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CHOSUN UNIVERSITY

February 2024
Ph.D. Dissertation

Biochemical characterization, antioxidant and anticancer mechanism of medicinally effective protease from *Bacillus siamensis* with prominent therapeutic applications

Graduate School of Chosun University

Department of Pharmacy

Hasan Tarek

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Biochemical characterization, antioxidant and anticancer mechanism of medicinally effective protease from *Bacillus siamensis* with prominent therapeutic applications

*Bacillus siamensis*가 생산하는 단백분해효소의 생화학적 특성규명, 항산화 및 항암 메커니즘, 의학적 활용 연구

February 23, 2024

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Biochemical characterization, antioxidant and anticancer mechanism of medicinally effective protease from *Bacillus siamensis* with prominent therapeutic applications

Advisor: Prof. Jin Cheol Yoo

This dissertation is submitted to the Graduate School of

Chosun University in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

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This is to certify that the Ph.D. dissertation of Hasan Tarek has successfully met the dissertation requirements of Chosun University

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Abbreviations

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

AMP: Antimicrobial peptide

AU: Arbitrary Unit

AUC: Area Under the Curve

CLSM: Confocal Laser Microscopy

DCFDA: 2,7-Dichlorofluoroscin Diacetate

DMEM: Dulbecco's Modified Eagle Medium

DFP: Di isopropyl fluorophosphate

DMSO: Dimethyl Sulfoxide

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetra acetic acid

LAB: Lactic Acid Bacteria

CFU: Colony Forming Unit

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MH: Muller-Hinton

MHA: Muller Hinton Agar

MHB: Muller Hinton Broth

MIC: Minimum Inhibitory Concentration

MRS: Media-De Man-Rogosa-Sharpe

MTT: 3-(4, 5-dimethyltheazol-2-yl)-2, 5-diphenyl tetrazolium bromide

PI: Propidium Iodide

ROS: Reactive Oxygen species

TPTZ: 2,4,6-Tris(2-pyridyl)-s-triazine

PMSF: Phenylmethylsulphonyl fluoride

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Abstract

Biochemical characterization, antioxidant and anticancer mechanism of medicinally effective protease from *Bacillus siamensis* with prominent therapeutic applications

Hasan Tarek

Advisor: Prof. Jin Cheol Yoo

Department of Pharmacy

Graduate School of Chosun University

Proteases are important enzymes that contribute to a range of essential biological functions and have a significant possibility for commercial applications. In this work, we reported the purification and biochemical description of a detergent-stable and multifunctional protease enzyme (SH21) produced by *Bacillus siamensis* CSB55 isolated from Korean fermented vegetable kimchi. SH21 was purified to obtain homogeneity via ammonium sulfate precipitation (40-80%), Sepharose CL-6B, and Sephadex G-75 column. By analyzing SDS-PAGE and zymogram, it was determined the molecular size was around 25 kDa. The NH_2-terminal sequence of purified SH21 was QTGGSFFEPFNSYNSGLWQKANGYS. The enzymatic activity was almost entirely inhibited in the presence of PMSF and DFP, which specified that it is a member of serine protease family. SH21 showed excellent activity with a wide scale of pH and temperature, with its maximum

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pH of (9.0) and temperature of 55 °C. The enzyme had estimated K_m and V_{max} values of 0.197 mg/mL and 1.22×10^3 U/mg, separately. In addition, it preserved good activity with different organic solvents, surfactants, and other reagents. This enzyme also showed good antimicrobial activity that was evaluated by MIC against several pathogenic bacteria. It exhibited strong antibiofilm activity as determined by MBIC and MBEC assay and degraded the biofilms which were analyzed by confocal microscopic study. SH21 displayed very powerful antioxidant and ROS (reactive oxygen species) production inhibition activity in a dose-dependent approach. The mRNA expressions and protein levels of antioxidant enzymes including SOD1 (superoxide dismutase 1), CAT (catalase), and GPx-1 (glutathione peroxidase 1) were enhanced in the SH21-treated sample. SH21 also boosted the translational and transcriptional activities of NF-E2-related factor 2 (Nrf2) with the subsequent development of detoxifying enzyme heme oxygenase-1 (HO-1). In addition, SH21 showed potential anti-inflammatory activity via inhibition of nitric oxide (NO) and proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, generation in LPS stimulated RAW 264.7 cells. At concentrations of 60, 80, and 100 μg/mL, SH21 potentially suppressed nitric oxide synthase (iNOS) and cytokine gene expressions. Furthermore, SH21 significantly released lactate dehydrogenase (LDH) enzyme in cancer cell supernatant in a dose-dependent approach and exhibited effective anticancer activity against three tested cancer cell lines,

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including cells HL-60, A549, and Hela. Our results suggest that SH21 has effective antimicrobial, antioxidant, anti-inflammatory, and anticancer effects and could be an excellent therapeutic agent against inflammation-related diseases.

Keywords: Protease SH21, SDS-PAGE, Antioxidant, Anticancer, Anti-biofilm, Mechanism of action, Confocal microscopy.

