37 ºC for 20 min. The tube can be stored at -20 ºC for storage or -80 ºC for long term storage.

4.27 Real-Time RT-PCR to study tubulin mRNA level:

Prepared cDNA was used to study the gene level of tubulin after subtilisin treatment. cDNAs were amplified by real time PCR using tubulin specific primer (forward primer 5'-CGGGCAGTGTTTGTAGACTTGG- 3', reverse primer 5'-CTCCTTGCCAATGGTG-TAGTGC- 3') using SYBER green Master Mix. Actin (forward primer 5'-GGACTTCGAGCAAGAGATGG- 3', reverse primer 5'-AGCACTGTGTTGGCGTACAG-3') was used as an endogenous control. Amplification was performed with 1 µl of cDNA, 50 pM Fwd and Rev primers and 10 µl SYBER green Master Mix was in 7500 Fast Real Time system (Applied Biosystem, USA). Relative gene expression of tubulin was calculated using ΔΔCT method and the statistical analysis was done based on triplicate PCR set of each sample.

4.28 Subtilisin labelling:

To determine whether subtilisin enters into the MCF-7 cells or acts through cell surface receptors, subtilisin was labelled with Alexa-Fluor 568 labeling kit (Thermo Fisher, cat-A10238) according to the manufacturer protocol. The kit contains- reactive dye (magnetic stir bar present in the vial), sodium bi-carbonate, purification column and collection tube. 1 ml dH_2O was added to the provided sodium bi-carbonate vial to achieve 1M sodium bi-carbonate solution. Purified subtilisin (1 mg in 500 µl) was mixed with 50 µl of 1M sodium bi-carbonate and the mixture was transferred to the vial of reactive dye. Invert and stir the mixture for one hour at room temperature. Tagged protein was collected and free dye was removed by gel filtration spin column provided with the kit. Purification column was spin at 1000 x g for 2 min to discard the storage buffer and then the 500 µl reaction mixture placed to the column. Spin column placed onto the collection tube and spin at 1000 x g for 2 min to collect the dye tagged protein.

4.29 si-RNA transfection and PARKIN knockdown:

Expression of PARKIN was blocked by transfection of PARKIN si-RNA construct (Santa Cruz Biotechnology cat- sc-42158) in MCF-7 cell line using Lipofectamine-3000 (Thermo-scientific) as per manufacturer's protocol. MCF-7 cells were seeded onto 6 well tissue culture plate such a way that it can reach 60% confluence at the day of transfection. For each well 40 pmol of PARKIN si-RNA mixed with 2 µl of Lipofectamine-3000 reagent in 800 µl OptiMEM medium and incubated at room temperature for 20 min. Culture media was aspirated from the well and washed with PBS. Then the si-RNA mixture was transferred to the well and the culture plate was incubated at 37 ºC for 6 hrs. After 6 hrs optiMEM media was removed from the well and complete growth media was added. The gene silencing was observed after 24 hrs of post-transfection by RT PCR and western blot.

4.30 Proteasome activity measurement:

In order to measure the proteasomal activity of whole cells after subtilisin treatment, we used the Proteasome activity assay kit (Abcam, cat-ab107921). The kit contains- proteasome assay buffer, proteasome substrate, proteasome inhibitor, AMC standard (1 mM) and positive control (Jurkar cell lysate). The very first step was the preparation of AMC standard by diluting 1mM AMC from the stock solution to 0.1mM AMC (10 µl AMC from the stock was mixed with 990 µl of assay buffer). For the preparation of standard curves, AMC was diluted from 0.1 mM to different levels as follows:

Table 4.6: AMC standard dilution method for standard curve preparation

Standard	Volume of Standard (µl)	Assay Buffer (µl)	Final volume standard in well (µl)	AMC concentration
1	0	300	100	0 pmol/well
2	6	294	100	20 pmol/well
3	12	288	100	40 pmol/well
4	18	282	100	60 pmol/well
5	24	276	100	80 pmol/well
6	30	270	100	100 pmol/well

AMC is a fluorescent dye which has 350 nm excitation and 440 nm emission spectra. 100 µl of each AMC dilution was taken in a 96 well microplate in duplicate and the fluorescence reading was taken at 350/440 nm. The average absorbance reading (RFU) in the Y-axis was plotted against concentration of AMC in the X-axis.

The second step was preparation of cell lysate for the assay. MCF-7 cells were treated with subtilisin (5 µg/ml) for 1, 3, 6 and 24 hrs and following treatment cells were harvested by centrifugation at 1000 rpm for 10 min. Cells were washed with cold 1x PBS and resuspended in 100 µl of 0.4% NP-40 by pipetting up and down. Resuspended cells were incubated for 30

min and then the lysate was collected by centrifugation at 14,000 rpm for 5 min. Prepared cell lysate was used for the measurement of proteasome activity and all the samples was assayed in duplicate. 20 µl of sample lysate was mixed with 80 µl of assay buffer. 10 µl positive

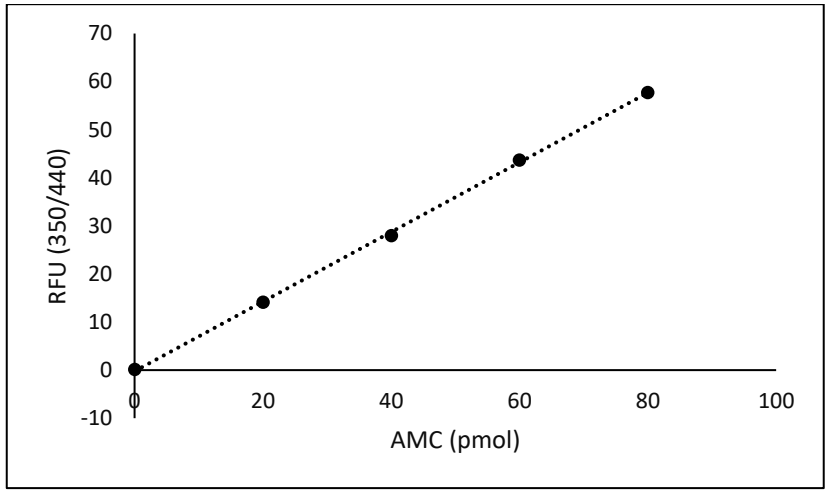


Fig 4.4: AMC standard curve

control was taken and the volume was adjusted to 100 µl by adding 90 µl assay buffer. Sample solution and positive control solution were taken and labelled as sample well and positive control well respectively in a 96-well microplate. The assay was performed in presence of inhibitor or without inhibitor so pair of samples well and positive control well was taken. The inhibitor to the well was added as followed:

Reagent	Positive control wells		Sample wells	
	With inhibitor (µl)	Without inhibitor (µl)	With inhibitor (µl)	Without inhibitor (µl)
Proteasome inhibitor	1	0	1	0
Assay Buffer	0	1	0	1

1 µl proteasome substrate (Succ-LLVY-AMC in DMSO) was added to each sample well and positive control well. Following addition solution was mixed by pipetting and incubated at 37 ºC. After 30 min incubation (T_1), fluorescence (Ex/Em-350/440 nm) was measured in a fluorometer (Thermo Scientific). The plate was kept at 37 ºC for another 30 min (T_2) and the reading was measured again. The absorbance value (RFU) of samples generated from the proteasomal activity was calculated by the following formula:

$$\Delta RFU = (RFU_2 - iRFU_2) - (RFU_1 - iRFU_1)$$

[RFU_1 = absorbance at T_1; RFU_2 = absorbance at T_2; iRFU = reading of inhibitor well]

The ΔRFU value of the samples were plotted on the AMC standard curve and the AMC concentration (generated by the proteasomal activity of the sample lysate by digested 'Succ-LLVY-AMC' peptide) was noted as B. After that the proteasomal activity was measured bby the following formula:

$$\text{Proteasome activity} = \left(\frac{B}{(T_{2-}T_1) * V} \right) * D$$

B = Amount of AMC in the sample well (pmol); V = Sample volume added into the reaction well (µL); T1 = Time (min) of the first reading (RFU1 and iRFU1); T2 = Time (min) of the first reading (RFU2 and iRFU2); D = Sample dilution factor.

4.31 DNA-ladder assay:

DNA ladder assay was used to study the fragmentation of DNA. Fragmentation of the DNA occurs during some pathophysiological condition of the cells such as apoptosis. If the genomic DNA is isolated from the apoptotic cells and run on SDS it will be visualised in gel like a ladder. Thus, this assay is called DNA ladder assay.

Procedure:

Genomic DNA was isolated from untreated and subtilisin (5 µg/ml) treated (for 6 and 18 hrs) MCF-7 cells by genomic DNA isolation kit (Qiagen, Germany). Isolated DNA was then resolved in 8% agarose gel electrophoresis and the DNA ladder was observed under Bio-Rad gel documentation system.

4.32 EAC induced mice model for studying anti-cancer activity of subtilisin:

The anti-cancer effect of subtilisin was observed *in-vivo* by using Erhlich ascites mammary carcinoma (EAC) induced mice model. 10^6 number of EAC cells were injected intraperitoneally to Swiss Albino mice (22-25 gm body weight) to develop liquid tumor in the mice. After 1 week of inoculation animals were divided into three groups with 10 animals in each group. First group was non-tumor bearing group (no cells were inoculated in this group), second group was tumor bearing group and third group was subtilisin treated group. 5 ug of subtilisin was injected intraperitonially once in a week for three successive weeks to the subtilisin treated group and tumor bearing groups were injected with buffer. Following treatments survival of animals were observed for 45 days. Survival rate was calculated as- Number of live animals in a group/ number of initial animals in that group X 100.

4.33 Statistical analysis:

All the experiments were performed in triplicate and the data was represented as mean ± standard deviation (SD). Statistical analysis between two experimental conditions was done by Students's t-test using Microsoft excel. P-value < 0.05 was considered as statistically significant for all the experiments.

CHAPTER 5

RESULTS

5.1 Screening of environmental bacterial isolates for protease activity:

The culture supernatants of more than 140 environmental microbes were screened for protease secretion by azocasein assay (Table; Fig 5.1). Five strains (DIU-144, RAJ-43, DHS-96, BSF-26 and BSF-17) showed significant protease activity and were further tested for apoptotic activity on cancer cells. The culture supernatant of *V. cholerae* O1 ElTor strain C6709 was selected as a positive control for protease activity.

Table 5.1: Protease secretion screening of bacterial isolates

Serial No.	Strain number	Azocasein assay result (OD at 440 nm)		
		Experimental sample	Negative Control	Positive Control
1.	DIU-210	0.203	0.110	0.98
2.	DIU-172	0.225	0.110	0.98
3.	DIU-211	0.081	0.110	0.98
4.	DIU-100	0.280	0.110	0.98
5.	DIU-174	0.216	0.110	0.98
6.	DIU-181	0.046	0.110	0.98
7.	DIU-189	0.202	0.110	0.98
8.	DIU-204	0.313	0.110	0.98
9.	DIU-176	0.240	0.110	0.98
10.	DIU-175	0.125	0.110	0.98
11.	DIU-204	0.087	0.133	1.233
12.	DIU-238	0.131	0.133	1.233
13.	DIU-227	0.119	0.133	1.233
14.	DIU-109	0.120	0.133	1.233
15.	DIU-104	0.329	0.133	1.233
16.	DIU-108	0.373	0.133	1.233

Serial No.	Strain number	Azocasein assay result (OD at 440 nm)		
		Experimental sample	Negative Control	Positive Control
17.	DIU-106	0.209	0.133	1.233
18.	DIU-110	0.399	0.133	1.233
19.	DIU-107	0.241	0.133	1.233
20.	DIU-137	0.200	0.064	1.052
21.	DIU-134	0.295	0.064	1.052
22.	DIU-133	0.074	0.064	1.052
23.	DIU-131	0.139	0.064	1.052
24.	DIU-138	0.302	0.064	1.052
25.	DIU-141	0.168	0.064	1.052
26.	GOL-135	0.126	0.064	1.052
27.	GDP-127	0.230	0.064	1.052
28.	RAJ-128	0.100	0.064	1.052
29.	RAJ-121	0.143	0.090	0.721
30.	RAJ-122	0.044	0.090	0.721
31.	BEP-123	0.159	0.090	0.721
32.	BEP-124	0.098	0.090	0.721
33.	BEP-143	0.337	0.090	0.721
34.	BEP-126	0.221	0.090	0.721
35.	RAJ-178	0.090	0.090	0.721
36.	RAJ-190	0.223	0.090	0.721
37.	GOL-93	0.097	0.087	0.779
38.	GOL-112	0.099	0.087	0.779
39.	GOL-113	0.117	0.087	0.779
40.	BSF-115	0.284	0.087	0.779
41.	BSF-116	0.113	0.087	0.779

Serial No.	Strain number	Azocasein assay result (OD at 440 nm)		
		Experimental sample	Negative Control	Positive Control
42.	BSF-117	0.129	0.087	0.779
43.	DIU-119	0.123	0.087	0.779
44.	DIU-98	0.226	0.055	0.739
45.	DIU-92	0.079	0.077	0.750
46.	DIU-94	0.134	0.077	0.750
47.	BSF-96	0.306	0.077	0.750
48.	BSF-98	0.276	0.077	0.750
49.	BSF-105	0.178	0.077	0.750
50.	RAJ-83	0.182	0.128	0.788
51.	RAJ-85	0.128	0.128	0.788
52.	RAJ-86	0.178	0.128	0.788
53.	RAJ-88	0.169	0.128	0.788
54.	RAL-89	0.145	0.128	0.788
55.	GOL-102	0.181	0.128	0.788
56.	GOL-111	0.339	0.128	0.788
57.	GOL-87	0.286	0.132	0.741
58.	BSF-95	0.178	0.132	0.741
59.	DIU-171	0.074	0.055	0.739
60.	DIU-177	0.165	0.055	0.739
61.	DIU- 206	0.007	0.023	0.831
62.	DIU-144	0.687	0.066	0.843
63.	DIU- 239	0.102	0.135	1.431
64.	DIU- 247	0.084	0.135	1.431
65.	DIU- 228	0.137	0.135	1.431
66.	DIU-241	0.083	0.135	1.431

Serial No.	Strain number	Azocasein assay result (OD at 440 nm)		
		Experimental sample	Negative Control	Positive Control
67.	DIU-233	0.215	0.135	1.431
68.	DIU-244	0.182	0.135	1.431
69.	GOL-145	0.161	0.135	1.431
70.	DIU-237	0.092	0.016	1.385
71.	DIU-245	0.049	0.016	1.385
72.	DIU-235	0.104	0.016	1.385
73.	DIU-260	0.083	0.016	1.385
74.	GDL-170	0.136	0.016	1.385
75.	DIU-136	0.157	0.016	1.385
76.	BSF-16	0.063	0.051	1.441
77.	RAJ-52	0.103	0.051	1.441
78.	RAJ-72	0.093	0.051	1.441
79.	RAJ-74	0.107	0.051	1.441
80.	RAJ-84	0.156	0.051	1.441
81.	RAJ-44	0.063	0.051	1.441
82.	RAJ-40	0.136	0.051	1.441
83.	RAJ-39	0.157	0.051	1.441
84.	RAJ-60	0.220	0.101	1.290
85.	RAJ-80	0.083	0.100	1.327
86.	BSF-21	0.027	0.100	1.327
87.	RAJ-77	0.031	0.101	1.290
88.	BSF-15	0.032	0.101	1.290
89.	RAJ-73	0.101	0.101	1.290
90.	RAJ-64	0.149	0.100	1.327
91.	BSF-19	0.248	0.013	1.238

Serial No.	Strain number	Azocasein assay result (OD at 440 nm)		
		Experimental sample	Negative Control	Positive Control
92.	DHS-96	1.342	0.013	1.238
93.	BSF-26	1.144	0.013	1.238
94.	BSF-14	0.375	0.013	1.238
95.	RAJ-47	0.056	0.013	1.238
96.	BSF-22	0.427	0.013	1.238
97.	RAJ-49	0.018	0.013	1.238
98.	BSF-25	0.037	0.013	1.238
99.	RAJ-63	0.179	0.047	1.497
100.	BSF-23	0.295	0.047	1.497
101.	BSF-18	0.168	0.047	1.497
102.	BSF-17	1.369	0.047	1.497
103.	RAJ-81	0.116	0.142	1.582
104.	RAJ-48	0.213	0.142	1.582
105.	RAJ-45	0.134	0.142	1.582
106.	BSF-4	0.232	0.142	1.582
107.	BSF-20	0.264	0.142	1.582
108.	RAJ-58	0.268	0.142	1.582
109.	RAJ-43	1.272	0.142	1.582
110.	BSF-12	0.150	0.142	1.582
111.	RAJ-71	0.379	0.142	1.582
112.	BSF-7	0.263	0.084	0.980
113.	BSF-30	0.099	0.084	0.980
114.	BSF-6	0.132	0.084	0.980
115.	RAJ-75	0.163	0.084	0.980
116.	RAJ-68	0.064	0.084	0.980

Serial No.	Strain number	Azocasein assay result (OD at 440 nm)		
		Experimental sample	Negative Control	Positive Control
117.	RAJ-51	0.087	0.084	0.980
118.	RAJ-59	0.092	0.084	0.980
119.	BSF-36	0.087	0.084	0.980
120.	BSF-34	0.213	0.084	0.980
121.	RAJ-72	0.029	0.084	0.980
122.	BSF-13	0.122	0.084	0.980
123.	BSF-58	0.102	0.013	1.394
124.	BSF-29	0.096	0.013	1.394
125.	BSF-5	0.132	0.013	1.394
126.	RAJ-41	0.076	0.013	1.394
127.	BSF-8	0.312	0.013	1.394
128.	RAJ-76	0.166	0.013	1.394
129.	RAJ-70	0.086	0.253	0.983
130.	BSF-2	0.017	0.253	0.983
131.	BSF-3	0.099	0.253	0.983
132.	RAJ-82	0.072	0.253	0.983
133.	RAJ-74	0.018	0.253	0.983
134.	BSF-37	0.302	0.253	0.983
135.	BSF-28	0.479	0.018	1.586
136.	RAJ-38	0.227	0.018	1.586
137.	RAJ-67	0.055	0.018	1.586
138.	RAJ-55	0.684	0.018	1.586
139.	DIU-248	0.019	0.020	1.212
140.	RAJ-55	0.280	0.020	1.212
141.	RAJ-66	0.115	0.020	1.212

Serial No.	Strain number	Azocasein assay result (OD at 440 nm)		
		Experimental sample	Negative Control	Positive Control
142.	RAJ-247	0.009	0.020	1.212
143.	RAJ-225	0.037	0.020	1.212
144.	BSF-13	0.122	0.088	1.711
145.	DIU-249	0.048	0.088	1.711
146.	DIU-92	0.079	0.088	1.711
147.	DIU-130	0.152	0.088	1.711
148.	BSF-22	0.427	0.088	1.711
149.	DIU-172	0.225	0.088	1.711
150.	BSF-21	0.027	0.088	1.711
151.	RAJ-57	0.0406	0.088	1.711
152.	RAJ-87	0.036	0.088	1.711

(Green highlighted represented as protease positive)

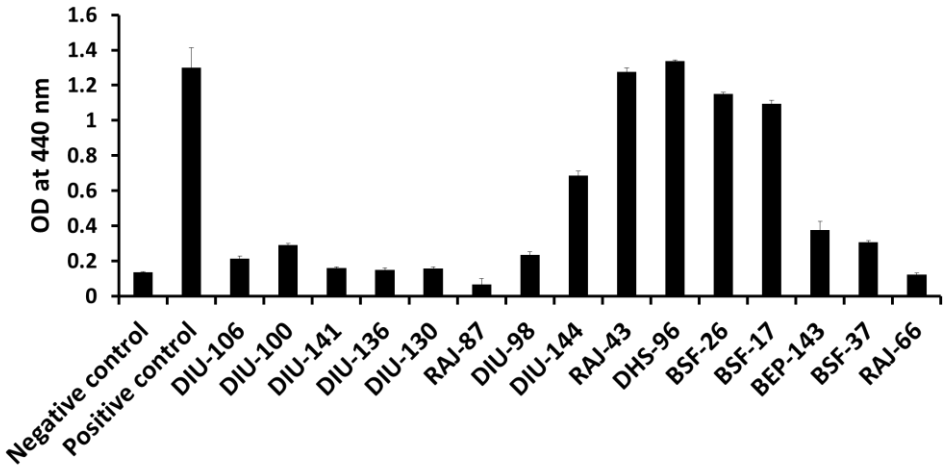


Fig 5.1: Azocasein assay of the culture supernatant of some representative strains. Culture supernatant of *Vibrio cholerae* ElTor strain C6709 was used as positive control and nutrient broth was used as negative control.

5.2 Culture supernatant of DHS-96 strain induced apoptosis in cancer cell:

To study the apoptotic effect, HT-29 (colon cancer) cells were incubated with 200 µl of sterile culture supernatant of protease positive strains (DIU-144, RAJ-43, DHS-96, BSF-26 and BSF-17) for 24 hrs. Apoptosis was observed by Annexin-V/PI staining. Our results showed that, out of five strains, only after treatment with protease positive culture supernatant of DHS-96, there is significant increase in Annexin-V^+/PI^+ cells, indicating apoptosis (Fig 5.2 & 5.3). This suggests that, only DHS-96 can induce apoptotic effect on HT-29 cancer cell and thus it was further selected for purification of protease and to study whether the protease has anti-cancer properties.

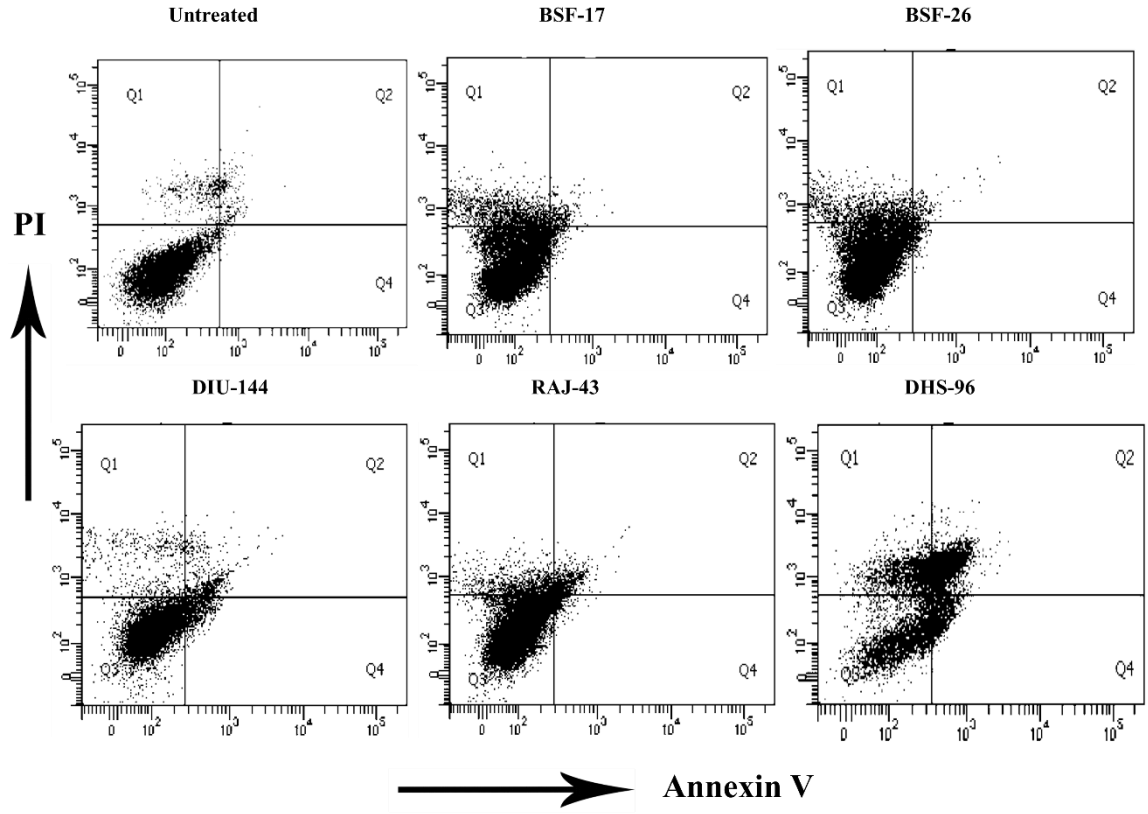


Fig 5.2: Screening of protease positive bacterial isolates for apoptotic activity on HT-29 cells. Flow-cytometric analysis with the culture supernatant of the protease positive strains was done to study apoptosis in HT-29 cells. In each display the lower left quadrant is for normal cells (Annexin V-/PI-), lower right quadrant is for early apoptotic cells (Annexin

V+/PI-), upper right is for late apoptotic cells (Annexin V+/PI+) and upper left is for necrotic population (Annexin V-/PI+).

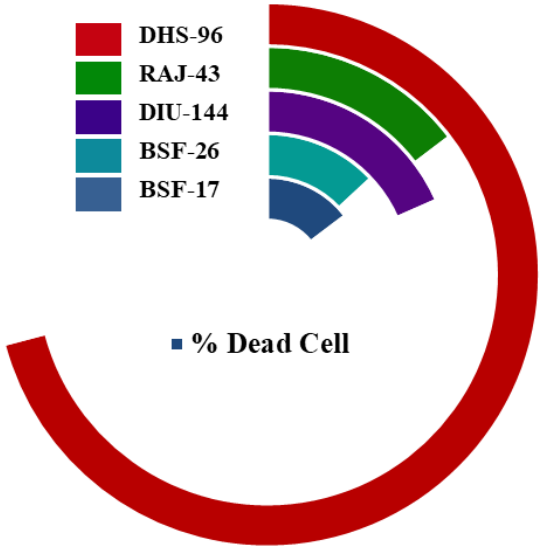


Fig 5.3: Statistical representation of figure 5.2. After flow cytometric analysis % of dead cells showed in a doughnut chart

5.3 Purification of the secreted protease from DHS-96:

To purify the secreted protease from the cell free culture supernatant of DHS-96 strain, proteins present in supernatant was precipitated using 80% ammonium sulphate. After precipitation crude protein sample was dissolved in 25 mM Tris-HCl (pH-7.4) and dialysed against the same buffer, concentrated and run-on DE-52 column. The flow-through fraction was eluted with 25 mM Tris-HCl (pH-7.4) and bound fractions were eluted with 0.1 M, 0.2 M, 0.3 M and 0.5 M NaCl (Fig 5.4). All the fractions were checked for protease activity, only the unbound (flow-through) fraction showed significant protease activity in azocasein assay (Fig. 5.5). The flow through fraction from DE-52 column was pooled, concentrated and further loaded on Sephadex G-75 gel filtration column. The two peaks eluted from the G-75 gel filtration column were collected and concentrated and tested for protease activity. The second peak in G-75 column showed protease activity (Fig 5.6 A & B)

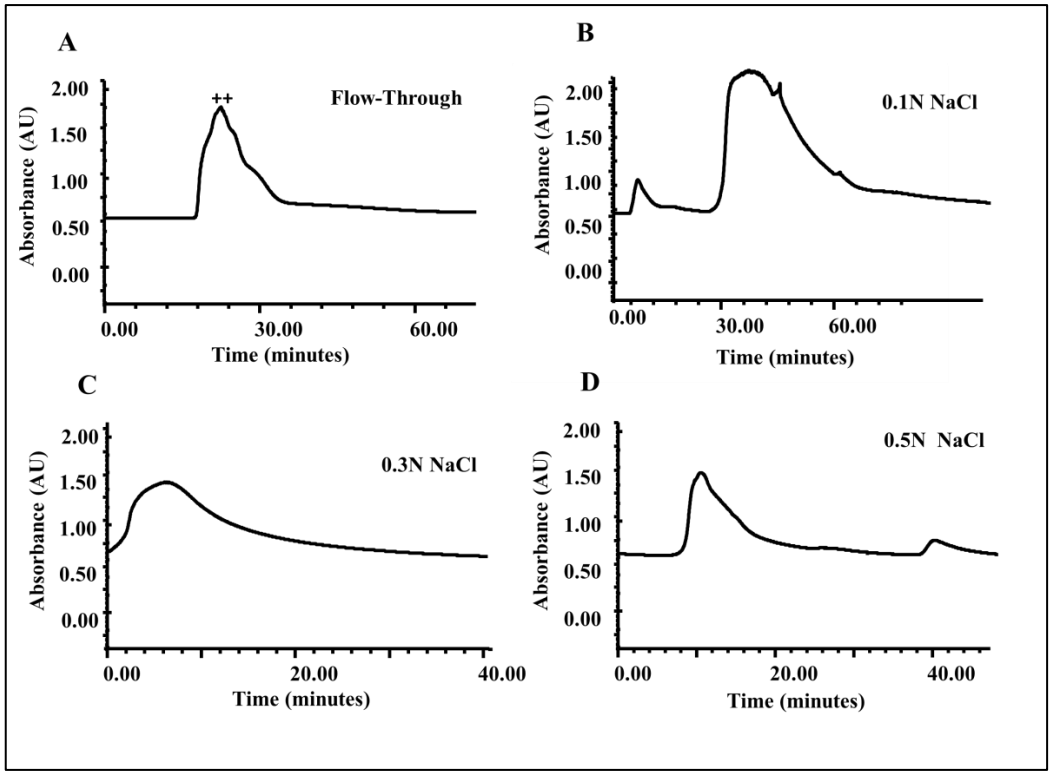


Fig 5.4: Purification of crude protein from DHS-96 bacterial isolate isolate. Chromatographic profile of ammonium sulphate precipitated crude proteins from culture supernatant of DHS-96 strain, loaded onto an anion-exchange DE-52 column. (**A)**, represents proteins in non-binding fraction of DE-52, (**B), (C)** and (**D)** are 0.1N, 0.3N & 0.5N NaCl eluted fractions respectively. '++' indicates fraction positive for protease activity.

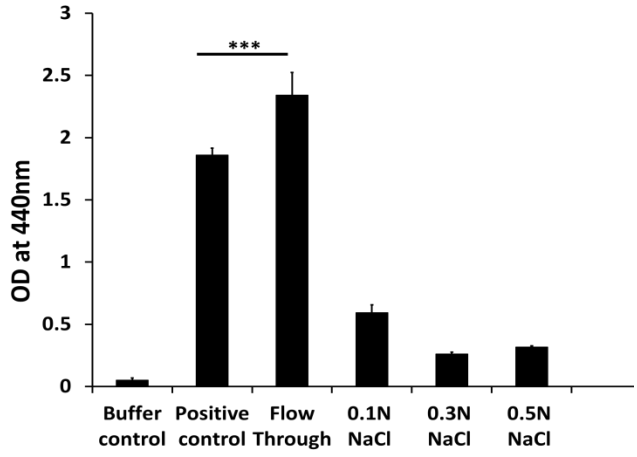


Fig 5.5: Azocasein assay of different DE-52 fraction. Error bar in each graphical representation represents standard error from three different replicates. *, p< 0.05, **, p< 0.01, ***, p<0.0005

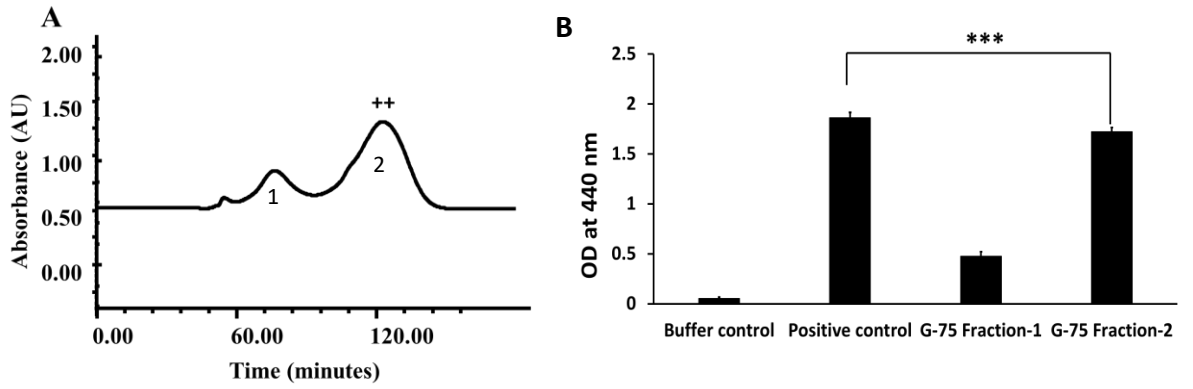


Fig 5.6: (A). Gel filtration chromatographic profile of DE-52 flow through fraction. '++' indicates fraction positive for protease activity. (**B). Azocasein assay of the fractions eluted from gel filtration.** Error bar in graphical representation represent standard error from three different replicates. *, p< 0.05, **, p< 0.01, ***, p<0.0005

5.4 Protein identification:

The second fraction of G-75 column showed the presence of two bands at 29 kDa and at 42 kDa in SDS-PAGE and in Native PAGE it showed presence of a single band (Fig 5.7).

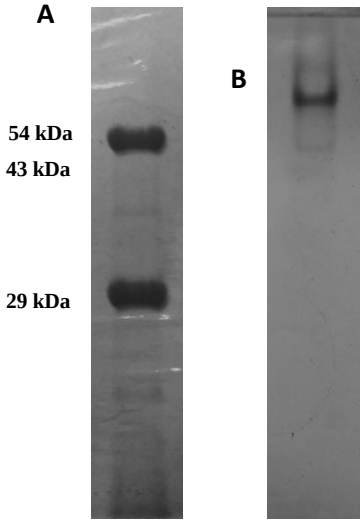


Fig 5.7: PAGE profile of protease purified fraction. (**A).** SDS-PAGE profile. (**B).** NATIVE-PAGE profile

Both the bands in SDS-PAGE and Native PAGE were further processed and analysed in Mass Spectrometry. The fragment peptide sequence generated from Mass spectrometry data was analysed and search in in the NCBI database, UniProt database. The highest matching peptide sequence was taken into account and it showed homology with 'subtilisin' from

Bacillus sp. The 42 kDa and 29 kDa bands in SDS-PAGE are the precursor and mature forms of subtilisin respectively. Subtilisin proteases have highly conserved peptidase S8 family of active site and the domain contains conserved amino acid D at 139, H at 171 and S at 328 positions. Peptide sequence homology search showed 100% sequence alignment with the conserved peptidase S8 domain of subtilisin (Fig. 2I).

>sp|P00782.1|Full=**Subtilisin** Novo

1	MRGKKVWISL	LFALALIFTM	AFGSTSSAQA	AGKSNGEKKY	IVGFKQTMST	MSAAKKKDVI
31	SEKGGKVQKQ	FKYVDAASAT	LNEKAVKELK	KDPSVAYVEE	DHVAHAYAQS	VPYGVSQIKA
61	PALHSQGYTG	SNVKVAVIDS	GIDSSHPDLK	VAGGASMVPS	ETNPFQDNNS	HGTHVAGTVA
91	ALNNSIGVLG	VAPSASLYAV	KVLGADGSGQ	YSWIINGIEW	AIANNMDVIN	MSLGGPSGSA
121	ALKAAVDKAV	ASGVVVVAAA	GNEGTSGSSS	TVGYPGKYPS	VIAVGAVDSS	NQRASFSSVG
151	PELDVMAPGV	SIQSTLPGNK	YGAYNGTSMA	SPHVAGAAAL	ILSKHPNWTN	TQVRSSLENT
180	TTKLGDSFYY	GKGLINVQAA	AQ			

Fig 5.8: Protein sequence of subtilisin. Peptide sequences obtained from Mass-spectometry was searched in the database and the homology sequence is highlighted as yellow and the conserved peptidase S8 domain is highlighted as red.

5.6 Characterization of purified subtilisin:

5.6.1 Effect of inhibitor on protease activity:

Subtilisin is a serine protease having serine at the catalytic site. After purification and identification of the protease, to confirm the protease type, metalloprotease inhibitor EDTA and serine protease inhibitor PMSF was used. The catalytic activity of the protease was completely inhibited in presence of 10 mM PMSF but in presence of EDTA protease activity remained unchanged (Fig 5.9). This confirms that purified protease subtilisin is a serine protease as reported earlier.

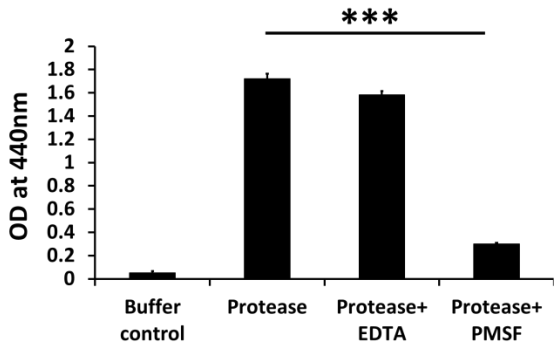


Fig 5.9: Effect of protease inhibitor on proteolytic activity of purified protease. Error bar in graphical representation represent standard error from three different replicates. *, p< 0.05, **, p< 0.01, ***, p<0.0005

5.6.2 Effect of pH on protease activity:

The catalytic activity of protease was observed in a wide pH range of 2-12, however, the activity is more at alkaline pH range of 8-12 (Fig 5.10). The optimum activity of subtilisin in alkaline pH further corroborates the finding that DHS-96 was isolated from alkaline environment.

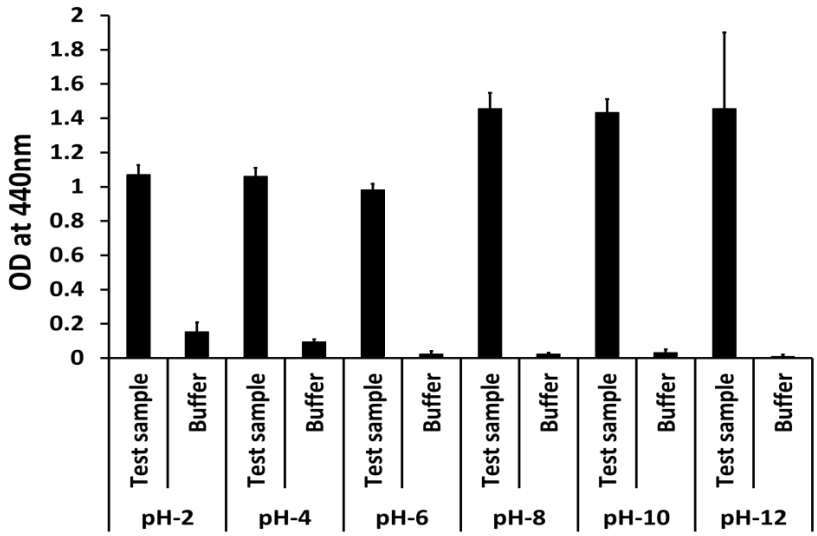


Fig 5.10: Proteolytic activity of purified protease on different pH

5.6.3 Effect of temperature on protease activity:

Protease activity was studied at different temperature and it was found that the activity was low at 25ºC and optimum activity was observed at temperature range in between 40-50 ºC. The protease activity decreased almost 50% at 60ºC and there was no activity at 70ºC or more temperature (Fig 5.11).

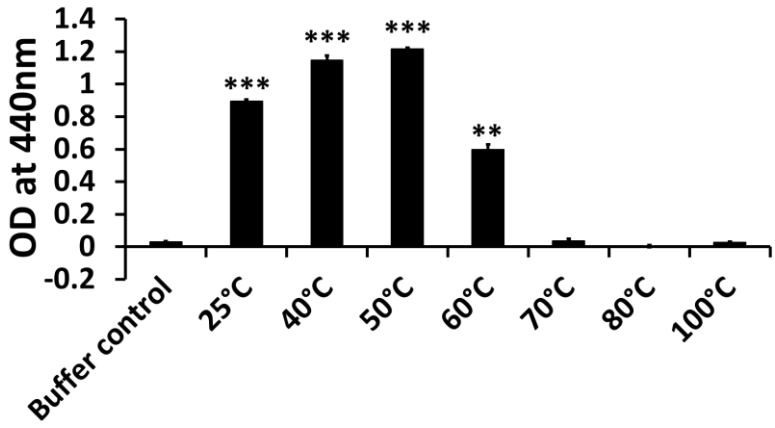


Fig 5.11: Effect of different temperature on the proteolytic activity of purified protease. Error bar in graphical representation represent standard error from three different replicates. *, p< 0.05, **, p< 0.01, ***, p<0.0005

5.7 Identification of the bacterial isolate DHS-96:

To identify the bacterial isolate DHS-96, 16S rRNA was sequenced. The sequence generated from the 16S rRNA sequencing was BLAST to search for similar sequence. The BLAST result in NCBI data base revealed that the sequence has 83.12% similarity with *Bacillus subtilis* (Accession number NR_102783.2) and 82.95% similarity with *Bacillus amyloliquefaciens* (Accession number NR_116022.1) (Fig. 5.12A & 5.13). Bioinformatics analysis showed that the subtilisin gene sequence present in both the bacterial species is not

similar to each other (79% similarity found). Thus, to distinguish the closest neighbour and to identify DHS-96, we have further designed 'subtilisin' gene specific primers from both *B. subtilis* and *B. amyloliquefaciens*. Subtilisin gene amplification was only observed when *B. amyloliquefaciens* specific primers were used (Fig 5.12B). These results suggest that the DHS-96 strain belongs to *Bacillus amyloliquefaciens*.

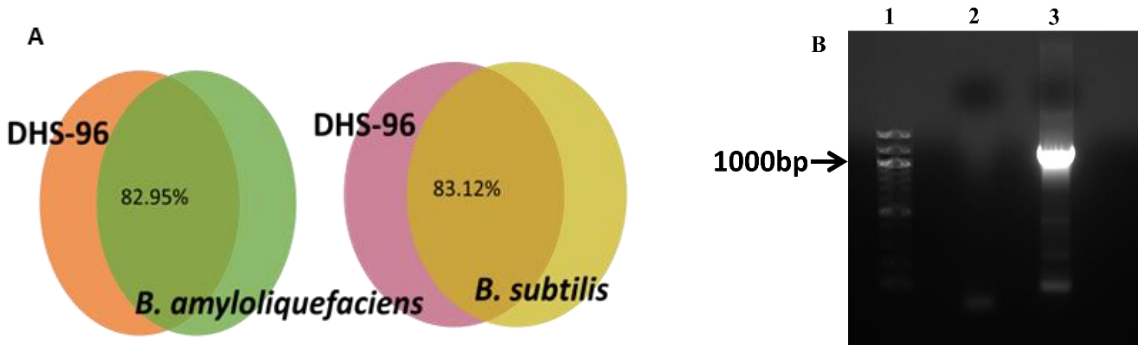


Fig 5.12: Identification of bacterial isolate DHS-96. (**A)**. Percentage similarity of 16S-rRNA sequence between two closely related *Bacillus* species and DHS-96. (**B).** Agarose gel electrophoresis showing amplified subtilisin gene. Lane-1, 1 kb plus DNA-ladder; Lane-2, PCR sample amplified with *B. subtilis* specific primer; Lane-3, PCR sample amplified with *B. amyloliquefaciens* specific primer.

5.8 Purified subtilisin showed cytotoxicity against cancer cells:

Cytotoxic effect of subtilisin was studied in MCF-7 cells. MTT assay showed the dose dependent reduction of viable cells in response to protease treatment. This result indicates that subtilisin have cytotoxic effect on MCF-7 cells. The IC_{50} value was calculated from the MTT assay and it was found that 50% of the cells were dead in between 3-5 µg of protease treatment (Fig 5.14). To further confirm MCF-7 and HT-29 cancer cells were treated with 3 and 5 µg of subtilisin and the cytotoxic effect was studied with flow-cytometric analysis by

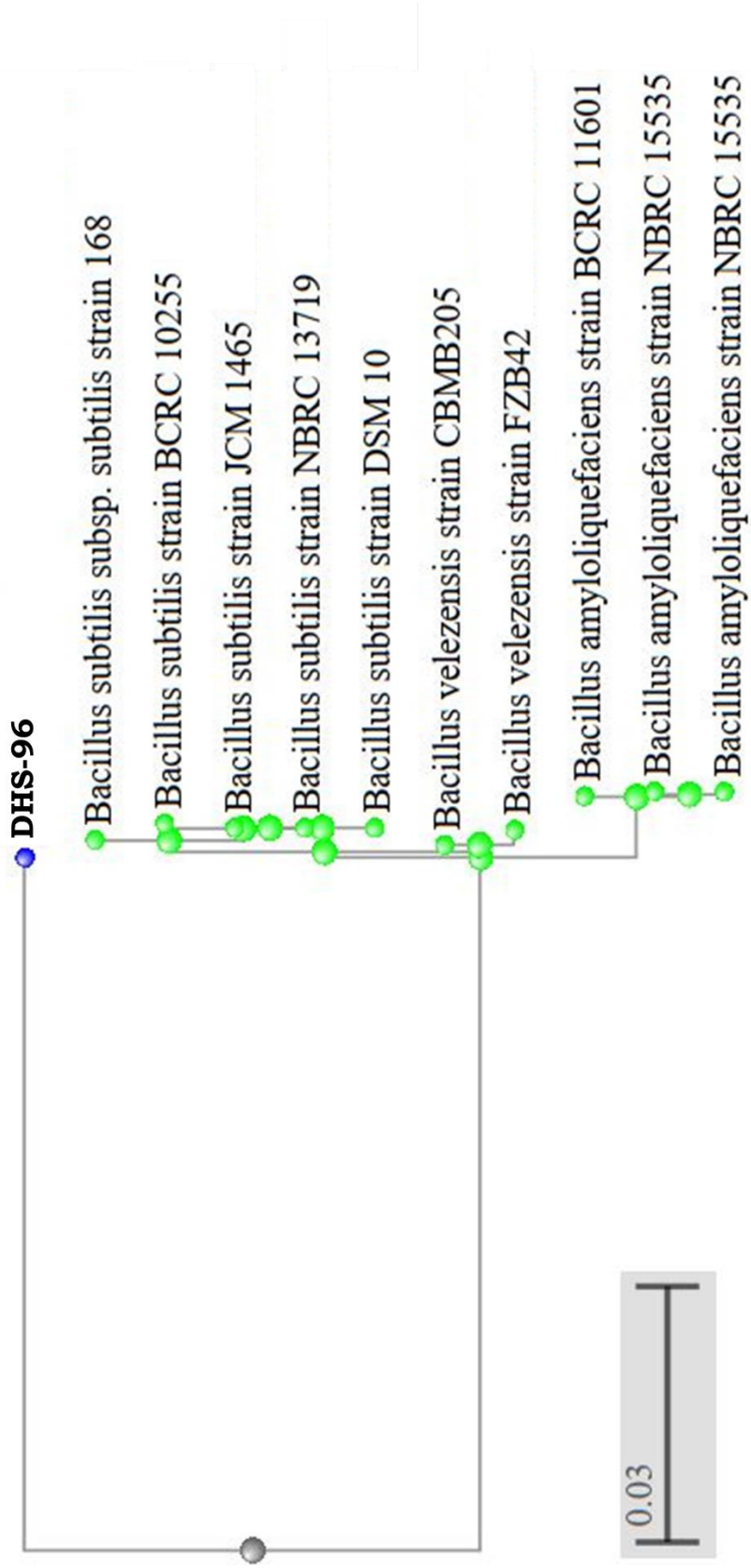


Fig 5.13: Phylogenetic analysis based on 16S-rRNA sequencing of bacterial isolate DHS-96

using Annexin-V/PI staining. Annexin-V binds with phosphatidyl-serine on the cell membrane, which is translocated at the extracellular side in the apoptotic cell and PI is a membrane impermeable agent, which stains dead cells or necrotic cells, where membrane integrity is lost [213,214]. After treatment with subtilisin, it was observed that MCF-7 had a greater number of cells in the Annexin-V^{+}/PI^{-} quadrant, which indicates early apoptosis, and HT-29 had a greater number of PI positive cells, which indicate necrosis. Inhibition of subtilisin with PMSF inhibited the effects of subtilisin on both the cell lines (Fig 5.15A & B). To compare the effect of subtilisin on normal cells, Mouse peritoneal macrophages and normal human breast cells MCF10A were used. Apoptosis was not observed in mouse peritoneal macrophages or normal human breast cells MCF-10A (Fig 5.15C & D) at similar concentrations of subtilisin (3 µg and 5 µg) treatment. The results above indicate that 3 and 5 µg of subtilisin treatment induce apoptosis in MCF-7 cells, but necrosis in HT-29 cells. Though subtilisin showed cytotoxic effect on both the cancer cells but it doesn't have any effect on normal cells.

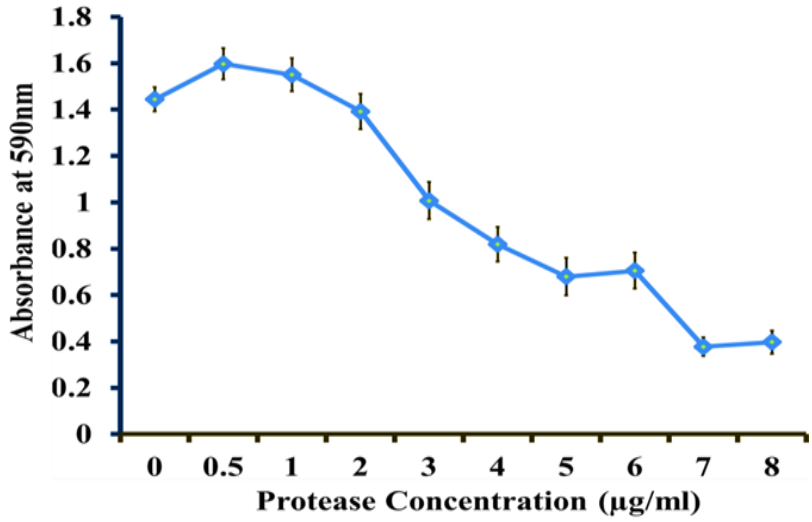


Fig 5.14: Determination of cytotoxic activity of subtilisin on MCF-7 cells using MTT assay

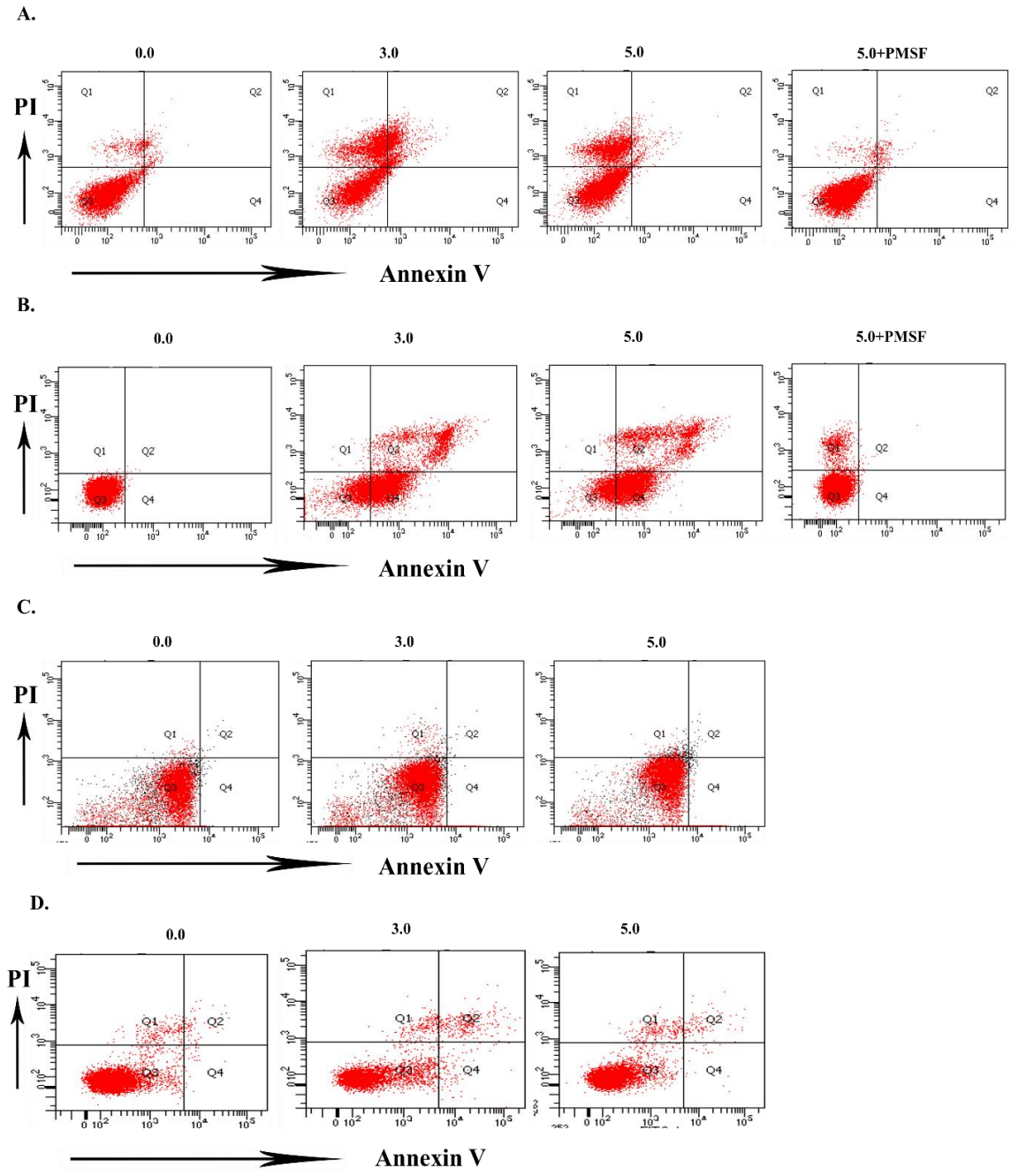


Fig 5.15: Flow cytometric analysis of purified subtilisin treated cell lines. (**A**) Dose dependent effects of subtilisin showed necrosis in HT-29 colon cancer cells. Inhibition with PMSF reduces the effect. (**B**) Dose dependent effect of subtilisin treatment showed apoptosis in MCF-7 breast cancer cell line. Inhibition with PMSF reduced the effect. (**C**) Subtilisin treatment does not show any effect on mouse peritoneal macrophage and (**D**) normal human breast cell line (MCF10A).

5.9 Subtilisin treatment showed no changes in pro- and anti-apoptotic markers but it promotes tubulin downregulation in MCF-7 cells:

Since we were more interested in apoptosis, we have selected MCF-7 cells to identify the apoptotic pathway induced by subtilisin. To check if subtilisin could induce the intrinsic pathway of apoptosis both the pro (bax) and anti-apoptotic (bcl-2) markers were checked. Western blot experiment showed no changes in bax and bcl-2 level in MCF-7 cells after 5 µg

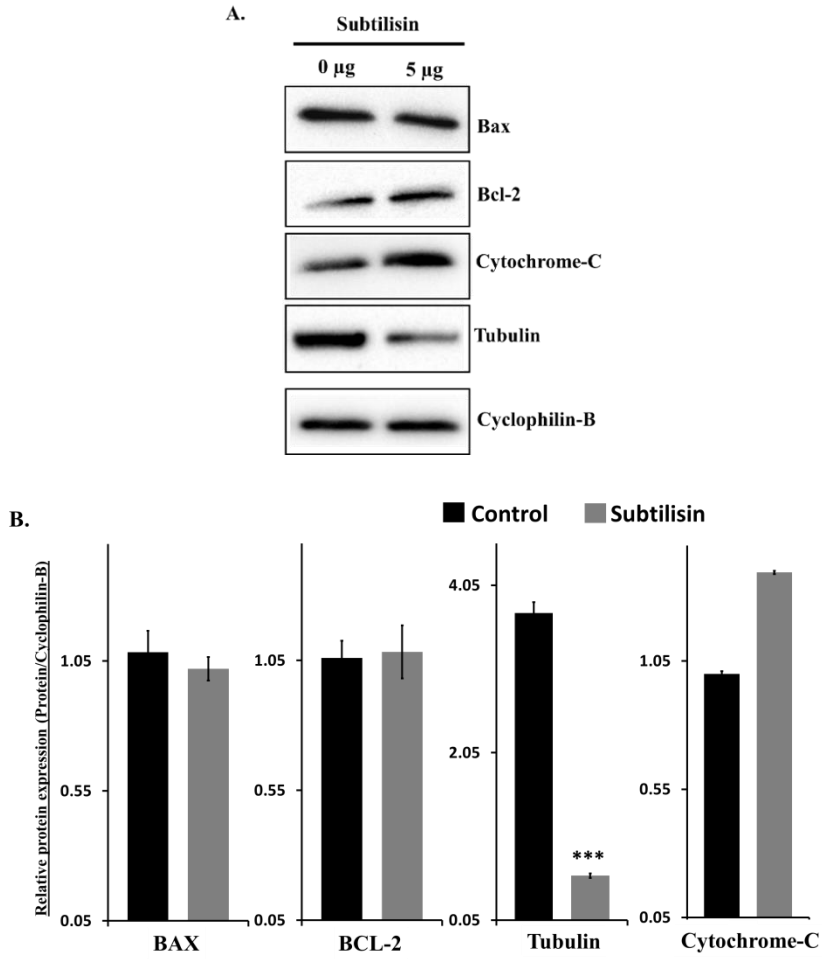


Fig 5.16: (A) Western blot analysis of pro and anti-apoptotic proteins and tubulin of MCF-7 cells after 24 hrs treatment with subtilisin. Cyclophilin-B was used as a loading control. **(B) Densitometric analysis of protein relative to cyclophilin-B quantified by ImageJ software using three experimental replicates from A.** Error bar in each graphical representation represent standard error from three different replicates. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0005$,

subtilisin treatment (Fig 5.16). Tubulin was initially used as a loading control in the western blot experiment but interestingly we observed significant decrease in the level of tubulin after 24 hrs of subtilisin treatment in MCF-7 cells. We have used other loading control in our experiment such as actin, GAPDH and cyclophilin-B. However, subtilisin did not show any effect on cyclophilin-B, for which it was used as a loading control for all our experiments. Furthermore, a time dependent response of subtilisin treatment on tubulin level was checked. After 6 hrs of subtilisin treatment, there was a significant decrease in tubulin level (Fig 5.17).

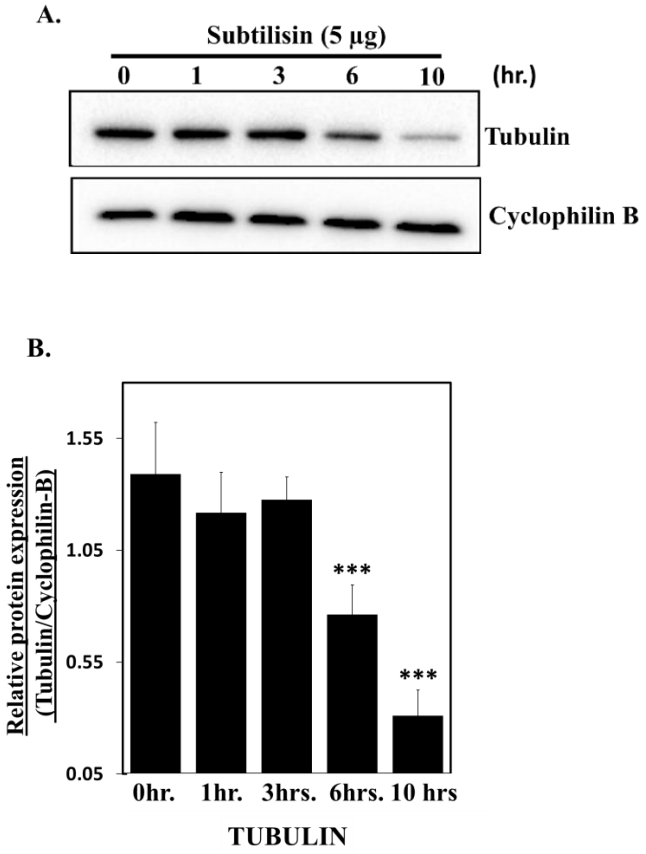


Fig 5.17: (A) Western blot analysis to observe tubulin level after subtilisin treatment on MCF-7 cells at different time intervals. **(B) Tubulin quantification relative to cyclophilin-B by Image-J software using three experimental replicates from A.** Error bar in each graphical representation represent standard error from three different replicates. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0005$,

In contrary, no change in tubulin level was observed in normal breast cell line MCF-10A after 24 hrs treatment with subtilisin (Fig 5.18A & B). In order to determine whether

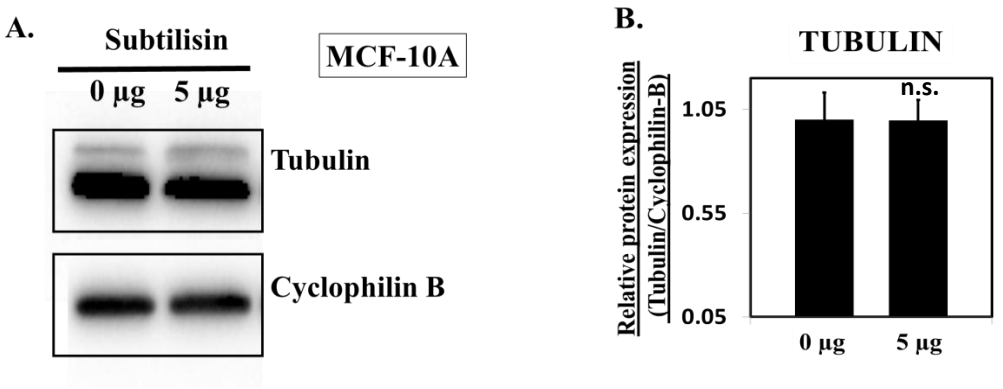


Fig 5.18: (A) Western blot analysis of tubulin after 24 h subtilisin treatment on MCF10A cells. (B) Densitometric representation of tubulin relative to cyclophilin-B from Fig A.

Subtilisin affects tubulin at mRNA level or protein level, quantitative real time PCR analysis was performed. Real time PCR data showed there is no difference of tubulin at mRNA level in subtilisin treated (6 hrs) and untreated control cells (Fig 5.19).

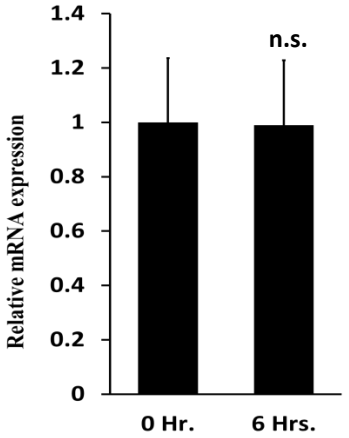


Fig 5.19: quantitative real time PCR analysis of tubulin in subtilisin treated (6 h) versus control cells (0 h). mRNA expression was normalised against actin gene expression (ΔCt = Ct tubulin- Ct actin). Error bar in each graphical representation represent standard error from three different replicates. n.s- non signifcant.

As we did not find any significant changes in mRNA level, it can be concluded that tubulin regulation by subtilisin is a post-transcriptional event.

5.10 Subtilisin induced ubiquitination and proteasomal mediated tubulin degradation:

MG-132, a proteasomal inhibitor was used to study the role of subtilisin-induced proteasomal mediated degradation of tubulin in MCF-7 cells. We found there was significant increase in tubulin levels in presence of 10 and 25 µM MG-132 (Fig 5.20) whereas in absence of MG-132 there was a significant decrease in tubulin level. The role of subtilisin in proteasomal

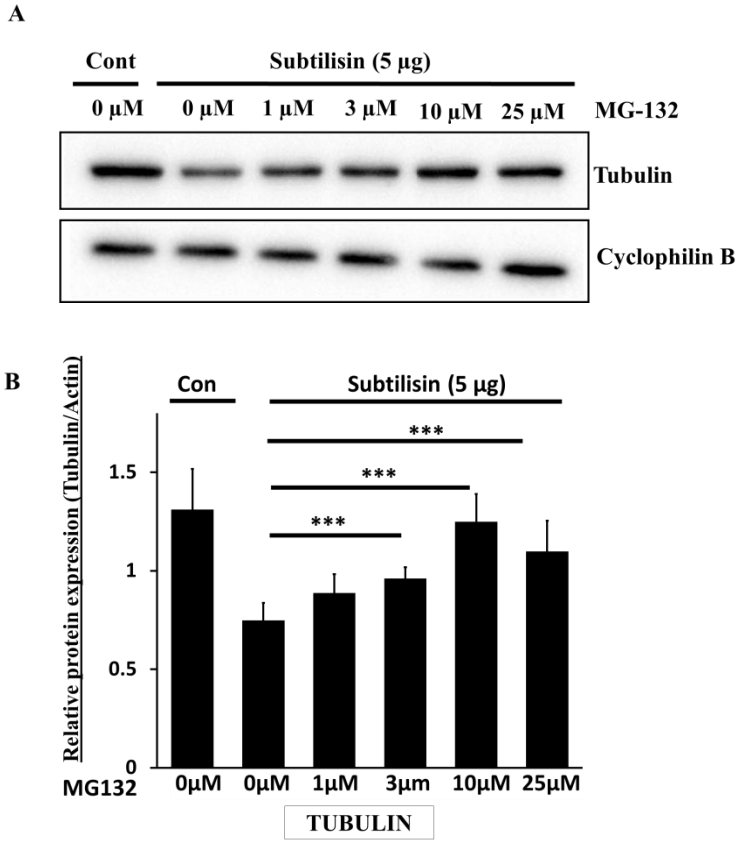


Fig 5.20: (A) Proteasome inhibitor MG-132 restores tubulin level in subtilisin treated MCF-7 cells. Western blot analysis of tubulin level with subtilisin treated at different doses of MG-132 on MCF-7 for 24 h. Cyclophilin-B was used as a loading control and the experiment was repeated three times. **(B) Relative expression of tubulin calculated by ImageJ software after normalization with cyclophilin-B.** Error bar represents standard error from three different biological replicates. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0005$.

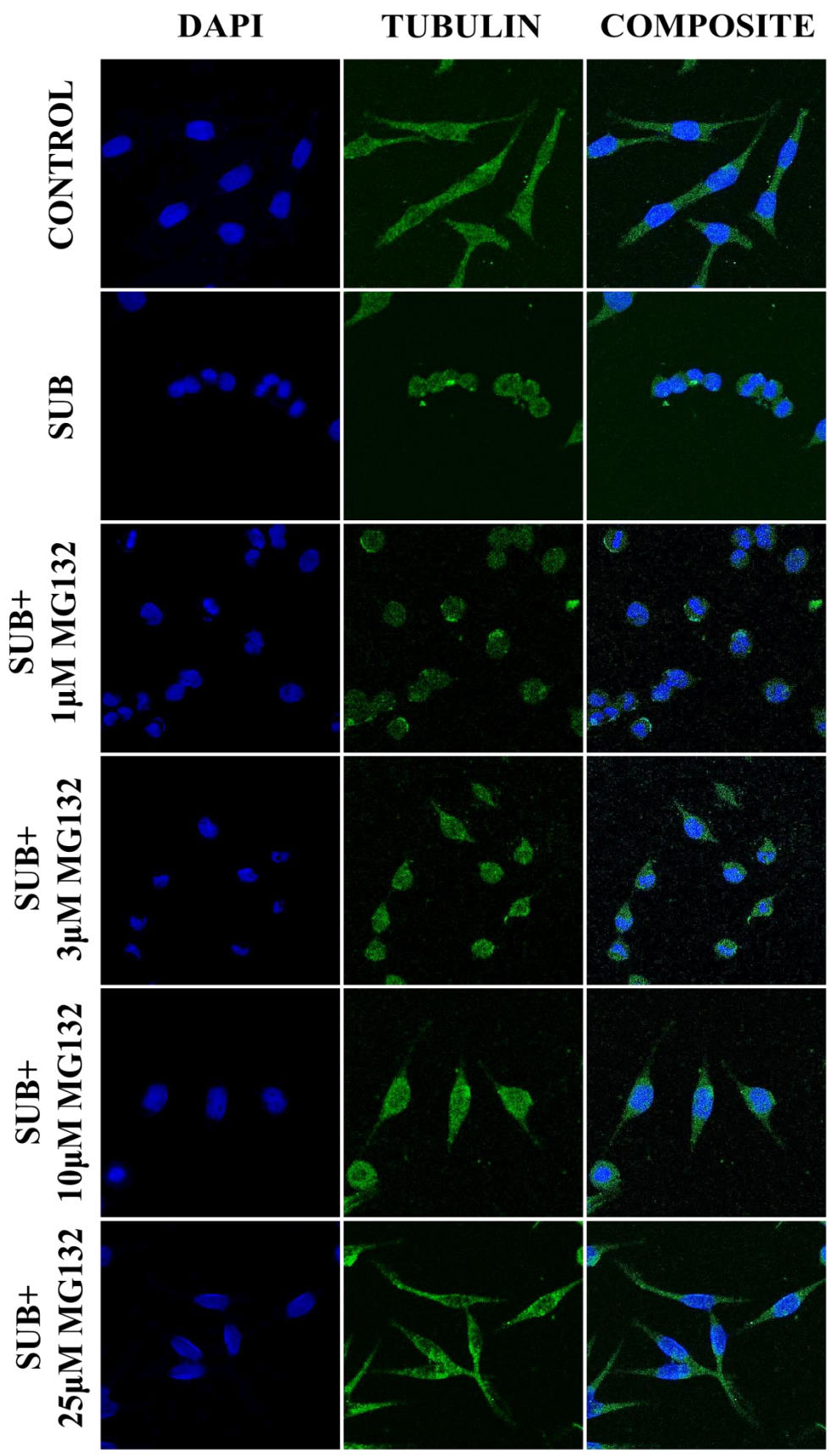


Fig 5.21: Immunofluorescence showed restoration of tubulin in presence of different doses of MG-132.

mediated tubulin degradation was further confirmed by immunofluorescence staining of subtilisin-treated MCF-7 cells in presence of proteasome inhibitor MG-132. The immunofluorescence staining of tubulin showed disruption of cytoskeletal structure of MCF-7 cells after subtilisin treatment. Disruption of cytoskeleton due to subtilisin was completely restored in presence of 10 and 25 µM MG-132 (Fig 5.21). The above findings indicate that the proteasome pathway takes part in tubulin degradation after subtilisin treatment. Any protein which will be degraded through proteasome is needed to be ubiquitinated first [215]. Thus, to test the ubiquitination of tubulin in subtilisin-treated MCF-7 cells, tubulin was immunoprecipitated with anti-tubulin antibody and as we predicted, ubiquitinated tubulin band was detected in western blot analysis. Ubiquitinated band was absent when only subtilisin-treated lysate was used but it gets accumulated in presence of proteasome inhibitor MG-132 (Fig 5.22). Collectively our results indicate that subtilisin induces ubiquitination of tubulin and promotes it's degradation through proteasomes in MCF-7 cells.

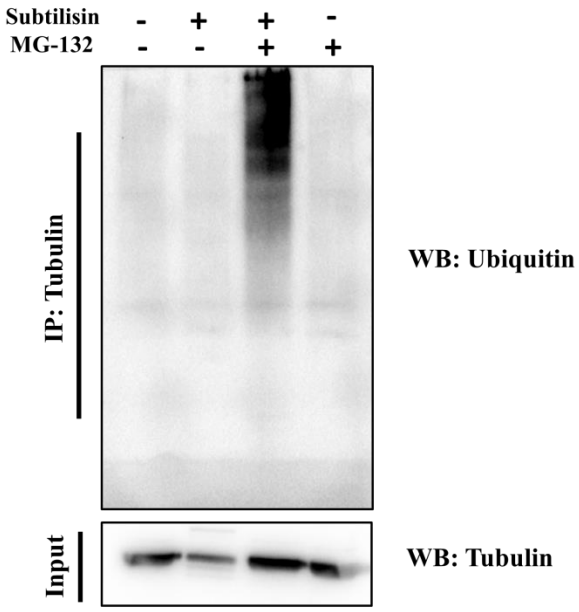


Fig 5.22: Subtilisin treatment (6 h) increased ubiquitination of tubulin in MCF-7 cells. Tubulin was immunoprecipitated (IP) from equal amount of cell lysate by anti-tubulin antibody. Western blot analysis was done by loading equal amount of IP sample and the blot was developed with anti-ubiquitin antibody. Input was developed by loading equal amount of cell lysate on which IP was done.

5.11 Subtilisin induced apoptosis was inhibited by Proteasome inhibitor MG-132:

Apoptosis in MCF-7 cells is examined in presence of a proteasome inhibitor to determine whether subtilisin induced apoptosis in MCF-7 cells is due to tubulin degradation. Our result showed that the proteasome inhibitor MG-132 significantly inhibited apoptosis caused by subtilisin in MCF-7 cells in a dose dependent manner (Fig 5.23). Particularly in presence of 10 and 25 µM of MG-132 there was a significant decrease in the number of apoptotic cells. These results indicate that apoptosis caused by subtilisin in MCF-7 cells occurs because of tubulin degradation.

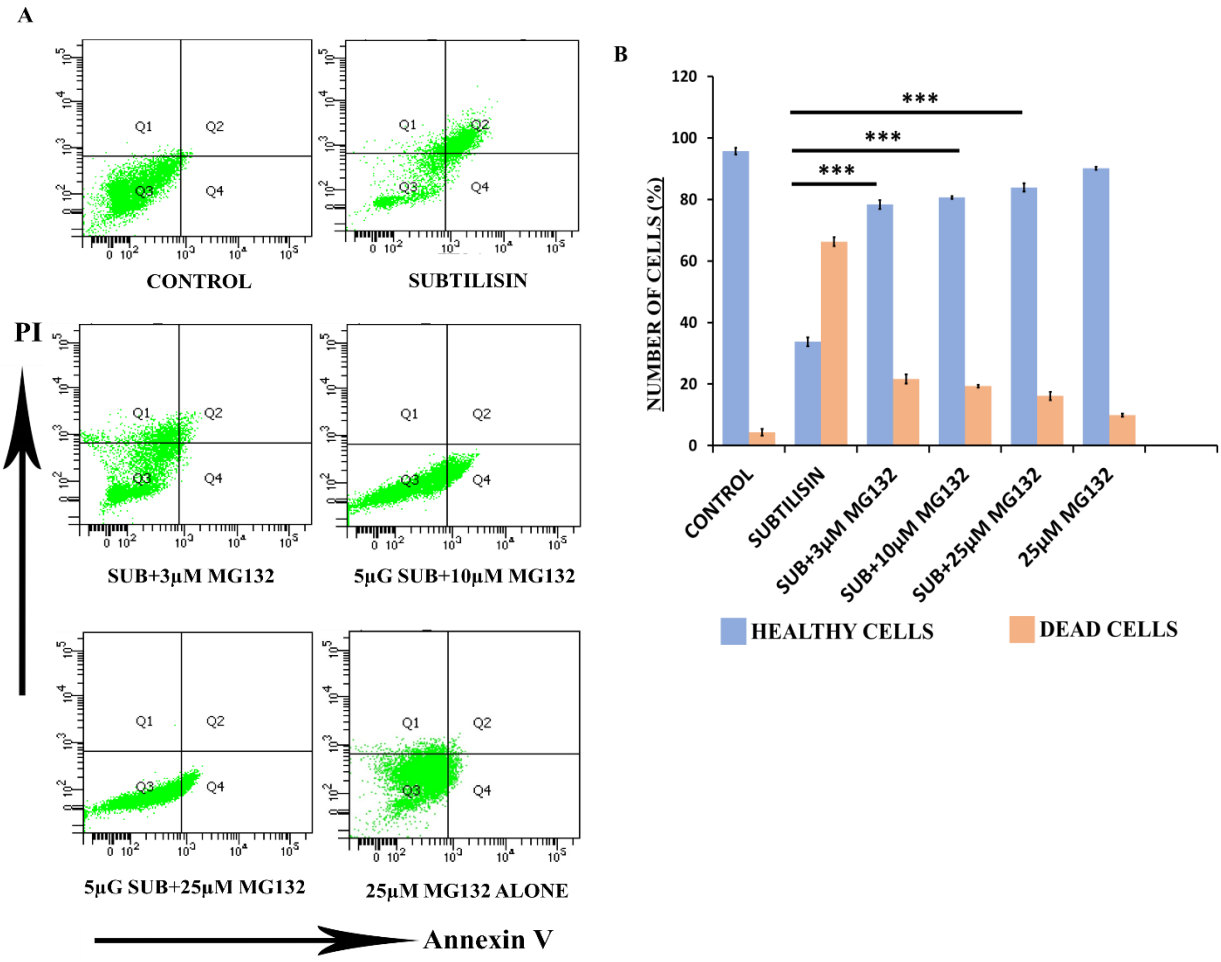


Fig 5.23: Fig. 6. Proteasome inhibitor MG-132 inhibits subtilisin induced apoptosis in MCF-7 cells. (A) Dose dependent effects of MG132 on subtilisin treated MCF-7 cells by flowcytometric analysis. There is signifcant increase in normal cells in presence of MG132 **(B)** Statistical representation of flow cytometric data from A. Error bar represents standard error from three different experimental replicates. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0005$.

5.12 Subtilisin showed cell surface interaction and was unable to directly degrade tubulin:

Cell free lysates of MCF-7 were prepared using 0.5% NP-40 and incubated with subtilisin (5 µg) for 6 hrs and 24 hrs. Our results showed that subtilisin did not degrade tubulin in cell free lysate of MCF-7 after 6 hrs. At 24 hrs there was complete degradation of tubulin and loading control cyclophilin-B (Fig 5.24A) indicating the degradation of total cell lysate.

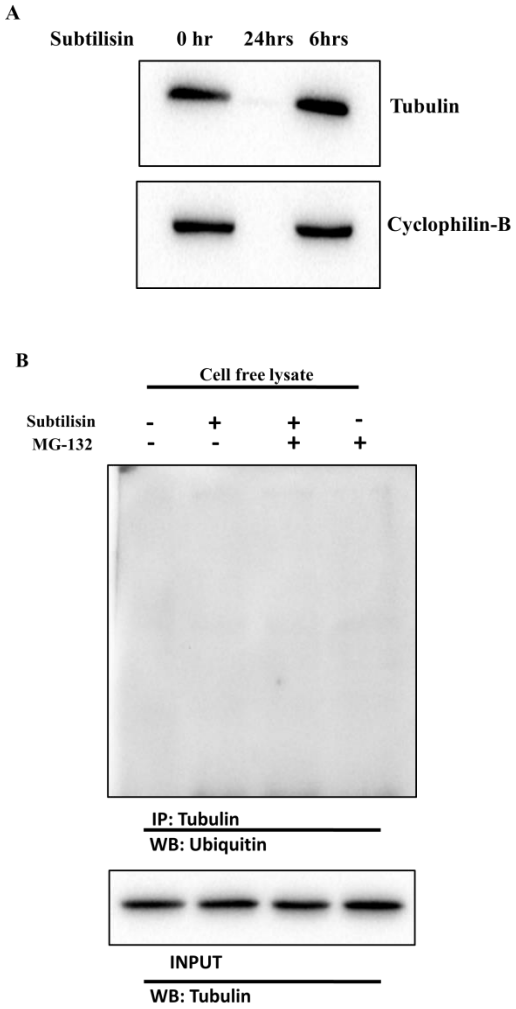


Fig 5.24: (A) Western blot analysis to observe tubulin degradation after 6 hrs and 24 hrs subtilisin treatment on cell free lysate of MCF-7 cells. (B) Effect of subtilisin treatment on tubulin ubiquitination in cell free lysate of MCF-7. Equal amount of cell free lysate was incubated with subtilisin in presence and absence of MG-132. Tubulin was immunoprecipitated and ubiquitination was observed by using anti-ubiquitn antibody.

Ubiquitination of tubulin by immunoprecipitation was not observed after 6 hrs treatment with subtilisin in cell free lysate of MCF-7 (Fig 5.24B). These results showed that subtilisin could not directly degrade tubulin, but it may interact with the cell surface of MCF-7 to induce ubiquitination and proteasomal degradation of tubulin. This was further confirmed by labelling of subtilisin with Alexa Fluor-568 and showing its interaction with MCF-7 cells by confocal microscopy. Data showed that Alexa-568-subtilisin was found to be present only on the cell surface and was absent in the cytoplasm of MCF-7 cells (Fig 5.25). These results indicate possible interaction of subtilisin with cell surface of MCF-7 cells.

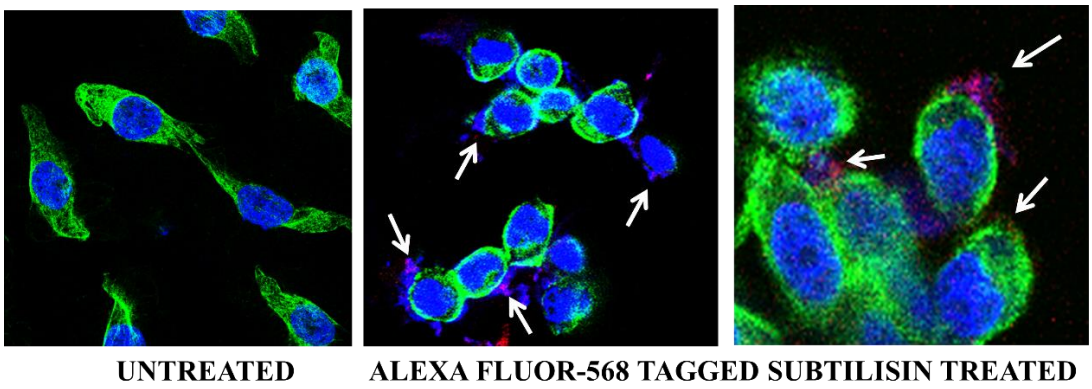


Fig 5.25: Representative confocal images of Alexa-568-subtilisin (orange) treated MCF-7 cells. White arrowhead showing subtilisin binds to the cell surface of MCF-7 cell. Cells cytoplasm was stained with DyLight-488 phalloidin (green) and nucleus was stained with DAPI (blue).

5.13 PARKIN interacted with tubulin and identified as an E3-ligase in subtilisin treated MCF-7 cells:

PARKIN has earlier been reported to act as an E3 ligase of tubulin and has been shown to play a role in ubiquitination and degradation of tubulin [216], so to test whether PARKIN could act as an E3 ligase in subtilisin treated cells, we immunoprecipitated tubulin from

subtilisin treated MCF-7 cells and study PARKIN interaction. Our immunoblot result exhibited increased interaction between PARKIN and tubulin at 3-6 hrs of subtilisin treatment in MCF-7 cells as detected in immune precipitation of tubulin by anti-tubulin antibody (Fig 5.26A). Further knockdown of PARKIN in MCF-7 cells was also performed to

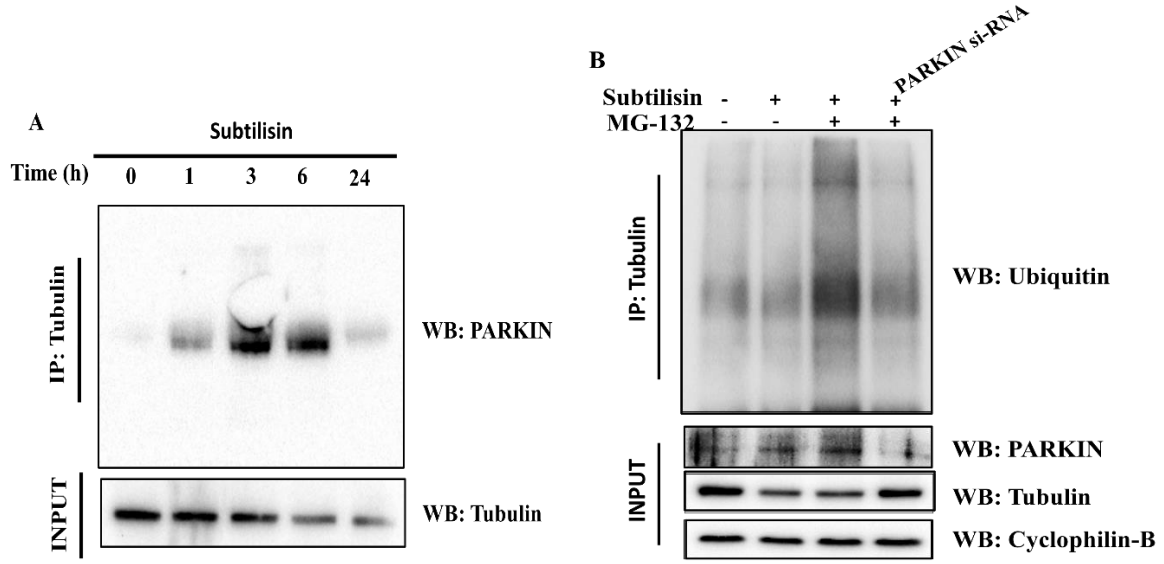


Fig 5.26: (A) Time dependent effects of subtilisin on PARKIN-tubulin interaction in MCF-7 cells. Tubulin was immunoprecipitated by anti-tubulin antibody and the blot was developed with anti-PARKIN antibody. **(B) PARKIN knock down reduces the ubiquitination of tubulin in MCF-7 cells after subtilisin treatment.**

determine whether this would result in reduced tubulin ubiquitination. We found that in presence of PARKIN-siRNA along with subtilisin and MG-132 there was a significant decrease in ubiquitination of tubulin (Fig 5.26B). These data confirms that PARKIN acts as an E3 ligase of tubulin in subtilisin treated MCF-7 cells and induce tubulin degradation. Then we asked whether continuous presence of subtilisin is necessary to sustain the signal of PARKIN-Tubulin interaction and degradation of tubulin. This was tested by withdrawing subtilisin from MCF-7 cells at different time point and examining PARKIN-Tubulin

interactions. We noticed 2 hrs of subtilisin treatment is enough for PARKIN-tubulin interaction (5.27). Therefore, we concluded that 2 hrs of subtilisin treatment can induce interaction of PARKIN with tubulin and after that in absence of subtilisin this interaction can persist.

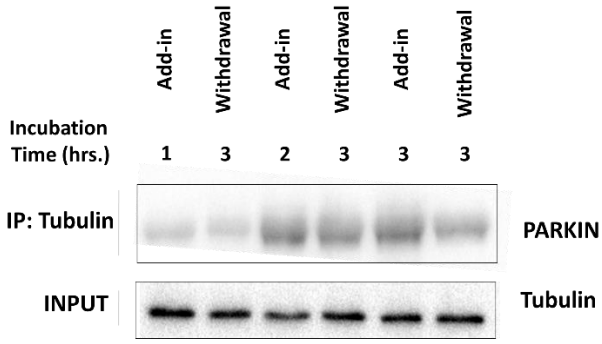


Fig 5.27: Subtilisin treatment for two hours is sufficient to sustain PARKIN-Tubulin interaction. Subtilisin treatment was withdrawn at different time points and following withdrawal, cells were incubated further for 3 hrs. Thereafter, tubulin was immunoprecipitated from cell lysate and interaction with PARKIN was studied.

5.14 Subtilisin induced ER-stress in MCF-7 cells:

It has been reported earlier that activation of the ubiquitin-proteasome system induces ER stress, which results in cellular apoptosis [40]. Similar with previous findings, we addressed if subtilisin can induce ER stress in the cell by activating whole cell proteasomes. To exhibit this, we tested the whole cell proteasome activity at 1, 3 and 6 hrs using whole cell proteasome activation kit. Our result showed that there was an increased proteasomal activity after 3 hrs of subtilisin treatment in MCF-7 cells (Fig 5.28). ER-stress activation can switch between ‘survival’ and ‘death’ pathway. The survival pathway maintains ER homeostasis so that cells can survive. BiP/GRP78 plays an important role in this survival pathway [40]. In the death pathway, ER-stress causes activation of IRE1α [40, 41], one of the important

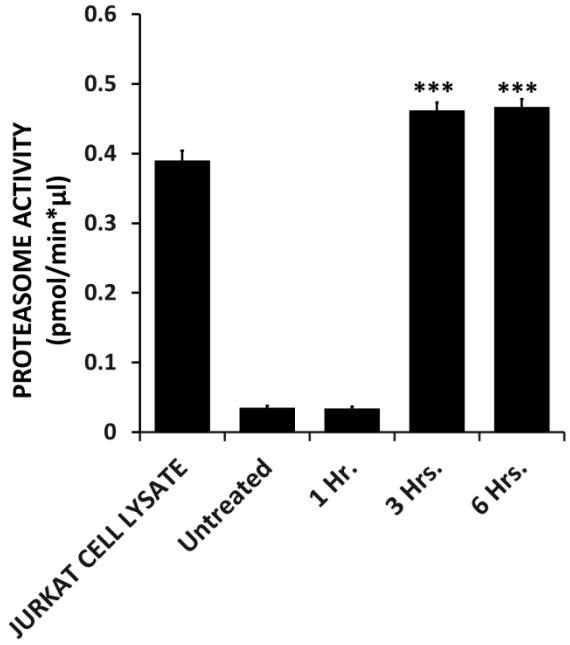


Fig 5.28: Subtilisin treatment induces whole cell proteasomal activity in MCF-7 cells. Relative fluorescence (350/440) was measured and proteasome activity was estimated from AMC standard curve. Statistical test performed using t-test. Error bar represents standard error from three different experimental replicates. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0005$.

components of the pathway. We observed ER stress in subtilisin treated cells by immnobloting different ER-stress markers. MCF-7 cells showed increased expression of ER-stress pro-death markers CHOP and IRE1α after 6 hrs of subtilisin treatment (Fig 5.29A). Increased expression of BiP was also observed at 24 hrs of treatment (Fig 5.29A). These findings suggest that subtilisin treatment can induce ER-stress in MCF-7 cells.

5.15 Subtilisin induced Caspase-7 activation and cleavage of PARP in MCF-7 cells:

To gain further insight into the pathway, we investigated caspase participation in subtilisin treated cells. Caspase-7 and caspase-3 are two important executioner caspases in the cells but caspase-3 is mutated in MCF-7 cells and according to previous report caspase 7 is the main executioner caspase in MCF-7 cells [42, 43]. Hence, we analysed caspase-7 activation in

subtilisin treated MCF-7 cells. Caspase-7 activation was observed at different time points by immunoblot analysis and significant level of cleaved caspase-7 appeared after 6 hrs of treatment with maximum activation in 24 hrs (Fig 5.29A). The dose dependent response in

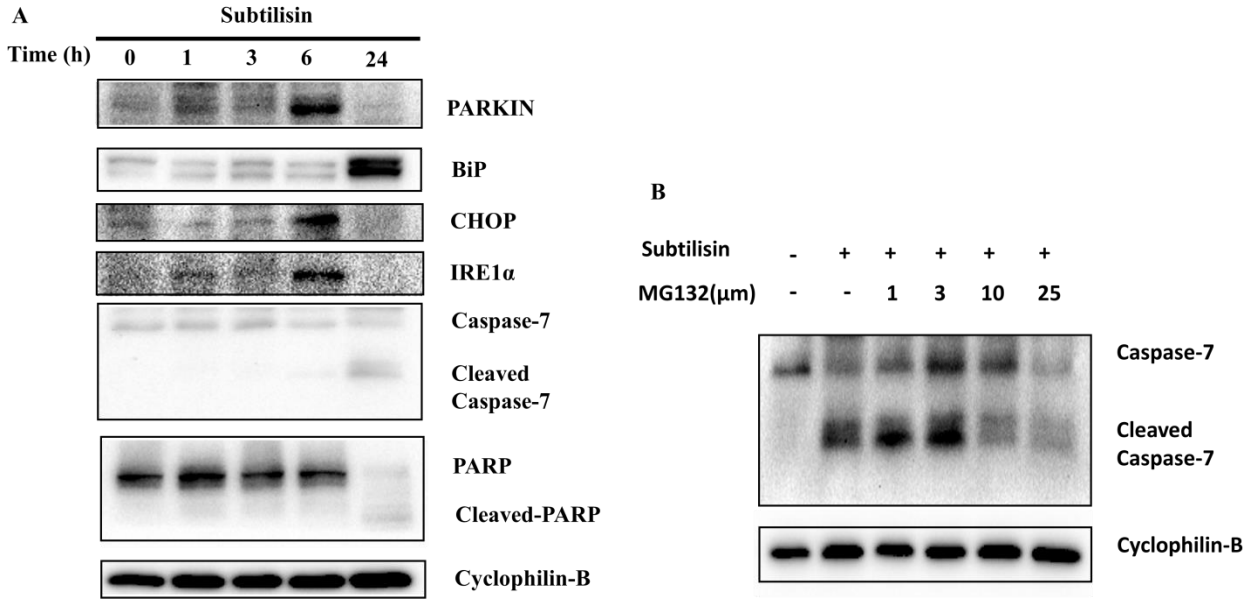


Fig 5.29: (A) Time dependent effects of subtilisin on PARKIN, BiP, CHOP, IRE1α, PARP and caspase-7. Cell lysates were taken from subtilisin treated MCF-7 cells at different time points and western blot was developed by using specifc antibody. **(B) Proteasome inhibitor inhibits caspase 7 activation in subtilisin treated MCF-7 cells.** MCF-7 cells were treated with subtilisin with different doses of MG-132. Cells lysates were collected and western blot of caspase-7 was observed to study effect of MG-132 on caspase-7 activation.

presence of MG-132 after 24 hrs of subtilisin treatment showed significant decrease in caspase-7 cleavage at 10 and 25 µM of MG-132 (Fig 5.29B), suggesting that activation of proteasomal pathway by subtilisin underlies its caspase-7 activation. We also investigated PARP which is a downstream target of caspase-7. Western blot analysis showed presence of cleaved-PARP after 24 hrs of subtilisin treatment in MCF-7 cells (Fig 5.29A). As DNA damage in apoptosis is related with PARP cleavage, DNA degradation status in subtilisin

treated MCF-7 cells was also investigated. Significant nuclear DNA degradation was found in subtilisin treated MCF-7 cells as shown by DNA ladder assay (Fig 5.30). Collectively our results indicate that subtilisin activated caspase-7, induced PARP cleavage and DNA damage in MCF-7 cells which is recognized as a hallmark of apoptosis.

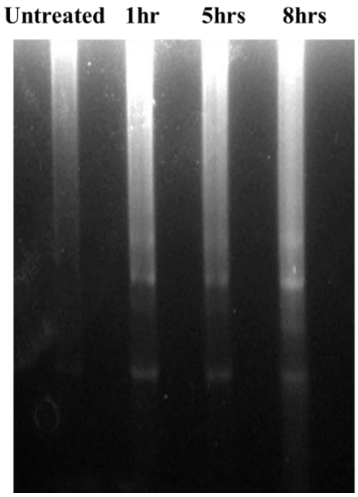


Fig 5.30: DNA ladder assay. MCF-7 cells treated with subtilisin or 1, 5 and 18 h and isolated genomic DNA was run on agarose gel.

5.16 Subtilisin treatment increases survival of EAC tumor bearing mice:

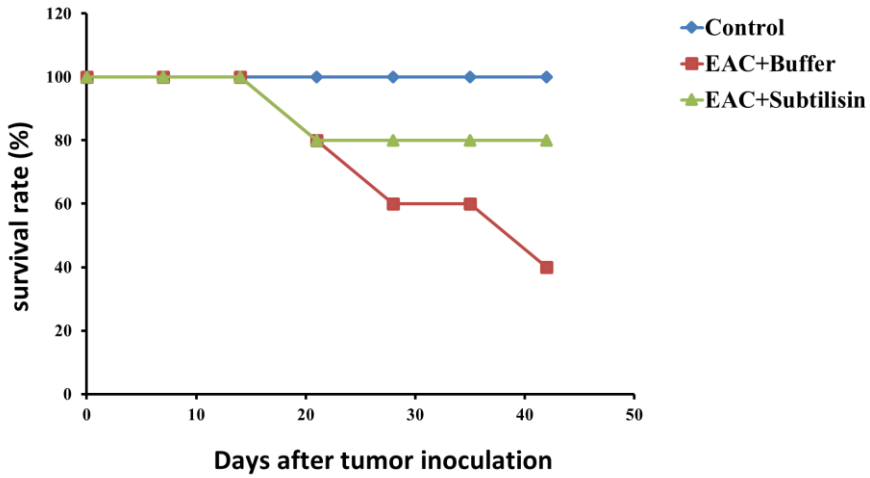


Fig 5.31: Subtilisin treatment showed significant increase in survival rate of EAC induced mice

EAC cells were inoculated intraperitoneally into Swiss albino mice to develop liquid tumor. After 1 week of inoculation mice were divided into three groups, non-tumor bearing control group, tumor bearing untreated group and subtilisin treated group. After 45 days of observation, it was found that only 40% animals were survived in tumor bearing untreated group but in comparison 80% animals were survived in subtilisin treated group (Fig 5.31). This data suggests that subtilisin treatment increase the survival rate of EAC tumor bearing mice.

CHAPTER 6

DISCUSSION

Cancer is a disease related with uncontrolled cell proliferation, with potential to invade other parts of the body and as a result it eventually kills the individual. Conventional therapies of cancer such as surgery, chemotherapy and radiation therapy have various side effects and may develop resistant to these treatments. To overcome these problems there is a need to develop alternative therapies to treat cancer. Treatments of cancer with molecules isolated from microbial origin shows great potential in cancer research. Earlier reports have shown that microbial proteases have anti-cancer activity and are found to be effective against cancer cell growth [20]. Treatment with these proteases reduced tumor size and can be a good candidate for regional treatment of solid tumor [42]. In our earlier studies we had reported that purified HAP secreted by *V. cholerae* showed apoptotic response in EAC breast cancer cell line and colon cancer cells. HAP increased ROS accumulation and triggered the intrinsic apoptotic pathway in cancer cells. In Swiss albino mice, one µg of HAP given at weekly intervals for three weeks significantly reduces tumour growth [217]. Activation and overexpression of PAR-1 was observed in breast cancer cells (EAC) after HAP treatment. HAP binds to PAR-1 and activates NF-kB and MAP kinase signalling pathways [44]. These signalling pathways enhanced the intrinsic apoptotic pathway in the cell. HAP activate PAR-1 and induces apoptosis in cancer cells without affecting healthy cells and makes it a promising anti-cancer agent. In another study we have reported that motility activating factor (MakA) secreted by *V. cholerae* reduced β-catenin level and induced apoptosis in colon cancer cells. Injection of MakA in colon cancer solid tumor model dramatically reduced tumor formation [218]. In this study we have isolated and purified a protease, showing homology with subtilisin from an environmental isolate DHS-96, identified as *Bacillus amyloliquefaciens*. All the bacterial strains were isolated from alkaline environment of salt farm of CSIR-Central Salt and Marine Research Institute, Gujrat India.

Subtilisin, is a bacterial secretory serine protease whose industrial applications are well appreciated. Due to its activity in wide pH range and wide temperature range, subtilisin was the first alkaline serine protease to be used in many biotechnological applications [219]. However, the therapeutic role of subtilisin as an anti-cancer drug has not been elucidated so far. Our study was initiated with the screening of environmental bacterial isolates for protease secretion and we found one of the isolate DHS-96 secreted a protease and also showed anti-cancer properties. The secretory protease from DHS-96 was isolated by ammonium sulphate precipitation and purified by DE-52 ion-exchange chromatography followed by Sephadex-G-75 gel filtration chromatography. The purified protease showed two bands (43 kDa and 29 kDa) in SDS-PAGE and a single band in NATIVE-PAGE. Treatment of cells with the purified protease induces apoptosis in MCF-7 breast cancer cells but not in normal breast cells (MCF10A) and mouse peritoneal macrophages. The characterization of purified protease showed inhibition by PMSF which showed it as a serine protease. The peptide sequencing of the protease showed homology with secretory protease subtilisin, having peptidase S8 domain of serine protease. The domain consists of conserved catalytic triad of amino acid D at 139, H at 171 and S at 328 positions. These findings corroborate with the earlier studies, that reported, subtilisin from *Bacillus amyloliquefaciens* secreted as 42 kDa precursor and 27.5 kDa matured form, in which 27.5 kDa showed as a stable form resulting in autoproteolytic cleavage of the precursor form [220]. The species of the bacteria DHS-96 was identified by 16S rRNA sequencing and the sequence showed 83.12% similarity with *Bacillus subtilis* and 82.95% similarity with *Bacillus amyloliquefaciens*. Amplification of 1.14 kb PCR product was only observed when *Bacillus amyloliquefaciens* specific primers were used and it confirmed that DHS-96 strain belongs to *Bacillus amyloliquefaciens*. Subtilisin is a protease, which belongs to subtilase superfamily and is found in wide range of organisms, such as bacteria, archaea and other eukaryotic micro-organisms [45]. Subtilisin

from Bacillus species is one of the most well studied commercial enzymes due to its use in modern detergent and waste management industry [221]. Mainly two types of subtilisin are used commercially, subtilisin Carlsberg from Bacillus licheniformis and subtilisin Novo or bacterial protease Nagase (BPN') from *Bacillus amyloliquefaciens* [222]. Among them, subtilisin from *Bacillus amyloliquefaciens* is less important commercially but protein sequences of both are highly conserved and these enzymes originate from a common ancestor [223]. Besides wide use in detergent industry, few reports have shown its role in basic research. Subtilisin has been used in combination with antibiotics to treat burn and wound injuries primarily based on its ability to remove specifically non-viable burn tissue without effecting viable tissue [224]. An accumulation of fibrin in the blood vessels can enhance thrombosis [225,226]. Earlier it has been reported that subtilisin have fibrinolytic activity and can be used as an oral fibrinolytic agent [227–229]. In a very recent study, engineered subtilisin has also been used to target active RAS, a small GTPase to treat cancer cells [47]. In this report we have studied the anti-cancer properties of subtilisin and demonstrated that subtilisin induced apoptosis in MCF-7 cells.

Apoptosis or programmed cell death is a complex biochemical mechanism in which pro-and anti-apoptotic proteins regulation occur [230]. The process can play crucial role in both physiological and pathological condition of the cell [231]. Various conditions can stimulate the event of apoptosis of a cell. It can be intracellular signal or extracellular signal. There are mainly two types of cellular signalling pathways activated in apoptosis, intrinsic pathway or mitochondrial pathway and extrinsic pathway or death receptor pathway [232]. In the final stage of apoptosis both the pathways activate the executioner caspases to execute the apoptosis [233]. Targeting apoptosis by using own cellular machinery is the main strategy to kill cancer cells. Most of the anti-cancer drug targeted apoptosis pathway to gain regulation of uncontrolled proliferation of cancer cells [234–236]. We examined the apoptosis of MCF-

7 cells by Annexin-V/PI staining after subtilisin treatment and found that subtilisin treatment significantly increase Annexin-V^+/PI^+ cells compared to untreated control cells. This confirms that subtilisin can activate apoptosis in MCF-7 cells. In order to investigate the signalling pathway, we also investigated the apoptotic makers of MCF-7 cells after subtilisin treatment. In the canonical intrinsic pathway of apoptosis, regulation of pro-apoptotic bax and anti-apoptotic bcl-2 is important [237]. Our experimental results showed that subtilisin did not induce the intrinsic pathway of apoptosis and there were no changes in the ratio of pro-apoptotic bax to anti-apoptotic bcl-2. Thus, the question arises as to whether subtilisin could activate apoptosis by activating another pathway or by regulating a different cellular component.

Tubulin was used as a loading control of our western blot experiment. It was observed that overnight incubation of MCF-7 cells with 5 µg of subtilisin could decrease tubulin level in the cells. Time dependent observation of tubulin after subtilisin treatment showed that after 6 hrs of treatment tubulin level decreased inside the cells. The subtilisin treatment could not regulate tubulin at mRNA level as we did not find any significant difference of the Ct value in subtilisin treated sample compared to untreated control. In order to study tubulin degradation, we investigated the ubiquitin-proteasome pathway, which is also one of the major cell degradation pathways.

The ubiquitin-proteasome pathway degrades majority of the intracellular proteins in eukaryotic cells. The targeted proteins which are needed to be degraded are tagged with ubiquitin and the ubiquitinated proteins are then degraded by 26S proteasome into smaller peptides [238]. Three enzymes E1, E2 and E3 are essential for degrading ubiquitin tagged proteins. E1-E2 helps to guide activated ubiquitin for ligation and E3 acts as a ligase, which can recognise the protein substrate and ligate it with ubiquitin [239]. MG-132 is widely used

as a proteasome inhibitor to study the role of ubiquitin-proteasome pathway in various cellular events, because of its high potency and selectivity against 26S proteasome complex [240]. Our studies showed that tubulin level was restored when we pre-treated MCF-7 cells with proteasome inhibitor MG-132. 10 µM and 25 µM doses of MG-132 completely restored tubulin, which indicates the involvement of proteasome in tubulin degradation after subtilisin treatment. Tubulin one of the major cytoskeletal proteins of the cells, tubulin degradation after subtilisin treatment led to cytoskeletal disruption of the MCF-7 cells. Confocal microscopy using antibody staining of tubulin showed that subtilisin treatment disrupt the cytoskeletal structure of MCF-7 cells and use of MG-132 could restore cytoskeletal structure in dose dependent manner. Any protein which is targeted for proteasomal degradation needs to be tagged with ubiquitin first. Co-immunoprecipitation experiment with tubulin, showed that tubulin is ubiquitinated in presence of MG-132 after subtilisin treatments. These results suggest that subtilisin degrades tubulin through ubiquitin-proteasomal mediated pathway in MCF-7 breast cancer cells. In an earlier report it has been shown that PARKIN, a protein E3 ligase linked to Parkinson's disease strongly binds with α/β tubulin heterodimer. This PARKIN-tubulin interaction activates ubiquitination and degrades tubulin [216]. Immunoprecipitation with tubulin showed interaction of PARKIN with tubulin in MCF-7 cells at 3 hrs and 6 hrs of subtilisin treatments. Additionally, si-RNA knock-down of PARKIN greatly reduced the tubulin ubiquitination. These results confirms that PARKIN acts as an E3 ligase of tubulin in subtilisin treated MCF-7 cells.

Our results showed that subtilisin treatment induced ubiquitin-proteasome mediated tubulin degradation in MCF-7 cells. Ubiquitin-proteasome system helps in maintaining protein homeostasis of our cells [241]. Different damaged or misfolded protein which are toxic to our cells can be cleared from the cells via this system. It is a well-established fact that some form of destabilization of the protein inside the cells could lead to activation of proteasome system

[242,243]. Although the mechanism by which subtilsin can destabilize tubulin so that it can be degraded by the cell's proteasome system has yet not identified. Our experiments revealed that subtilisin treatment could induce whole cell proteasomal activity of the MCF-7 and from 3 hrs onward the activity is significantly high in the treated cells when compared to untreated control cells.

Increased ubiquitin-proteasome activity leads to ER-stress in the cells [244]. The two major pathways in ER stress are the pro-death pathway and the pro-survival pathway. In pro-death pathway, pro-death factors (e.g. IRE1α) and in pro-survival pathway, pro-survival factor (GRPs protein) accumulates in the cells [245]. Subtilisin treatment induced the expression of pro death factors IRE1α and CHOP in MCF-7 cells. In the cellular apoptotic event execution of apoptosis occurs by PARP cleavage and DNA fragmentation of the cell [246,247]. The executioner caspase-3 and caspase-7 play important roles in this phase and if activated it can cleave different intra-cellular proteins and execute apoptosis. In MCF-7 cells caspase-3 is mutated [248]. Caspase-7 acts as an executioner caspase in MCF-7 cells and PARP is more efficiently cleaved by caspase-7 than caspase-3 [249]. In our study, we found, subtilisin treatment induced caspase-7 activation in MCF-7 cells but proteasome inhibitor MG-132 can revert back this activation in a dose dependent manner. We also showed subtislisin treatment activates PARP cleavage and nuclear DNA degradation, leading to apoptosis in MCF-7 cells.

Microtubule targeting agents (MTAs) bind to tubulin and disrupt the microtubule dynamics causing cell cycle arrest and apoptosis. Cancer therapeutics that use MTAs have been shown to have great potential. Various tubulin targeting drugs (paclitaxel, docetaxel and vinblastine) have been used successfully in cancer treatments and are shown to be very effective against cancer [203]. A microtubule targeting small molecule T0070907, causes tubulin degradation through proteasomal mediated pathway. Molecules like T007097, can be utilised to decrease

cellular tubulin levels, suggesting that induced tubulin degradation could lead to therapies that target tubulin [250]. Subtilisin can be used as MTA, since our study has shown that subtilisin could induce tubulin degradation in MCF-7 cells through ubiquitin-proteasomal dependent pathway. Subtilisin may induce tubulin degradation in MCF-7 cells through some unknown cell surface receptor and further studies are in progress to identify the receptor responsible for subtilisin mediated apoptotic induction. Further *in-vivo* studies are required to understand the complete properties of subtilisin as an anti-cancer molecule.

The potential of subtilisin from *Bacillus amyloliquefaciens* as an anti-cancer drug and its mechanism of action have been established in this study. Subtilisin induces apoptosis only in cancer cells but does not affect the normal cells. Subtilisin activates ubiquitination of tubulin by enhancing PARKIN-tubulin interaction, resulting in tubulin degradation that induces apoptosis in cancer cells. To execute apoptosis, subtilisin induces caspase-7 activity, PARP cleavage and nuclear DNA damage in the cells. Our results show that subtilisin can be a good therapeutic candidate for cancer treatment.

SUMMARY

- In an attempt to purify novel proteases having anti-cancer activity, in this study we have purified secreted protease subtilisin from environmental isolate *Bacillus amyloliquefaciens*. The bacterial isolate was purified from salt firm of CSIR-Central Salt and Marine Research Institute (Lat. 21◦47′51′N and Long. 72◦7′38″E) Gujrat, India.
- Secreted protease was purified from the environmental isolate by ammonium sulphate precipitation, ion-exchange chromatography followed by gel filtration chromatography. Purified protease showed two bands (43 kDa and 29 kDa) in SDS-PAGE and a single band in NATIVE-PAGE. Mass spectrometric analysis of the protease identified it as subtilisin. The bacterial isolate was identified by 16S-rRNA sequencing followed by gene specific PCR. The identification revealed the isolate was *Bacillus amyloliquefaciens*.
- Subtilisin is an alkaline secretory protease widely used in commercial industry including detergent, leather, food industry for long time. However, the study subtilisin in anti-cancer research have not ben explored earlier. This study is the first to identify molecular mechanism through which subtilisin might prevent cancer.
- In this study we showed that subtilisin could kill MCF-7 and HT-9 cancer cells but did not show any effect on normal MCF-10A cells and mouse peritoneal macrophages.
- It was found that subtilisin induced apoptosis in MCF-7 breast cancer cells and the apoptosis was inhibited when serine protease inhibitor PMSF was used.
- The pathway analysis revealed that subtilisin activate tubulin degradation of MCF-7 cells while there was no change at mRNA level. Our experimental data found that tubulin degradation occurs after 6 hrs of subtilisin treatment.

- An analysis of tubulin degradation found proteasome inhibitor MG-132 could inhibit subtilisin induced tubulin degradation in a dose dependent manner. When MCF-7 cells were pre-treated with dose of 10 μM and 25 μM of MG-132, degradation of tubulin was inhibited. Imaging of the cells showed subtilisin treatment disrupt the cytoskeletal structure of the cells but it was restored in a dose dependent way when proteasome inhibitor MG-132 was used. Our result also showed that apoptosis after subtilisin treatment significantly inhibited when proteasome inhibitor MG-132 used.
- Whether subtilisin could induce tubulin degradation through direct interaction or there is any cell surface mediated interaction taking place, we tagged subtilisin with Alexa Fluor-568 and analysed its interactions with MCF-7 cells. We found subtilisin could not enter into the cells rather it present outside the cell surface. Incubation of subtilisin with cell free lysate of MCF-7 also showed no degradation of tubulin after incubation. These all indicate possible interaction of subtilisin with cell surface of MCF-7 cells.
- Targeted protein that degraded through proteasome pathway needs to be ubiquitinated first. Immunoprecipitation experiment revealed that subtilisin treatment induced ubiquitination of tubulin in MCF-7 cells and PARKIN, one of the known E3 ligase of tubulin interacted after 3 hrs of subtilisin treatment, which can last for 6 hrs post treatment. Knock down of PARKIN by si-RNA showed significant decrease in ubiquitination and increase in tubulin level in the cells. Our result showed if we withdraw subtilisin after 2 hrs of treatment, PARKIN-Tubulin interaction could persist.
- There was significant increase in whole cell proteasomal activity was found after 3 hrs of subtilisin treatment. As ER-stress is linked with proteasomal activation, we

tested ER-stress after subtilisin treatment in MCF-7 cells. We found ER-stress marker BiP, CHOP, IRE1α was upregulated following subtilisin treatment inside the cells.

- To study the executioner phase of apoptosis caused by subtilisin treatment, our result showed there was activation of executioner caspase-7 in MCF-7 cells. Activation of caspase-7 was inhibited in a dose dependent manner when proteasome inhibitor MG-132 was used. PARP, which is a downstream target of caspase-7, was also found cleaved after subtilisin treatment. DNA degradation, which is a hallmark of apoptosis was also investigated in MCF-7 cells after subtilisin treatment. Significant nuclear degradation was found in subtilisin treated MCF-7 cells.
- In animal experiments subtilisin treatment could increase the survival of EAC induced liquid tumor bearing mice. Our result showed 80% animals survived in subtilisin treated group compared to untreated group.

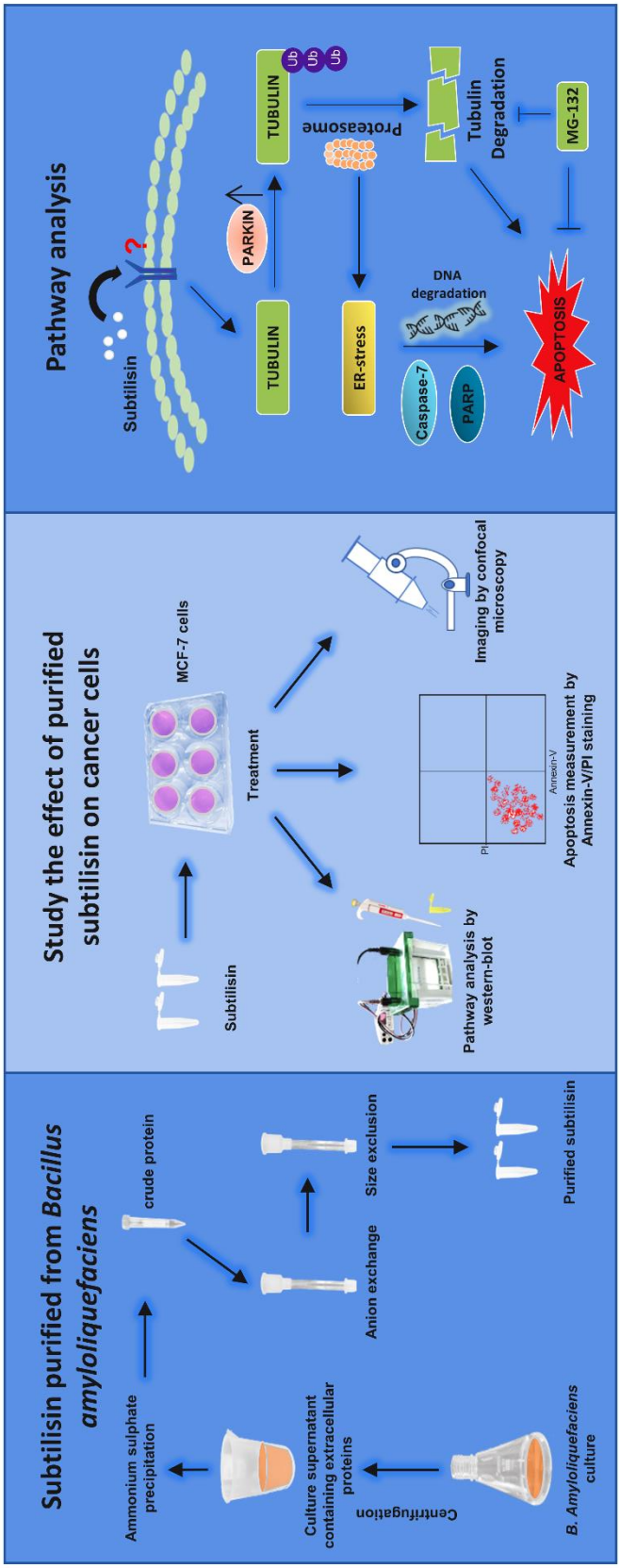


Fig: Graphical representation of experimental designing and analysis of the study

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Subtilisin from *Bacillus amyloliquefaciens* induces apoptosis in breast cancer cells through ubiquitin-proteasome-mediated tubulin degradation

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ABSTRACT

To search for novel proteases from environmental isolates which can induce apoptosis in cancer cells, we have purified subtilisin from *Bacillus amyloliquefaciens* and studied its anti-cancer properties. Subtilisin induced apoptosis in colon (HT29) and breast (MCF7) cancer cells but showed no effect on mouse peritoneal macrophages and normal breast cells (MCF10A). Western blot analysis showed that Bax, Bcl-2 level remained unchanged but tubulin level decreased significantly. Subtilisin does not induce the intrinsic pathway of apoptosis, rather it induced tubulin degradation in MCF-7 cells, whereas in normal cells (MCF-10A) tubulin degradation was not observed. Subtilisin activates ubiquitination and proteasomal-mediated tubulin degradation which was completely restored in presence of proteasome inhibitor MG-132. We further observed PARKIN, one of the known E3-ligase, is overexpressed and interacts with tubulin in subtilisin treated cells. Knockdown of PARKIN effectively downregulates ubiquitination and inhibits degradation of tubulin. PARKIN activation and tubulin degradation lead to ER-stress which in turn activates caspase-7 and PARP cleavage, thus guiding the subtilisin treated cells towards apoptosis. To our knowledge this is the first report of subtilisin induced apoptosis in cancer cells by proteasomal degradation of tubulin.

1. Introduction

Cancer remains the main cause of death worldwide along with cardiovascular disease [1,2]. Various endogenous and exogenous stimuli can cause normal cells to change their molecular and cellular makeup, leading to uncontrolled growth of cells that transform normal cells into cancerous cells [2–4]. Early diagnosis, chemotherapy, radiation and surgical therapy can decrease mortality associated with cancer [5,6]. Though several approaches have been used to treat cancer, due to the limitation of current therapeutic options, one of the goals of effective cancer therapy is to search for anticancer drugs.

Natural products from plants, environment and microorganisms have helped us in the fight against cancer and over 60 % of today's anticancer compounds originate from these sources [7–9]. Natural products are advantageous as they do not cause problems that are associated with chemotherapy, such as multi-drug resistance, toxicity and lack of specificity which results in the destruction of both cancer and normal cells [10–14]. Microbes have become an important source for natural product which can be used as drugs. Among the advantages of using microorganisms is the abundance of species which can be a source of structurally diverse natural products. These products can be purified in a relatively cost-efficient manner compared to chemically synthetic drugs [15,16]. Many microorganisms have been screened for anti-cancer drugs and studies have shown that it causes apoptosis and can inhibit cell proliferation [17,18]. Bacterial toxins used for cancer therapies showed an effect on cellular proliferation, differentiation and apoptosis in cancer cells [19]. *Clostridium perfringens* enterotoxin (CPE) secreted by *Clostridium perfringens* causes cytotoxicity on pancreatic cancer cells and prevents tumor growth *in vivo* [20,21]. Similarly, the *Bordetella pertussis* adenylate cyclase (AC) toxin has been shown to trigger apoptosis in different malignant cells [22,23]. Botulinum neurotoxin type-A from *Clostridium botulinum* showed an apoptotic effect on human prostate cancer cells and is used in the treatment of benign prostate hyperplasia [24,25]. Azurin secreted by *Pseudomonas aeruginosa* inhibits cancer cell growth by forming a complex with p53 and inhibiting the EphB2 signaling pathway in the cell. Peptides generated from azurin have also

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been shown to be effective against breast cancer cells, melanoma cells and oral squamous carcinoma cells [26–30]. *Vibrio cholerae* cytolysin, motility-associated factor A (MakA) caused cytotoxic effects on cancer cells without affecting the normal cells. MakA injection in mice significantly slowed the progression of solid tumor formation in colon cancer-induced mice model [31]. Proteases isolated from microbial sources can also act as therapeutics against cancer. A protease secreted from *Serratia marcescens* has anti-cancer properties [32]. In our earlier studies we showed that *Vibrio cholerae* hemagglutinin protease (HAP) increased cellular ROS levels, triggered the intrinsic apoptotic pathway through PAR-1 activation, and reduced tumor growth in a mouse model [33,34].

In search for similar microbial proteases from environmental sources which can induce cell death by apoptosis in cancer cells, we identified and purified an extracellular protease subtilisin from an environmental isolate *Bacillus amyloliquefaciens*. Though commercial use of subtilisin has been extensively studied [46], its anti-cancer properties have not been addressed earlier. In the present study, we evaluated the anti-cancer properties of subtilisin and the mechanism of its action was assessed.

2. Materials and methods

2.1. Chemicals and reagents

Azocasein, ammonium sulphate, TCA, PMSF, EDTA, 1, 10-phenanthroline and other general chemicals, unless otherwise specified, were from Sigma-Aldrich (USA). Nutrient broth from Difco-BD (USA), penicillin-streptomycin, fetal bovine serum (FBS), minimum essential medium (MEM), trypsin, and Dulbecco's modified Eagle's medium (DMEM) were from Gibco (Rockville, MD), mammary epithelial cell growth basal medium (MEBM) and mammary epithelial cell growth medium (MEGM) were from Lonza (Swizerland). DNA ladder, BCA assay kit and DreamTaq green mastermix from ThermoFisher (USA), protein ladder from Genetix (India). Annexin V-PI staining kit from BD-Bioscience (USA), genomic DNA isolation kit was from Qiagen (Germany), PARKIN si-RNA was from Santa Cruz Biotechnology and proteasome activity assay kit from Abcam (UK). All the primary antibodies, protein A/G agarose beads and prolong anti-fade DAPI were from Cell signaling technology (USA). Anti-tubulin antibody and all the secondary antibodies were from Santa-Cruz Biotechnology (USA). Primers used here were purchased from IDT (USA).

2.2. Bacterial isolates and growth condition

A total of 140 bacterial isolates from salt farm of CSIR-CSMRI, Bhavnagar, Gujrat (Lat. 21°47′51N and Long. 72°7′38″E) were screened for protease activity. The bacterial isolates were stored at -80 °C in 25 % glycerol. To screen for protease activity bacterial isolates were revived in nutrient broth (NB) (containing 2 % NaCl, pH -8) and incubated at 37 °C till the OD reached 0.6. The pre-culture was inoculated in nutrient broth at a ratio of 1:100 and incubated overnight. The culture supernatant was used for protease screening.

2.3. Purification and identification of subtilisin

Subtilisin was purified from culture supernatant of DHS-96. The organism was grown in 600 ml NB for 18 h at 37 °C shaker incubator. The culture was centrifuged at 12,000 rpm for 10 min at 4 °C, the supernatant was collected and salted out using 80 % saturated ammonium sulphate and kept at 4 °C for 2 days to precipitate the protein which was collected by centrifugation at 14,000 rpm at 4 °C for 10 min. The pellet was dissolved in 25 mM Tris-HCl, pH -8 and dialysed (HIMEDIA- dialysis membrane, cat- LA395-30MT) against the same buffer for 48 h at 4 °C. After dialysis the protein solution was concentrated using speed-vac vacuum centrifugation and purified using anion exchange DE-52 column (2.5 × 20 cm), pre-equilibrated with 25 mM Tris-HCl pH-8. The non-binding fraction was pooled, concentrated and assayed for protease activity. DE-52 bound proteins were eluted with an increasing concentration of NaCl (0.1 M to 0.5 M). The eluted fractions were pooled, dialysed and concentrated. Protease activity was detected in the proteins eluted in the non-binding fraction, which was then further purified by Sephadex G-75 gel filtration chromatographic column (1.5 × 30 cm) with 25 mM Tris-HCl, pH-8. Protease active fractions were eluted, concentrated, and analysed by NATIVE and SDS-PAGE. The major bands from SDS and NATIVE-PAGE were sent to cCAMP (Paid Mass spectrometry facility of NCBS Bangalore, India) for MALDI-ToF mass spectrometry. Peptide sequence generated from MS/MS spectra was searched in NCBI database for homology alignment.

2.4. Azocasein assay

Azocasein was used as a substrate to determine the protease activity both for the culture supernatant of bacterial isolates and for the proteins eluted during purification as described in our earlier study [35]. Briefly, protein samples or culture supernatant from individual bacterial isolate was mixed with 0.1 % azocasein and incubated for 1 h at 37 °C. The reaction was stopped with 10 % TCA (Tri-Chloro Acetic Acid) and centrifuged for 10 min at 10,000 rpm. The supernatant was mixed with 500 mM NaOH and absorbance was measured at 440 nm. NB and purified *Vibrio cholerae* HAP were used as negative and positive control respectively.

2.5. Biochemical characterization of the protease

In order to study the nature of subtilisin, known inhibitors of protease, PMSF (10 mM) and EDTA (10 mM) were used. 100 mM stock of PMSF and 500 mM stock of EDTA were prepared in sterile water. Inhibition of protease activity was carried out using 5 μg of purified protease incubated with inhibitors at 37 °C and after incubation for 1 h, azocasein assay was performed.

To measure the optimum pH for protease activity, subtilisin was dialysed against buffers ranging from pH 2 to 12 and the activity of the protease was determined using azocasein assay. For pH 2–5, 25 mM acetate buffer, for pH 6–7, 25 mM PBS buffer and for pH 8–12, 25 mM Tris-HCl buffer were used to achieve different pH.

To find out the optimum temperature for protease activity, 5 μg of subtilisin was incubated at different temperatures: 25 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C and 100 °C for 60 min and protease activity was performed by azocasein assay.

2.6. 16S-rRNA sequencing and subtilisin gene specific PCR for bacterial identification

16S-rRNA sequencing was carried out for identification of the species. Genomic DNA was isolated and 16S-rDNA was amplified (Forward primer (Fwd) 5′ – GGATGAGCCCGCGGCCTA – 3′, reverse primer (Rev) 5′ – CGGTGTGTACAAGGCCCGGGAACG – 3′). Purified PCR product was sequenced bi-directionally and the data was aligned and analysed to identify the bacteria and its closest neighbour. 16S-rRNA sequencing identified two closely related species, *Bacillus subtilis* and *Bacillus amyloliquefaciens.* To differentiate between the two species subtilisin gene was amplified using species-specific primers. Two sets of primers from *Bacillus subtilis* (Fwd 5′–AGCGGGATCCATGAGAAGCAAAAAATTGT GGATC– 3′, Rev. 5′–AATACTCGAGCGTTGTGCAGCTGCTTGTACGTT– 3′) and *Bacillus amyloliquefaciens* (Fwd 5′–AGCGGGATCCATGAGAG GCAAAAGTATGATCAGT–3′, Rev. 5′–AATACTCGAGCGCTGAGCTGCCG CCTGTACGT– 3′) were used. For amplification, total genomic DNA was isolated from overnight grown bacterial culture using gram-positive bacterial genomic DNA isolation kit (Qiagen) as described in the manufacturer's instructions. Amplification reaction was performed with DreamTaq green mastermix in an automated thermal cycler (Bio-Rad, USA) under the following conditions. 10 min initial denaturation at

95 °C followed by 35 cycles of 1 min denaturation at 95 °C, 30 s annealing at 52 °C, followed by 1 min extension at 72 °C and final 10 min extension at 72 °C.

2.7. Cell culture and treatment

Human breast cancer cell line MCF-7 was cultured and maintained in MEM, human colon cancer cell line HT-29 and mouse peritoneal macrophages cells were cultured in DMEM supplemented with 10 % FBS, 100 μg/ml Streptomycin sulphate and 100 U/ml penicillin-G at 37 °C in presence of 5 % CO_2. MCF10A, a non-tumorigenic breast epithelial cell line, was grown in MEBM along with MEGM-bullet kit (containing BPE, hEGF, Insulin, hydrocortisone) and 100 ng/ml cholera toxin. Mouse peritoneal macrophages were isolated from BALB/c mice using Phosphate Buffer Saline (PBS) and cultured in DMEM, with 10 % FBS, 100 μg/ml Streptomycin and 100 U/ml penicillin-G. MCF10A cell was purchased from American Type Culture Collection (ATCC). MCF-7 and HT-29 cells were provided by Chittaranjan National Cancer Institute (CNCI), Kolkata.

For treatment, six-well tissue culture plates were seeded with 10^6 numbers of HT-29 and MCF-7 cells. After 70 % confluency, cells were washed with 1× PBS twice and starved for 6 h in serum-free medium. HT-29 cells were treated with filter sterilized bacterial culture supernatant and MCF-7 cells were treated with different doses of subtilisin (0, 3, 5 μg/ml) for 24 h for flow cytometric analysis. MCF-7 cells were treated with 5 μg/ml of subtilisin for immunoblot, immunoprecipitation, immunofluorescence, Real-Time PCR and proteasome activity measurement. For treatment with proteasome inhibitor MG-132, MCF-cells were pre-incubated with MG-132 for 1 h prior to treatment with subtilisin.

2.8. Apoptosis assessment by flow cytometric analysis

A quantitative assessment of apoptosis was done using an apoptosis detection kit (BD-bioscience, USA), where cells were stained with FITC conjugated annexinV/propidine iodide (PI) and analysed in flow cytometer. Six-well tissue culture plates (Corning, USA) were seeded with 1×10^6 MCF-7 and HT-29 cells. Cells with 70 % confluency were washed once with 1× PBS and starved for 6 h in serum-free medium. HT-29 cells were treated with filter sterilized bacterial culture supernatant and MCF-7 cells were treated with subtilisin in different doses (0, 3, 5 μg/ml) for 24 h. To observe apoptosis in presence of proteasome inhibitor, MCF-7 cells were pre-incubated with different doses of MG-132 (1, 3, 10, 25 μM). After treatment, cells were pelleted down and washed with 1× PBS. The cells were re-suspended in 1× binding buffer (provided with BD annexin-V kit) and transferred into 2 ml FACS tube. Annexin V (2 μl) and PI (1 μl) were added to each tube. Following incubation for 5 min at RT in dark, samples were analysed in FACS Aria II using 'Cell Quest' software.

2.9. Western blot analysis

Following treatment with subtilisin, cells were lysed with RIPA lysis buffer (Thermo Scientific). The proteins in the lysates were estimated and loaded equally onto an 12 % SDS-PAGE for separation. The separated proteins were then electrophoretically transferred to polyvinylidene membrane (PVDF), blocked with 5 % dry skimmed milk prepared in Tris-buffered-saline (TBS) and incubated overnight at 4 °C with specific primary antibodies (1:500). Membranes were washed thrice using TBS-Twin-20 (TBST) and incubated with species specific HRP-conjugated secondary antibodies (1:2000) for 1.5 h at room temperature (RT). Proteins were visualised by Bio-Rad gel documentation system using HRP substrate. Cyclophilin-B was used as loading control in each set of experiment.

2.10. Immunoprecipitation

Immunoprecipitation was done as described earlier [36]. Subtilisin (5 μg/ml) and/or MG-132 (10 μM) treated or untreated cells were lysed by NP-40 lysis buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1 mM sodium fluoride, 0.5 % Nonidet P-40 (NP-40), 50 mM β-glycerophosphate, 10 % glycerol] on ice. The proteins in the lysates were estimated and 500 μg of proteins from each lysate was incubated overnight with α and β-tubulin specific antibody (1:50) at 4 °C in an end-to-end rotor with mild rocking. Tubulin antibody incubated lysate was incubated with Protein A/G agarose beads for 4 h with gentle rocking followed by centrifugation at 3824 RCF at 4 °C for 3 min. A/G beads with immune-complexes were washed with NP-40 lysis buffer to remove any non-specific bindings. Beads were re-suspended in 2×-SDS-PAGE loading buffer and boiled for 10 min before loading to SDS-PAGE. Immunoblotting was done using anti-ubiquitin (1:500) antibody to study tubulin ubiquitination and anti-PARKIN (1:500) antibody, to observe tubulin-PARKIN interaction.

2.11. Subtilisin labelling

To determine whether subtilisin enters into the MCF-7 cells or acts through cell surface receptors, subtilisin (1 mg in 500 μl) was labelled with Alexa-Fluor 568 labeling kit (Thermo Fisher, cat-A10238). Purified subtilisin was incubated with Alexa-Fluor 568 dye (containing succinimidyl ester or tetrafluorophenyl ester moieties) in bicarbonate buffer for 1 h at RT. Free dye was removed by gel filtration spin column provided with the kit.

2.12. Immunofluorescence

MCF-7 (10^5 cells) seeded onto a sterile cover-slip in a six-well plate was washed with 1× PBS following treatment. The cells were fixed with acetone/ethanol (1:1) and blocked with 5 % FBS in PBS. Anti-tubulin antibody (1:500; Santa-cruz Biotechnology) was used to incubate cells overnight at 4 °C. The cells were washed with PBS containing 5 % FBS and further incubated with a FITC-conjugated secondary antibody (1:1000; Santa-cruz Biotechnology) for 1.5 h at RT. Following incubation, cells were washed with PBS, nuclei were stained with DAPI-ProLong gold antifade reagent and observed under confocal microscope (Zeiss-LSM510).

2.13. Real-Time RT-PCR to study tubulin mRNA level

Total RNA from subtilisin treated MCF-7 cells was isolated using TRIzol reagent. One microgram of RNA from each sample was used for the first strand cDNA synthesis using Invitrogen-Retroscript reverse transcription kit. cDNAs were amplified by real time PCR using tubulin specific primer (forward primer 5′-CGGGCAGTGTTTGTAGACTTGG- 3′, reverse primer 5′-CTCCTTGCCAATGGTGTAGTGC- 3′) using SYBER green Master Mix. Actin (forward primer 5′-GGACTTCGAGCAAGAGATGG- 3′, reverse primer 5′-AGCACTGTGTTGGCGTACAG- 3′) was used as an endogenous control. Amplification reaction mixture with 1 μl of cDNA, 50 pM Fwd and Rev. primers and 10 μl SYBER green Master Mix was used for the real time PCR in 7500 Fast Real Time system (Applied Biosystem, USA). Relative gene expression of tubulin was calculated using ΔΔCT method and the statistical analysis was done based on triplicate PCR set of each sample.

2.14. Si-RNA transfection and PARKIN knockdown

Expression of PARKIN was blocked by transfection of PARKIN si-RNA construct in 60 % confluent MCF-7 cell line using Lipofectamine-3000 (Thermo-scientific) as per manufacturer's protocol. MCF-7 cells were transfected for 24 h and thereafter used for determination of ubiquitination of tubulin.

2.15. Proteasome activity measurement

MCF-7 cells, treated with subtilisin (5 μg/ml) for 1, 3, 6 and 24 h were washed with 1× PBS and incubated with 100 μl of 0.4 % NP-40 for 30 min. The protein lysates were collected by centrifugation at 14,000 rpm for 5 min. Prepared cell lysates were used for measurement of proteasome activity by using Proteasome activity assay kit (Abcam, USA) as per manufacturer's protocol. Sample lysate were incubated with proteasome substrate (Succ-LLVY-AMC in DMSO) for 20 min with proper positive (Jurkat Cell lysate with significant proteasome activity) and negative control (lysate treated with proteasome inhibitor MG-132). After incubation, fluorescence (Ex/Em-350/440 nm) was measured in a fluorometer (Thermo Scientific).

2.16. DNA-ladder assay

Genomic DNA was isolated from untreated and subtilisin (5 μg/ml) treated (for 6 and 18 h) MCF-7 cells by genomic DNA isolation kit (Qiagen, Germany). Isolated DNA was then resolved in 8 % agarose gel electrophoresis and the DNA ladder was observed under Bio-Rad gel documentation system.

2.17. Statistical analysis

All the experiments were performed in triplicate and the data was represented as mean ± standard deviation (SD). Statistical analysis between two experimental conditions was done by Students's *t*-test using Microsoft excel. *P*-value <0.05 was considered as statistically significant for all the experiments.

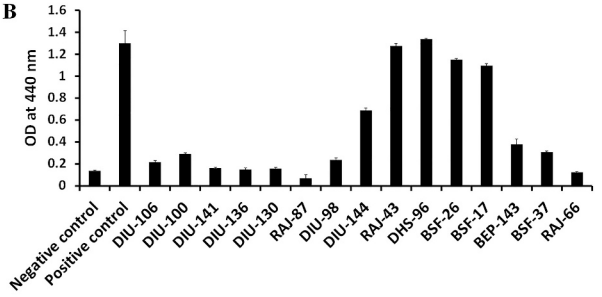
3. Results

3.1. Screening of environmental bacterial isolates for protease activity

The culture supernatants of 140 environmental microbes were assayed for protease activity by azocasein assay (Fig. 1A & B). Five strains (DIU-144, RAJ-43, DHS-96, BSF-26 and BSF-17) showed significant protease activity and were further tested for apoptotic activity on cancer cells (Fig. 1C). The culture supernatant of *V. cholerae* O1 strain C6709 was selected as a positive control for protease activity.

A

No. of bacterial strains screened	No. of protease positive bacterial strains	No. of strains for which protease activity was inhibited by EDTA	No. of strains for which protease activity was inhibited by PMSF	No. of bacterial strains, induced cell death in cancer cells
140	5	0	5	1



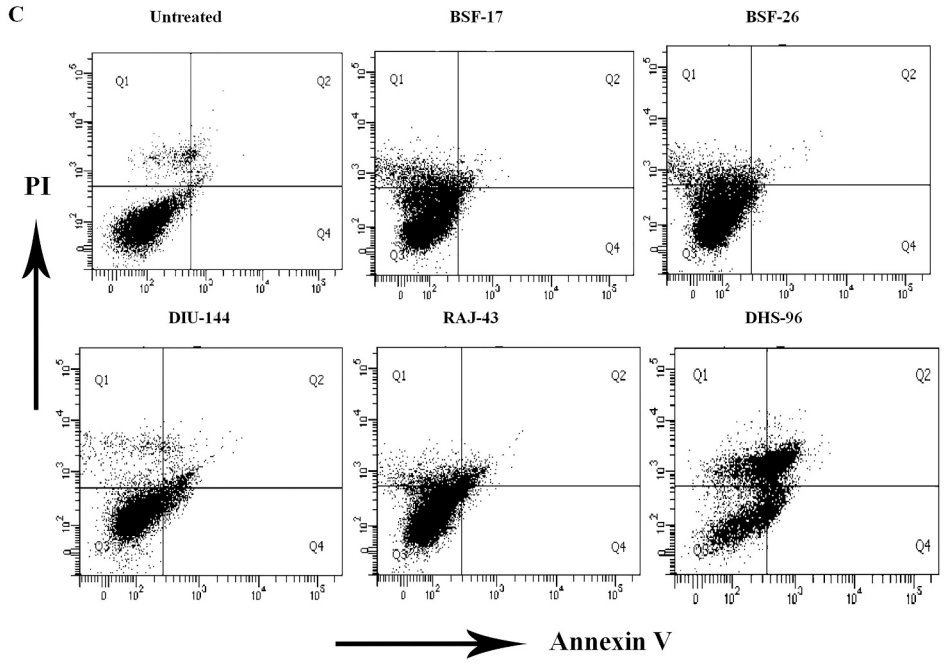


Fig. 1. Screening of bacterial isolates from environmental sources for protease and apoptotic activity.
(A) Total number of strains screened for protease activity, protease positive strains, inhibition of protease activity and their apoptotic response. (B) Azocasein assay to check the protease activity of representative strains. Culture supernatant of *Vibrio cholerae* EL Tor strain C6709 was used as positive control and nutrient broth was used as negative control. (C) Flow-cytometric analysis with the culture supernatant of the protease positive strains was done to study apoptosis in HT-29 cells. In each display the lower left quadrant is for normal cells (Annexin V-/PI-), lower right quadrant is for early apoptotic cells (Annexin V+/PI-), upper right is for late apoptotic cells (Annexin V+/PI+) and upper left is for necrotic population (Annexin V-/PI+).

3.2. Culture supernatant of DHS-96 strain induced apoptosis in cancer cell

To study the apoptotic effect, HT-29 (colon cancer) cells were incubated with 200 µl of sterile culture supernatant of protease positive strains (DIU-144, RAJ-43, DHS-96, BSF-26 and BSF-17) for 24 h. Apoptosis was observed by Annexin-V/PI staining. Our results showed that, out of the five protease positive strains tested for apoptosis, the culture supernatant of DHS-96 showed significant increase in Annexin-V^{+}/PI^{+} cells, indicating apoptosis (Fig. 1C). This suggests that, only DHS-96 can induce apoptotic effect on HT-29 cancer cell and was further selected for purification of protease and to study whether the protease has anti-cancer properties.

3.3. Purification and identification of the secreted protease from DHS-96

The cell free culture supernatant of DHS-96 strain was precipitated using 80 % ammonium sulphate, dialysed, concentrated and run-on DE-52 column. The flow-through fraction was eluted with 25 mM Tris-HCl (pH -7.4) and bound fractions were eluted with 0.1 M, 0.2 M, 0.3 M and 0.5 M NaCl. All the fractions were checked for protease activity, only the unbound (flow-through) fraction showed significant protease activity in azocasein assay (Fig. 2A-D, F). The flow through fraction from DE-52 column was pooled, concentrated and further loaded on Sephadex G-75 gel filtration column. The two peaks eluted from the G-75 gel filtration column were collected and concentrated and tested for protease activity (Fig. 2E). The second peak in G-75 column showed protease activity (Fig. 2F) and SDS-PAGE showed the presence of two bands

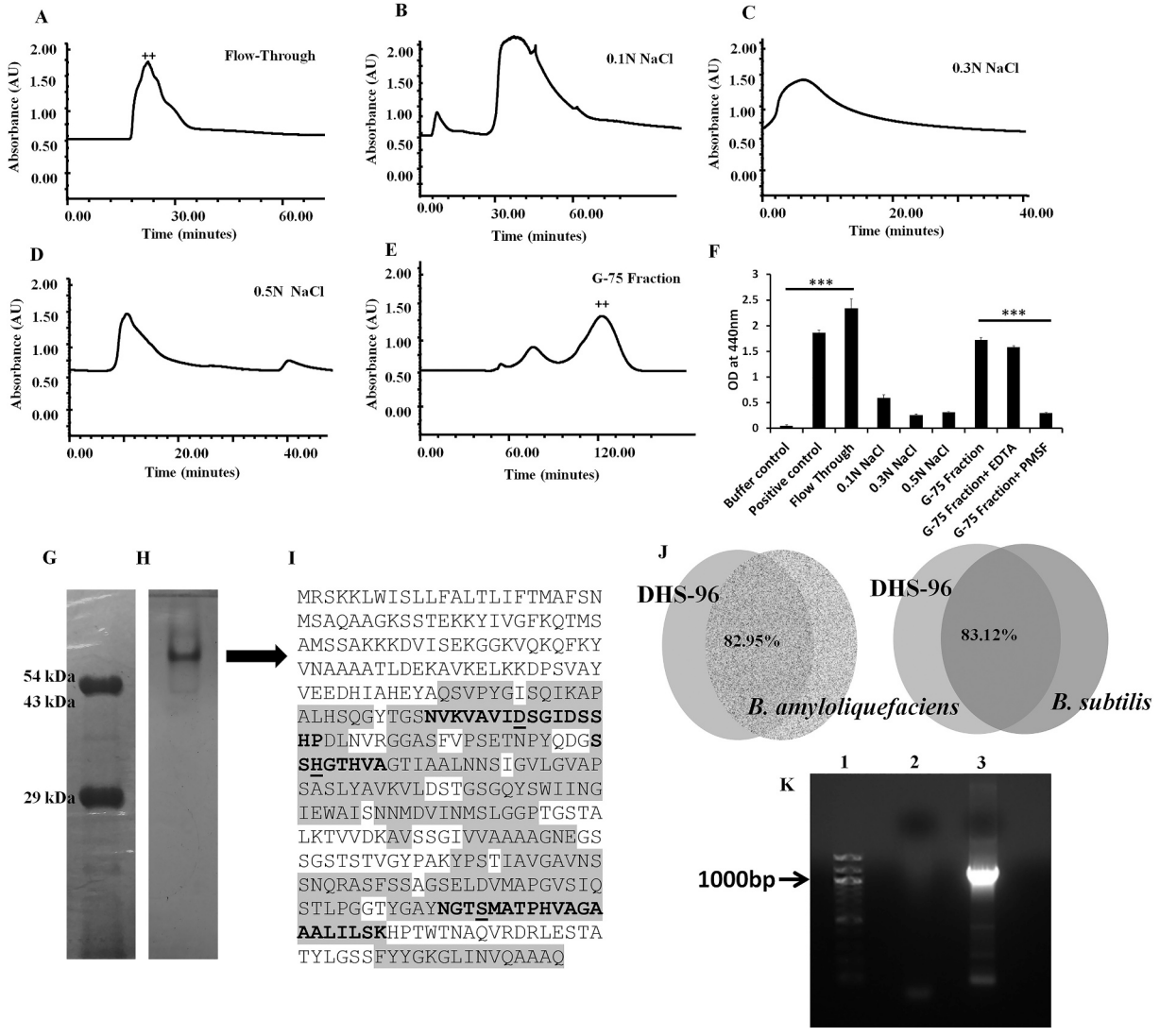


Fig. 2. Purification and identification of Subtilisin from *Bacillus amyloliquefaciens* strain.
(A-D) Chromatographic profile of ammonium sulphate precipitated crude proteins from culture supernatant of DHS-96 strain, loaded onto an anion-exchange DE-52 column. A, Represents proteins in non-binding fraction of DE-52, B,C and D are 0.1 N, 0.3 N & 0.5 N NaCl eluted fractions respectively. '++' indicates fraction positive for protease activity. (E) Chromatographic profile of proteins eluted from Sephadex G-75 column loaded with non-binding pooled and concentrated fraction of DE-52. Second peak shows presence of protease activity. (F) Azocasein assay to determine protease activity in all the fractions eluted from DE-52 and G-75 columns with Tris-Cl buffer as negative control & purified HAP from *V. cholerae* O1 as positive control. (G) SDS-PAGE (12 %) and (H) Native-PAGE (12 %) profile of proteins eluted in second peak of G-75 column. (I) The band on SDS and Native-PAGE were analysed by MS/MS peptide sequencing and showed homology with 'subtilisin'. The peptides showing homology with subtilisin are with background colour. The S8 domain of subtilisin is shown in bold with the catalytic triad of D, H and S (underlined). (J) Percentage similarity of 16S-rRNA sequence between two closely related Bacillus species and DHS-96. (K) Agarose gel electrophoresis showing amplified subtilisin gene. Lane-1, 1 kb plus DNA-ladder; Lane-2, PCR sample amplified with *B. subtilis* specific primer; Lane-3, PCR sample amplified with *B. amyloliquefaciens* specific primer.

29 kDa and 42 kDa and Native PAGE showed the presence of a single band (Fig. 2G and H). Both the bands in SDS-PAGE and the band in Native-PAGE were analysed in Mass Spectrometry. The peptide sequence generated from Mass spectrometry data was analysed and homologous protein was found in the NCBI database. The highest matching peptide sequence showed homology with 'subtilisin' from *Bacillus sp.* The 42 kDa and 29 kDa bands in SDS-PAGE are the precursor and mature forms of subtilisin respectively. Homology search also showed peptidase S8 domain with Asp, His and Ser catalytic triad also present in subtilisin (Fig. 2I).

3.4. Effect of protease inhibitors, pH and temperature on protease activity of subtilisin

Purified protease, identified as subtilisin was completely inhibited in presence of 10 mM PMSF. In presence of EDTA, protease activity remained unchanged (Fig. 2F). This indicates that purified protease subtilisin is a serine protease as reported earlier.

Protease activity was observed in a wide pH range of 2–12, however, the optimum activity was at alkaline pH of 8–12 (result not shown). The optimum activity of subtilisin in alkaline pH further corroborates the finding that DHS-96 was isolated from alkaline environment.

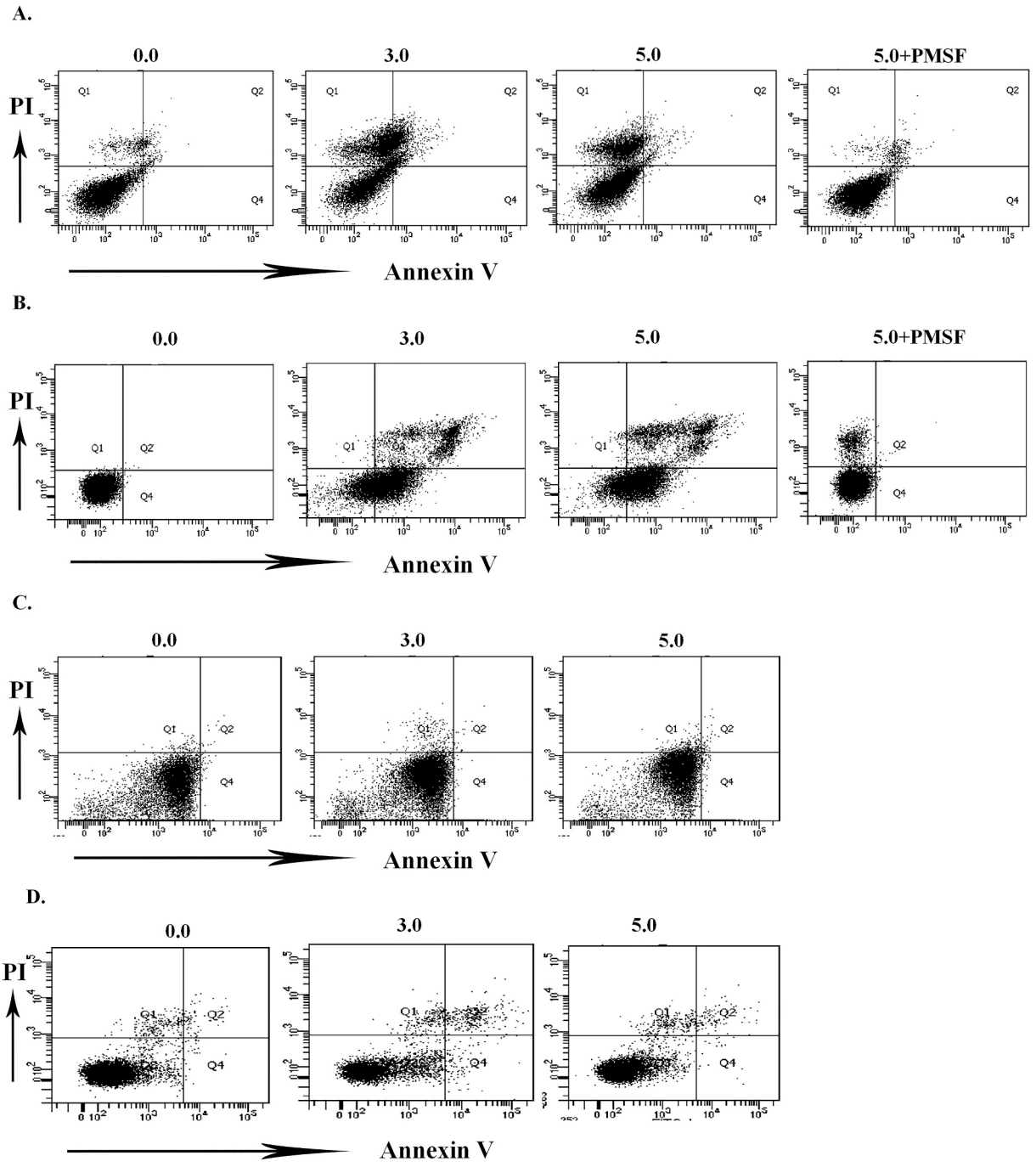


Fig. 3. Flow cytometric analysis of purified subtilisin treated cell lines.
(A) Dose dependent effects of subtilisin showed necrosis in HT-29 colon cancer cells. Inhibition with PMSF reduces the effect. (B) dose dependent effect of subtilisin treatment showed apoptosis in MCF-7 breast cancer cell line. Inhibition with PMSF reduced the effect. (C) subtilisin treatment does not show any effect on mouse peritoneal macrophage and (D) normal human breast cell line (MCF10A).

Protease activity was low at 25 °C, optimum activity was observed in 40–50 °C. The protease activity decreased almost 50 % at 60 °C and there was no activity at 70 °C (result not shown).

3.5. Identification of the isolate DHS-96

To identify the bacterial isolate DHS-96, 16S rRNA was sequenced. The BLAST result in NCBI data base revealed that the sequence has 83.12 % similarity with *Bacillus subtilis* (Accession number NR_102783.2) and 82.95 % similarity with *Bacillus amyloliquefaciens* (Accession number NR_116022.1) (Fig. 2J). Bioinformatics analysis showed that the subtilisin gene sequence present in both the bacterial species is not similar (data not shown) to each other (79 % similarity found). To distinguish the closest neighbour and to identify DHS-96, we have further designed 'subtilisin' gene specific primers from both *B. subtilis* and *B. amyloliquefaciens*. Subtilisin gene amplification was only observed when *B. amyloliquefaciens* specific primers were used (Fig. 2K). These results suggest that the DHS-96 strain belongs to *Bacillus amyloliquefaciens*.

3.6. Purified subtilisin showed cytotoxic effect in cancer cells

Cytotoxic effect of subtilisin was studied in MCF-7 and HT-29 cancer cells. Mouse peritoneal macrophages and normal human breast cells MCF10A were used as control. Cells were treated with 3 and 5 μg of subtilisin and the cytotoxic effect was studied with flow-cytometric

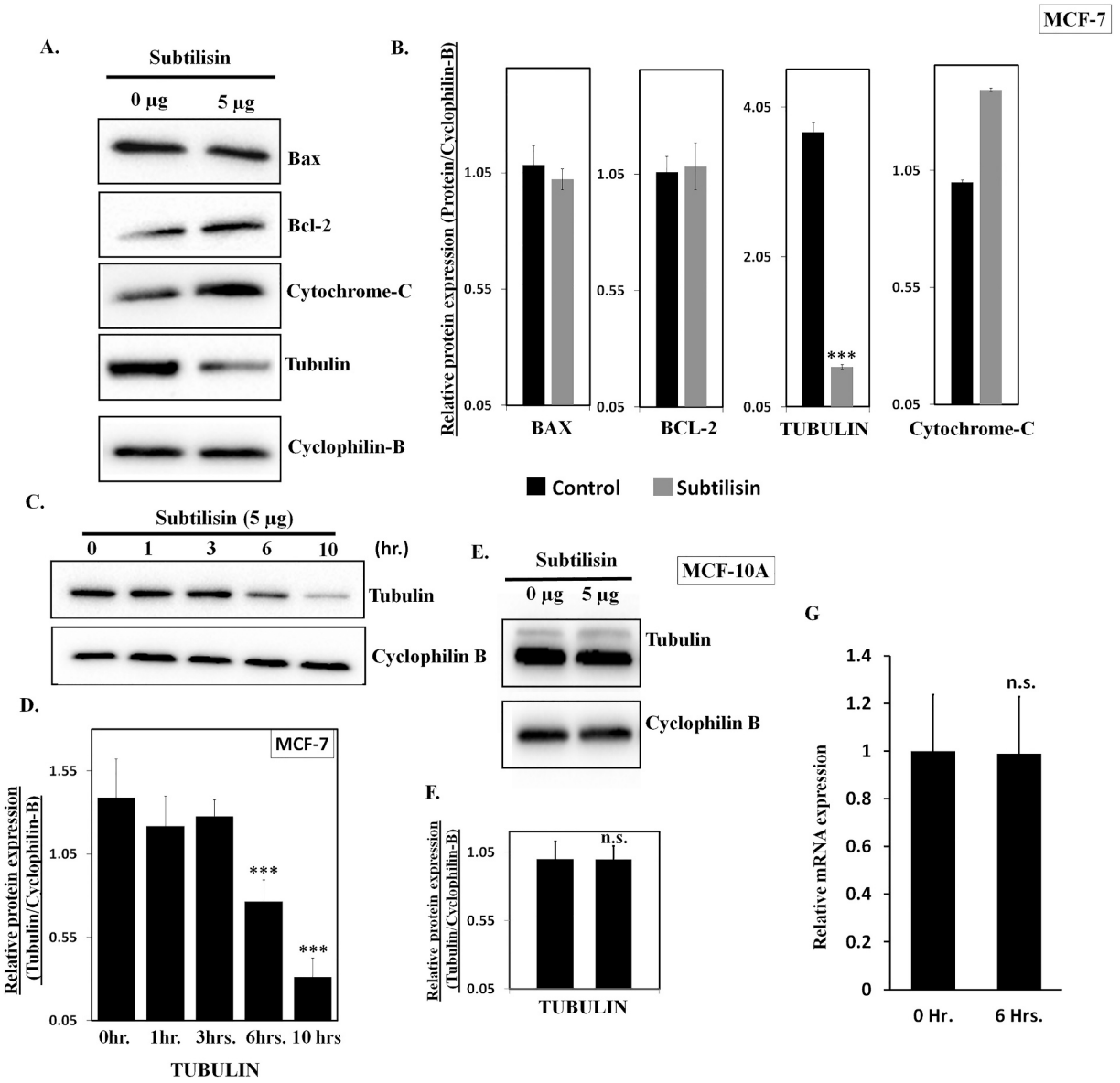


Fig. 4. Subtilisin from *Bacillus amyloliquefaciens* decreases tubulin level post-transcriptionally without affecting pro and anti-apoptotic proteins in MCF-7 cells. (A) Western blot analysis of pro and anti-apoptotic proteins and tubulin after 24 h treatment with subtilisin. Cyclophilin-B was used as a loading control. (B) Densitometric analysis of protein relative to cyclophilin-B quantified by ImageJ software using three experimental replicates from A. (C) Western blot analysis to observe tubulin level after subtilisin treatment on MCF-7 cells at different time intervals. Cyclophilin-B was used as a loading control. (D) Tubulin quantification relative to cyclophilin-B by Image-J software using three experimental replicates from C. (E) Western blot analysis to observe tubulin level after 24 h subtilisin treatment on MCF10A. (F) Densitometric representation of tubulin relative to cyclophilin-B from fig. E. (G) quantitative real time PCR analysis of tubulin gene shows no significant difference in subtilisin treated (6 h) *versus* control cells (0 h). mRNA expression was normalised against actin gene expression (ΔCt = Ct tubulin- Ct actin). Error bar in each graphical representation represent standard error from three different replicates. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0005$, n.s-non significant.

analysis by using Annexin-V/PI staining. Annexin-V binds with phosphatidyl-serine on the cell membrane, which is translocated at the extracellular side in the apoptotic cell and PI is a membrane impermeable agent, which stains necrotic cells, where membrane integrity is lost [37,38]. After treatment with subtilisin, MCF-7 cells showed Annexin-V^{+}/PI^{-} staining, which were identified as apoptotic cells (Fig. 3B) and HT-29 cells showed PI staining, which were identified as necrotic cells (Fig. 3A). PMSF inhibited the effects of subtilisin in both the cell lines (Fig. 3A and B). At similar concentrations (3 μg and 5 μg) of subtilisin showed no apoptotic response on mouse peritoneal macrophage (Fig. 3C) and normal human breast cell MCF10A (Fig. 3D).

3.7. Subtilisin treatment showed no changes in pro- and anti-apoptotic markers but it promotes tubulin downregulation in MCF-7 cells

To check if subtilisin could induce the intrinsic pathway of apoptosis both the pro (bax) and anti-apoptotic (bcl-2) markers were checked. Subtilisin (5 μg) showed no changes in bax and bcl-2 level in MCF-7 cells (Fig. 4A & B). There was an increase in cytochrome-c level in presence of subtilisin (Fig. 4A & B). Tubulin was initially used as a loading control in this study. Interestingly, we observed significant decrease in the level of tubulin after 24 h of subtilisin treatment in MCF-7 cells. However, subtilisin did not show any effect on cyclophilin-B (Fig. 4A & B) which was used as a loading control for all our experiments. Furthermore, a time dependent response of subtilisin treatment on tubulin level was checked. After 6 h of subtilisin treatment, there was a significant

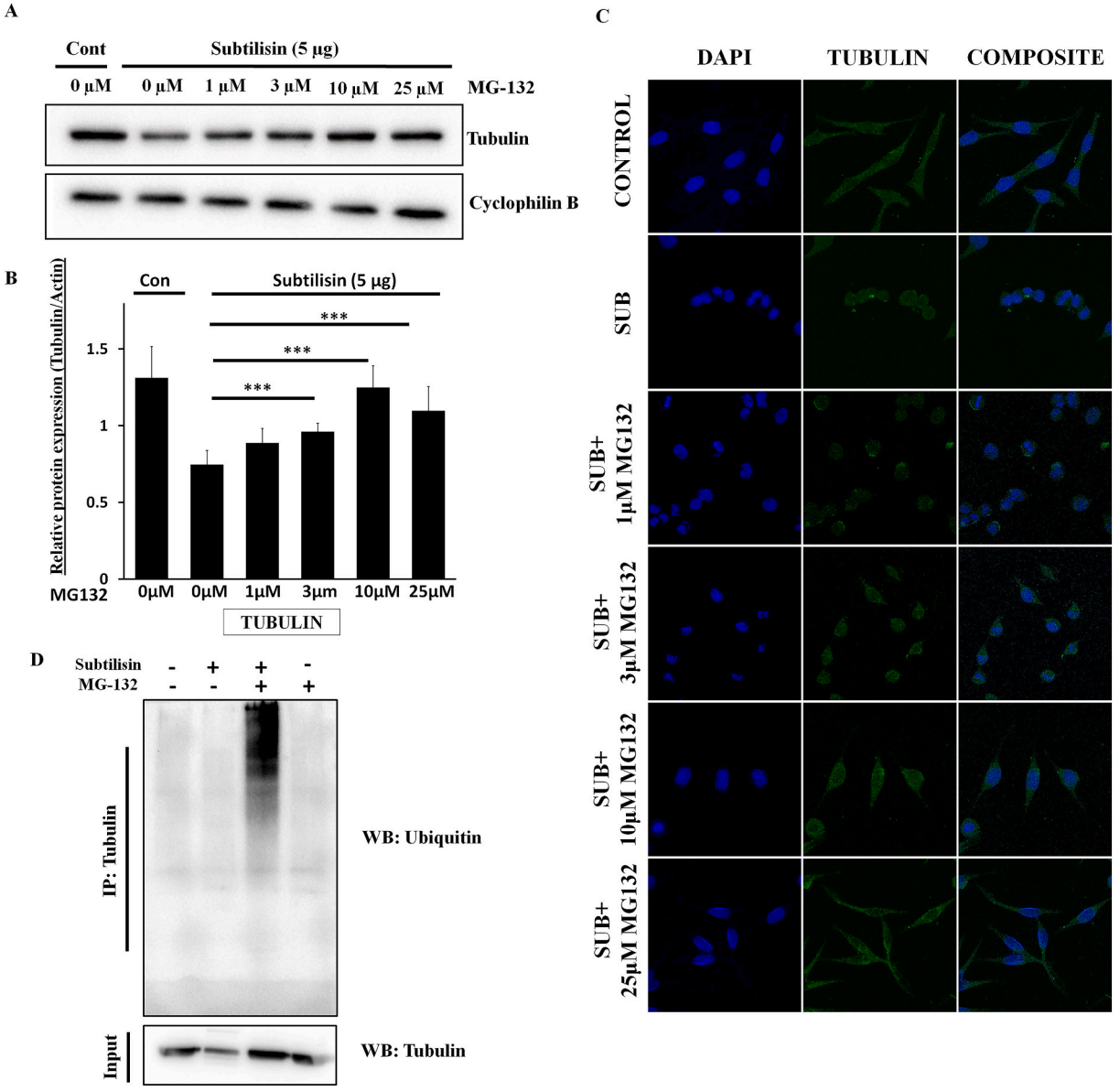


Fig. 5. Subtilisin induced tubulin degradation by ubiquitin-proteasomal mediated pathway in MCF-7 cells.
(A) Proteasome inhibitor MG-132 restores tubulin level in subtilisin treated MCF-7 cells. Western blot analysis of tubulin level with subtilisin treated at different doses of MG-132 on MCF-7 for 24 h. Cyclophilin-B was used as a loading control and the experiment was repeated three times. (B) Relative expression of tubulin calculated by ImageJ software after normalization with cyclophilin-B. Error bar represent standard error from three different biological replicates. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0005$. (C) Confocal microscopy showed restoration of tubulin in presence of different doses of MG-132. (D) Subtilisin treatment (6 h) increased ubiquitination of tubulin in MCF-7 cells. Cell lysate was taken from untreated, subtilisin treated with or without MG132 and only MG132 treated MCF-7 cells. Tubulin was immunoprecipitated (IP) from equal amount of cell lysate by anti-tubulin antibody. Western blot analysis was done by loading equal amount of IP sample and the blot was developed with anti-ubiquitin antibody. Input was developed by loading equal amount of cell lysate on which IP was done.

decrease in tubulin level (Fig. 4C & D). In contrary, no change in tubulin level was observed in normal breast cell line MCF10A after 24 h treatment with subtilisin (Fig. 4E & F). In order to determine whether subtilisin affects tubulin at mRNA level or protein level, quantitative real time PCR analysis was performed which showed no difference in subtilisin treated (6 h) and untreated control cells (Fig. 4G). As we did not find any significant changes in mRNA level, we can conclude that tubulin regulation by subtilisin is a post-transcriptional event.

3.8. Subtilisin induced ubiquitination and proteasomal mediated tubulin degradation

MG-132, a proteasomal inhibitor was used to study the role of subtilisin-induced proteasomal mediated degradation of tubulin in MCF-7 cells. MCF-7 cells showed significant increase in tubulin levels in presence of 10 and 25 μM MG-132 before treatment with 5 μg of subtilisin for 24 h (Fig. 5A & B) whereas in absence of MG-132 there was a significant decrease in tubulin level. The role of subtilisin in proteasomal mediated tubulin degradation was further confirmed by immunofluorescence staining of subtilisin-treated MCF-7 cells in presence of MG-132. Disruption of cytoskeleton due to subtilisin was completely restored in presence of 10 and 25 μM MG-132 (Fig. 5C).

To test the ubiquitination of tubulin in subtilisin-treated MCF-7 cells, tubulin was immunoprecipitated with anti-tubulin antibody and ubiquitinated tubulin band was detected (Fig. 5D) using anti-ubiquitin antibody. Ubiquitinated band was absent when only subtilisin-treated lysate was used but it gets accumulated in presence of proteasome inhibitor MG-132 (Fig. 5D). Our results showed subtilisin induced ubiquitination and proteasomal mediated tubulin degradation.

3.9. Subtilisin induced apoptosis was inhibited by proteasome inhibitor MG-132

The proteasome inhibitor MG-132 inhibited apoptosis in MCF-7 cells (Fig. 6A & B) in a dose dependent manner. In presence of 10 and 25 μM MG-132 there was a significant decrease in the number of apoptotic cells when treated with subtilisin. However, no effect was observed with 25 μM MG-132 in absence of subtilisin.

3.10. Subtilisin showed cell surface interaction and did not directly degrade tubulin

Cell free lysates of MCF-7 were prepared using 0.5 % NP-40 and incubated with subtilisin (5 μg) for 6 h and 24 h. Our results showed that subtilisin did not degrade tubulin in cell free lysate of MCF-7 after 6 h but at 24 h there was complete degradation of tubulin and loading control cyclophilin-B (Fig. 7A). Ubiquitination of tubulin by immunoprecipitation was not observed after 6 h treatment with subtilisin in cell free lysate of MCF-7 (Fig. 7B).These results showed that subtilisin could not directly degrade tubulin, but it may interact with the cell surface of MCF-7 to induce ubiquitination and proteasomal degradation of tubulin. This was further confirmed by labelling subtilisin with Alexa Fluor-568

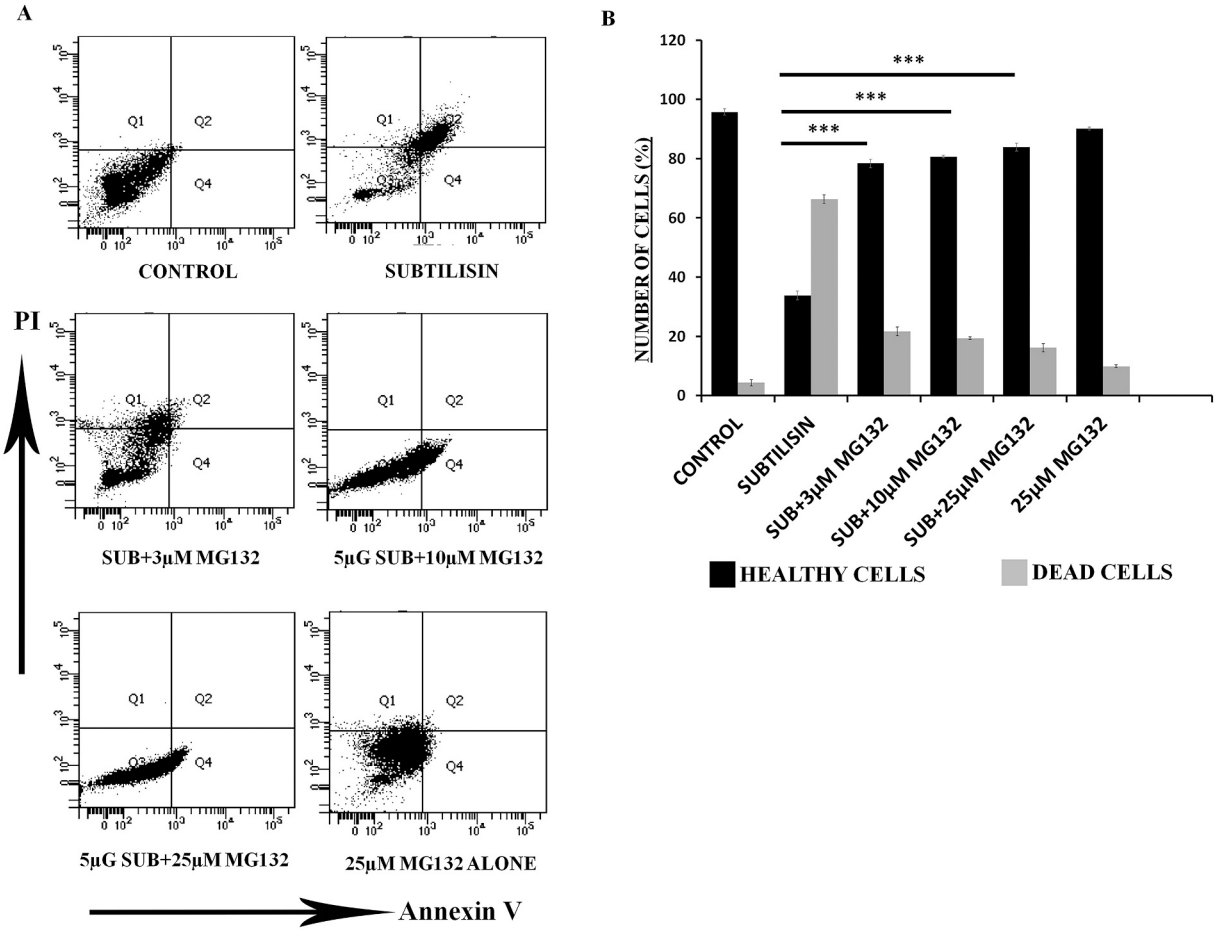


Fig. 6. Proteasome inhibitor MG-132 inhibits subtilisin induced apoptosis in MCF-7 cells.
(A) Dose dependent effects of MG132 on subtilisin treated MCF-7 cells by flowcytometric analysis. There is significant increase in normal cells in presence of MG132 (B) Statistical representation of flow cytometric data from A. Error bar represents standard error from three different experimental replicates. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0005$.

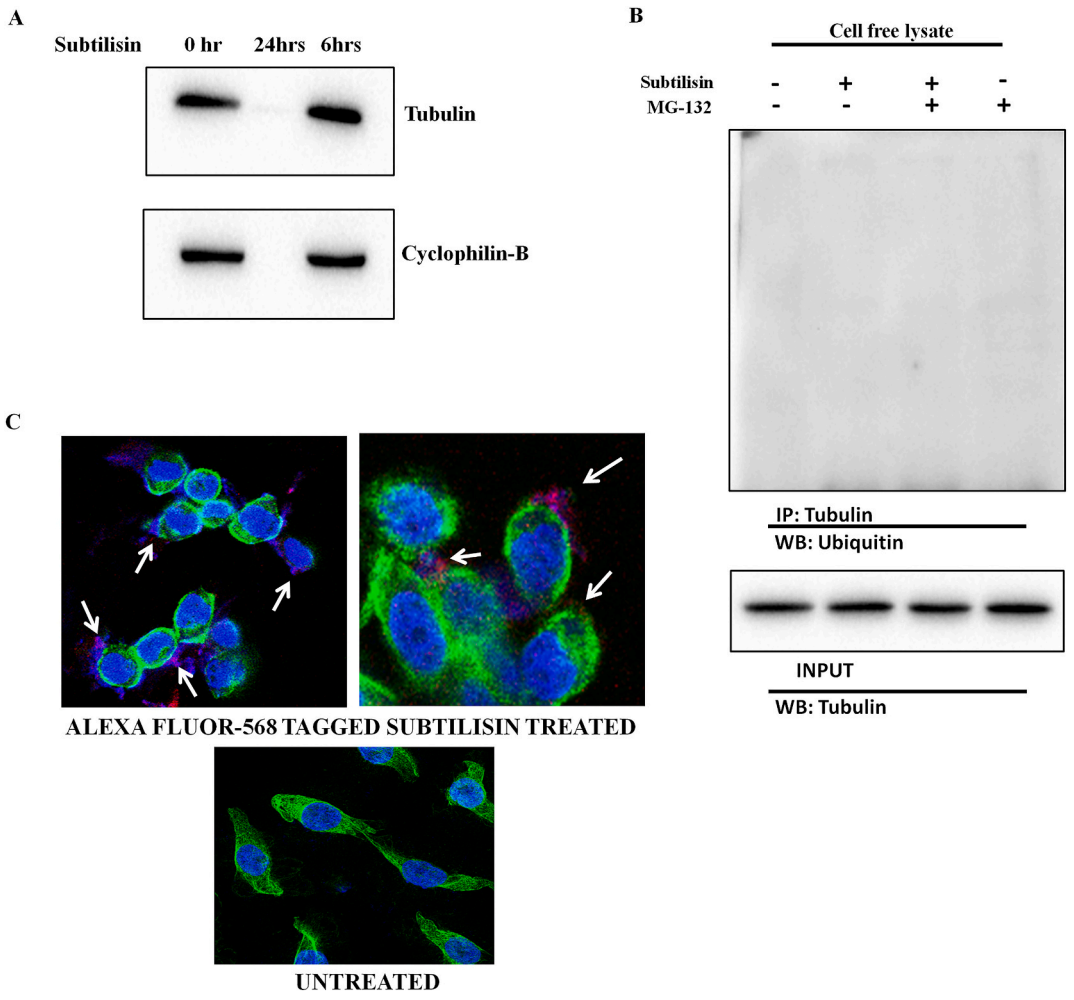


Fig. 7. Subtilisin does not directly degrade tubulin in cell free lysate of MCF-7 cells.
(A) Western blot analysis to observe tubulin degradation after 6 h and 24 h subtilisin treatment on cell free lysate of MCF-7 cells. (B) Effect of subtilisin treatment on tubulin ubiquitination in cell free lysate of MCF-7. Equal amount of cell free lysate was incubated with subtilisin in presence and absence of MG-132. Tubulin was immunoprecipitated and ubiquitination was observed by using anti-ubiquitn antibody. (C) Representative confocal images of Alexa-568-subtilisin (orange) treated MCF-7 cells. White arrowhead showing subtilisin binds to the cell surface of MCF-7 cell. Cells cytoplasm was stained with DyLight-488 phalloidin (green) and nucleus was stained with DAPI (blue).

and showing its interaction with MCF-7 cells by confocal microscopy. Alexa-568-subtilisin was found to be present on cell surface of MCF-7 cells and was absent in the cytoplasm of MCF-7 cells (Fig. 7C). These results indicate possible interaction of subtilisin with cell surface of MCF-7.

3.11. The E3 ubiquitin ligase PARKIN interacts with tubulin and regulates its ubiquitination in subtilisin treated MCF-7 cells

PARKIN has earlier been reported to act as an E3 ligase of tubulin and has been shown to play a role in ubiquitination and degradation of tubulin [39]. Our results exhibited there is increased interaction between PARKIN and tubulin at 3–6 h of subtilisin treatment in MCF-7 cells as detected in immune precipitation of tubulin by anti-tubulin antibody (Fig. 8A). In addition, in presence of PARKIN-siRNA along with subtilisin and MG-132 there was a significant decrease in ubiquitination of tubulin (Fig. 8B). This confirms that PARKIN acts as an E3-ubiquitin ligase of tubulin in subtilisin treated MCF-7 cells.

3.12. Subtilisin induced ER-stress in MCF-7 cells

Earlier studies have shown that activation of ubiquitin-proteasome system can induce ER-stress leading to apoptosis [40]. The whole cell proteasome activity was measured at 1, 3 and 6 h using whole cell proteasome activation kit. Our result showed that there was an increased proteasomal activity after 3 h of subtilisin treatment in MCF-7 cells (Fig. 8D). ER-stress activation can switch between 'survival' and 'death' pathway. In survival pathway, ER homeostasis maintains and cell survives, BiP/GRP78 plays an important role in the survival pathway [40]. In death pathway, ER-stress causes activation of IRE1α [40,41]. MCF-7 cells showed increased expression of ER-stress pro-death markers CHOP and IRE1α after 6 h of subtilisin treatment (Fig. 8D). Increased expression of BiP was observed at 24 h of treatment (Fig. 8D). This suggests that subtilisin treatment induced ER-stress in MCF-7 cells.

3.13. Subtilisin induced Caspase-7 activation and cleavage of PARP in MCF-7 cells

Caspase-7 and caspase-3 are two important executioner caspases. Caspase-3 is mutated in MCF-7 cells [42,43]. Hence, we analysed caspase-7 activation in subtilisin treated MCF-7 cells. Caspase-7 activation was observed at different time points by immunoblot analysis and significant level of cleaved caspase-7 appeared after 6 h of treatment with maximum activation at 24 h (Fig. 8D). The dose dependent response in presence of MG-132 after 24 h of subtilisin treatment showed significant decrease in caspase-7 cleavage at 10 and 25 μM of

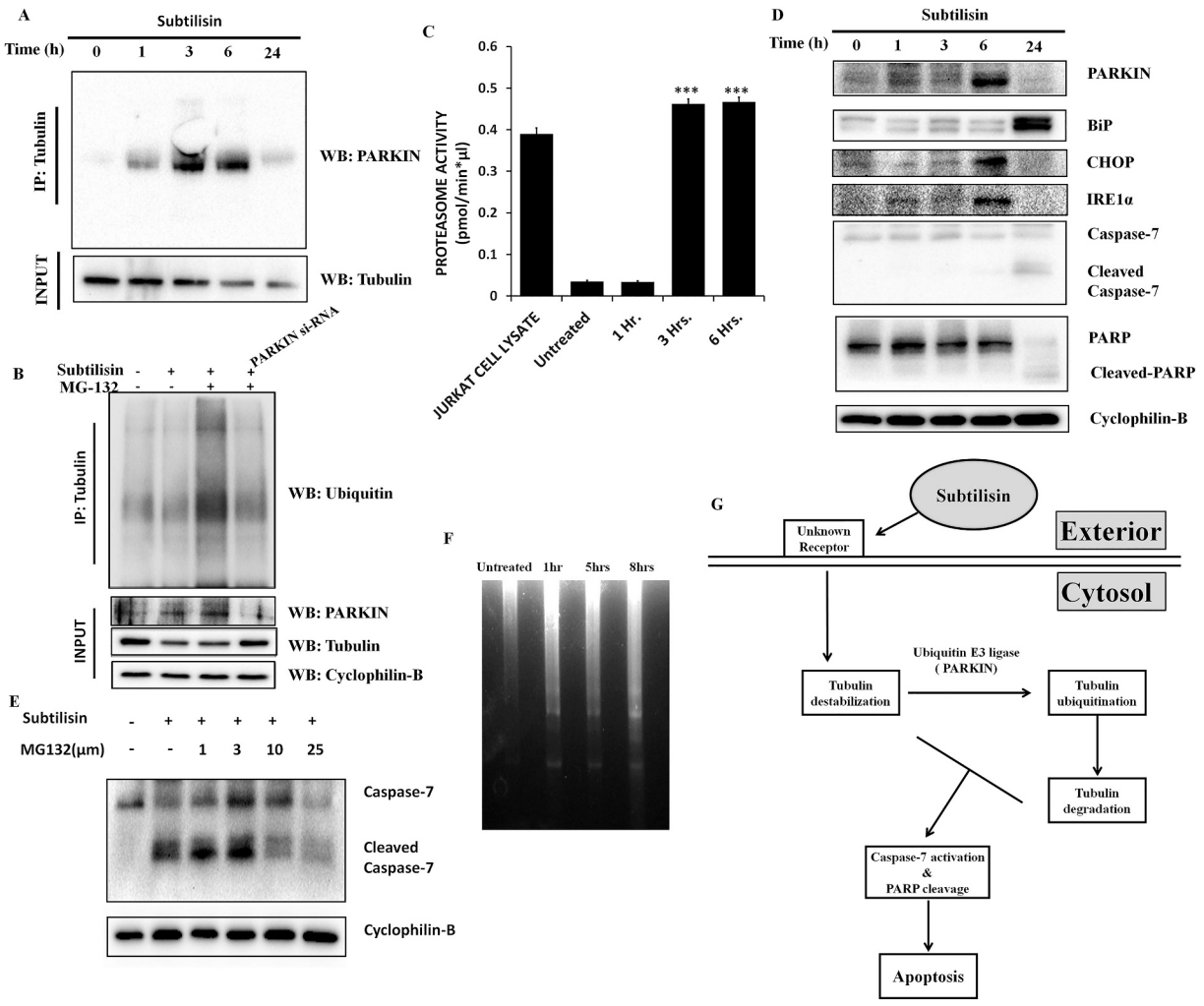


Fig. 8. PARKIN interacts with tubulin and induces ubiquitination of tubulin in subtilisin treated MCF-7 cells. Schematic representation of the pathway of apoptosis induced by subtilisin in MCF-7 cells.
(A) Time dependent effects of subtilisin on PARKIN-tubulin interaction in MCF-7 cells after immune precipitation of tubulin by anti-tubulin antibody. The blot was developed with anti-PARKIN antibody and the input by western blot with anti-tubulin antibody. (B) MCF-7 cells were treated with subtilisin with or without MG-132 and transiently transfected with PARKIN-siRNA in presence of MG-132. Cell lysates were collected and tubulin immunoprecipitated with anti-tubulin antibody. Western blot of ubiquitin was developed using anti-ubiquitin antibody and Input by western blot with anti-PARKIN, anti-tubulin and anti-cyclophilin-B antibody. (C) Proteasome activity was measured in subtilisin treated MCF-7 cells. Relative fluorescence (350/440) was measured and proteasome activity was estimated from AMC standard curve. Statistical test performed using *t*-test. Error bar represents standard error from three different experimental replicates. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0005$. (D) Time dependent effects of subtilisin on PARKIN, BiP, CHOP, IRE1α, PARP and caspase-7. Cell lysates were taken from subtilisin treated MCF-7 cells at different time points and western blot was developed by using specific antibody. (E) MCF-7 cells were treated with subtilisin with different doses of MG-132. Cells lysates were collected and western blot of caspase-7 was observed to study effect of MG-132 on caspase-7 activation. (F) DNA ladder assay. MCF-7 cells treated with subtilisin or 1, 5 and 18 h and isolated genomic DNA was run on agarose gel. (G) Schematic representation of apoptosis induction of MCF-7 cells by subtilisin treatment. Subtilisin induced tubulin ubiquitination by some unknown cell surface interaction. PARKIN acts as an E3 ligase in tubulin ubiquitination which leads to tubulin degradation and increases the whole cell proteasomal activation. These events induced caspase-7 and PARP activation leading to execution of apoptosis.

MG-132 (Fig. 8E), suggesting that activation of proteasomal pathway by subtilisin underlies its caspase-7 activation. We also investigated PARP which is a downstream target of caspase-7. Western blot analysis showed presence of cleaved-PARP after 24 h of subtilisin treatment in MCF-7 cells (Fig. 8D). As DNA damage in apoptosis is related with PARP cleavage, DNA degradation status in subtilisin treated MCF-7 cells was also investigated. Significant nuclear DNA degradation was found in subtilisin treated MCF-7 cells as shown by DNA ladder assay (Fig. 8F). Our results indicated that subtilisin activates caspase-7, PARP cleavage and subsequent DNA damage in MCF-7 cells which are recognized as hallmark of apoptosis (Fig. 8G).

4. Discussion

The protein studied here, subtilisin, is a bacterial secretory serine protease whose industrial applications are well appreciated. However, its therapeutic role as an anti-cancer drug has not been elucidated. This study was initiated with the screening of environmental microbes for protease activity and one of the isolates DHS-96 secreted a protease with potential anti-cancer properties. The purified protease which showed two bands (43 kDa and 29 kDa) in SDS-PAGE and single band in NATIVE-PAGE could induce apoptosis in MCF-7 breast cancer cells but not in normal breast cells (MCF10A) and mouse peritoneal macrophages. The purified protease showed inhibition by PMSF and was identified as subtilisin, having peptidase S8 domain of serine protease,

which contains the conserved catalytic triad of D at 139, H at 171 and S at 328 positions. These findings corroborate with the earlier studies that reported secretion of subtilisin from *Bacillus amyloliquefaciens* as 42 kDa precursor and 27.5 kDa matured form, in which 27.5 kDa showed as a stable form resulting in autoproteolytic cleavage of the precursor form [44]. The species of DHS-96 was identified by 16S rRNA sequencing that showed 83.12 % similarity with *Bacillus subtilis* and 82.95 % similarity with *Bacillus amyloliquefaciens*. However, this discrepancy was sorted out by PCR with primers designed from subtilisin gene sequence present in both the bacterial species. Amplification of 1.14 kb PCR product was only observed when *Bacillus amyloliquefaciens* specific primers were used which confirmed that DHS-96 strain belongs to *Bacillus amyloliquefaciens*.

Subtilisin is a protease that belongs to subtilase superfamily and is found in wide range of organisms, such as bacteria, archaea and other eukaryotic microorganisms [45]. Subtilisin from *Bacillus* species is one of the most well studied commercial enzymes due to its use in modern detergent and waste management industry [46]. Mainly two types of subtilisin are used commercially, subtilisin Carlsberg from *Bacillus licheniformis* and subtilisin Novo or bacterial protease Nagase (BPN') from *Bacillus amyloliquefaciens* [47]. Among these two types, subtilisin from *Bacillus amyloliquefaciens* is less important commercially but protein sequences of both are highly conserved and these two types originate from a common ancestor [48]. Besides wide use in detergent industry, few reports have shown its role in basic research [48]. Subtilisin has been used in combination with antibiotics to treat burn and wound injuries primarily based on its ability to remove specifically non-viable burn tissue without effecting viable tissue [49]. In a very recent study, engineered subtilisin has also been used to target active RAS, a small GTPase to treat cancer cells [50]. In this report, we have studied the anti-cancer properties of subtilisin and have demonstrated that subtilisin induced apoptosis in MCF-7 cells.

Apoptosis or programmed cell death is a complex biochemical mechanism in which pro-and anti-apoptotic proteins regulation occur [51]. In the canonical intrinsic pathway of apoptosis, regulation of pro-apoptotic bax and anti-apoptotic bcl-2 is important [52]. Our results showed that subtilisin did not induce the intrinsic pathway of apoptosis and there was no change in the ratio of pro-apoptotic bax to anti-apoptotic bcl-2. Interestingly, we observed that overnight incubation with 5 μg of subtilisin could degrade tubulin in MCF-7 cells and the degradation of tubulin was inhibited in the presence of proteasome inhibitor MG-132. The ubiquitin-proteasome pathway degrades majority of the intracellular proteins in eukaryotic cells. The targeted proteins which are needed to be degraded are tagged with ubiquitin and the ubiquitinated proteins are then degraded by 26S proteasome into smaller peptides [53]. Three enzymes E1, E2 and E3 are essential for degrading ubiquitin tagged proteins. E1-E2 helps to guide activated ubiquitin for ligation and E3 acts as a ligase, which can recognise the protein substrate and ligates it with ubiquitin [54]. MG-132 is widely used as a proteasome inhibitor to study the role of ubiquitin-proteasome pathway in various cellular events because of its high potency and selectivity against 26S proteasome complex [55]. Our studies showed that MG-132 completely restored tubulin which indicates the involvement of proteasome in tubulin degradation after subtilisin treatment. Confocal microscopy using antibody staining of tubulin also showed that MG-132 could restore cytoskeletal structure in subtilisin treated-MCF-7 cells. Co-immunoprecipitation experiment with tubulin showed that tubulin is ubiquitinated in presence of MG-132 after subtilisin treatment. These results suggest that subtilisin degrades tubulin through ubiquitin-proteasomal mediated pathway in MCF-7 breast cancer cells. In an earlier report it has been shown that PARKIN, a protein E3 ligase linked to Parkinson's disease strongly binds with α/β tubulin heterodimer. This PARKIN-tubulin interaction activates ubiquitination and degrades tubulin [39]. In this study, immunoprecipitation with tubulin showed interaction of PARKIN with tubulin in MCF-7 cells at 3 h and 6 h of subtilisin treatments. Additionally, si-RNA knock-down of PARKIN greatly reduced the tubulin ubiquitination. These results indicate that PARKIN acts as an E3 ligase of tubulin in subtilisin treated MCF-7 cells. In addition, we have also found that subtilisin induced whole cell proteasomal activity in MCF-7 cells. It has already been reported that increased ubiquitin-proteasome activity leads to ER-stress in the cells [40]. The two major pathways in ER stress are the pro-death pathway and the pro-survival pathway. In pro-death pathway, pro-death factors (*e.g.* IRE1α) and in pro-survival pathway, pro-survival factors (GRPs protein) accumulate in the cells [41]. We have found that subtilisin treatment induced the expression of pro-death factors IRE1α and CHOP in MCF-7 cells. Earlier studies have shown that in the cellular apoptotic event, execution of apoptosis occurs by PARP cleavage and DNA fragmentation of the cell [56,57]. The executioner caspase-3 and caspase-7 play important roles in this phase and if activated it can cleave different intra-cellular proteins and execute apoptosis. In MCF-7 cells caspase-3 is mutated [43]. Caspase-7 acts as an executioner caspase in MCF-7 cells and PARP, a downstream target of both the caspases is more efficiently cleaved by caspase-7 than caspase-3 [58]. In our study, we found that subtilisin treatment induced caspase-7 activation in MCF-7 cells but proteasome inhibitor MG-132 can revert back this activation in a dose dependent manner. We also showed subtislisin treatment activates PARP cleavage and nuclear DNA degradation, leading to apoptosis in MCF-7 cells.

Microtubule targeting agents (MTAs) bind to tubulin and disrupt the microtubule dynamics causing cell cycle arrest and apoptosis. Cancer therapeutics that uses MTAs are incredibly beneficial. Various tubulin targeting drugs (paclitaxel, docetaxel and vinblastine) have been used successfully in cancer treatments and are shown to be very effective against cancer [59]. Several MTAs are also reported that can act through degradation of tubulin to induce apoptosis such as T0070907 that causes tubulin degradation through proteasomal mediated pathway. Hence, molecules like T007097 can be used to decrease cellular tubulin levels, leading to their use in therapies against cancer [60]. In this context, it is worthy to state that subtilisin can also be used as a MTA as it could induce tubulin degradation in MCF-7 cells through ubiquitin-proteasomal dependent pathway.

Our results showed that subtilisin induces cell death in MCF-7 and HT-29 cells but not in normal mouse peritoneal macrophages and normal breast MCF10A cells. The role of any specific receptors targeted by subtilisin which may be present only in cancer cells but not in normal cells needs to be studied further. Though we have shown the anti-cancer effect of subtilisin on *in-vitro* cancer cell line, the *in-vivo* mouse model for regressing solid tumor and intra-peritoneal mouse model to study the survival kinetics of mice in treatment with subtilisin needs to be studied to further evaluate the role of subtilisin as an anti-cancer molecule.

5. Conclusion

The potential of subtilisin from *Bacillus amyloliquefaciens* as an anti-cancer drug and its mechanism of action have been established in this study. Subtilisin induces apoptosis only in cancer cells but does not affect the normal cells. Subtilisin activates ubiquitination of tubulin by enhancing PARKIN-tubulin interaction, resulting in tubulin degradation and subsequent apoptosis in cancer cells. To execute apoptosis, subtilisin induces caspase-7 activity, PARP cleavage and nuclear DNA damage in the cells. Based on our results, we conclude that subtilisin could be a potent therapeutic candidate for cancer treatment.

Abbreviations

Bicinchoninic acid BCA
Sodium chloride NaCl
Diethylaminoethyl-52 cellulose DE-52
Endoplasmic reticulum ER
Ethylenediamine tetraacetic acid EDTA
Fluorescence activated cell sorting FACS

Forward primer Fwd
Hydrochloric acid HCl
Human epithelial growth factor hEGF
Matrix-assisted laser desorption/ionization-Time of flight MALDI-ToF
Nutrient broth NB
N-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal MG-132
Nonidet P-40 NP-40
Optical density OD
Phosphate buffer saline PBS
Phenylmethylsulfonyl fluoride PMSF
Polyacrylamide gel electrophoresis PAGE
Polymerase chain reaction PCR
Polyvinylidene fluoride PVDF
Propidium iodide PI
Revers primer Rev
Reactive oxygen species ROS
Room temperature RT
Small interfering RNA Si-RNA
Sodium hydroxide NaOH
Sodium dodecyl sulfate SDS
Sodium chloride NaCl
Trichloroacetic acid TCA
Tris-buffered-saline-twin20 TBST
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid HEPES

CRediT authorship contribution statement

Nanda Singh: Conceptualization, Supervision, Resources, Methodology, Validation, Formal analysis, Investigation, Writing- original draft preparation, Visualization, Data Curation.

Rima Tapader: Conceptualization, Investigation, Methodology, Formal analysis.

Shruti Chatterjee: Resources.

Ananda Pal: Formal analysis, Flow-cytometric data acquisition.

Amit Pal: Conceptualization, Supervision, Funding acquisition, Project administration, Methodology, Investigation, Resources, Writing-original draft preparation, Writing- Review and Editing.

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