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국문 초록

*Bacillus siamensis*가 생산하는 단백분해효소의 생화학적 특성 규명, 항산화 및 항암 메커니즘, 의학적 활용 연구

하산 타렉

지도교수:유진철

조선대학교 약학대학 약학과

단백분해효소는 다양한 필수 생물학적 기능에 중요한 역할을 하며 상업적으로도 큰 가능성을 가진 효소입니다. 이 연구에서는 한국의 전통발효식품 김치에서 분리된 *Bacillus siamensis* CSB55가 생산하는 세제 안정성을 갖춘 다기능 단백분해효소(SH21)의 정제 및 생화학적 특성을 보고합니다. SH21은 황산 암모늄 침전 (40-80%), Sepharose CL-6B, Sephadex G-75 컬럼을 통해 순수하게 정제되었습니다. SDS-PAGE 및 zymogram분석으로 그 분자 크기는 약 25 kDa로 확인되었습니다. 정제된 SH21의 N말단 서열은 QTGGSFFEPFNSYNSGLWQKANGYS입니다. 이 효소의 활성은 PMSF 및 DFP의 존재 하에서 거의 완전히 억제되었으며, 이는 SH21이

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serine protease 계열에 속한다는 것을 나타냅니다. SH21은 다양한 pH와 온도 범위에서 뛰어난 활성을 보였으며, 최적 pH는 9.0, 최적 온도는 55 °C였습니다. 이 효소의 추정 K_m 및 V_{max} 값은 각각 0.197 mg/mL, 1.22×10^3 U/mg입니다. 또한, 다양한 유기 용매, 계면 활성제, 그리고 다른 시약들과 함께 좋은 활성을 유지했습니다. 이 효소는 다양한 병원균에 대한 MIC 측정을 통해 강력한 항균 활성을 나타냈습니다. MBIC 및 MBEC 실험을 통해 확인된 강력한 항생물막 활성도 보여주었으며, 공초점 현미경 연구를 통해 생물막을 분해하는 것이 확인되었습니다. SH21은 용량 의존적으로 강력한 항산화 및 ROS (reactive oxygen species) 생성 억제 활동을 보였습니다. SH21 처리된 샘플에서 SOD1(superoxide dismutase 1), CAT(catalase), GPx-1(glutathione peroxidase 1)과 같은 항산화 효소의 단백질 및 mRNA 수준이 향상되었습니다. 또한, SH21은 Nrf2의 번역 및 전사 활동을 촉진하며, 이에 따라 해독 효소인 heme oxygenase-1 (HO-1)발달을 이끌었습니다. 더욱이, SH21은 LPS로 자극된 RAW 264.7 세포에서 NO 및 사이토카인(예: TNF-α, IL-6, IL-1β) 생성을 억제하는 방식으로 항염증 활동을 보였습니다. 60, 80, 100 μg/mL의 농도에서 SH21은 질산화효소(iNOS) 및 사이토카인 유전자 발현을 효과적으로 억제했습니다. 또한, SH21은 용량 의존적으로 암 세포 배양액에서 젖산 탈수소 효소(LDH)의 방출을 촉진하며 HL-60, Hela, A549 등의

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테스트된 암 세피들에 대해 효과적인 항암 활성을 보였습니다. 이러한 연구 결과는 SH21이 항균, 항산화, 항염증 및 항암 효과를 갖추고 있으며, 염증 관련 질환에 대한 우수한 치료제가 될 수 있음을 시사합니다.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Kimchi, a traditional Korean fermented food

Kimchi, a tasty combination of fermented vegetables and spices with a sour and salted flavor, is essential in Korean cuisine. The kinds of materials and the way they are prepared greatly influence the flavor of the more than 200 different types of kimchi. Classic baechu style asks for radishes, napa cabbage, ginger, garlic, green onions and a particular red pepper; nevertheless, regional variations, seasonality, and cultural customs all affect the distinctive varieties of kimchi.

Figure 1.1. Distinct varieties of kimchi.

(Ref URL: https://www.gobizkorea.com/user/goods/frontGoodsDetail.do)

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Health benefits

Promotes Digestion

A great food to aid in digesting is kimchi. Because of the fermentation technique used in its manufacture, it is a source of probiotics. In addition to improving flavor, fermentation produces *Lactobacillus*, a good bacterium that the body needs to maintain a balanced population of gut microbes.

Regulates Cholesterol

Regularly kimchi eating positively lowers cholesterol levels. It is prepared with garlic, which is high in allicin and selenium. One important ingredient that lowers cholesterol is allicin, which also lowers the chance of coronary artery disease and other cardiovascular diseases.

Antioxidant Properties

Different types of kimchi are full of potent antioxidants, which act as natural scavengers. Together with the flavonoids and phenols it contains, these antioxidants provide a defense against oxidative stress.

Treats Atopic Dermatitis

Kimchi has multiple uses because of the *lactobacillus* bacteria that it contains. Its medicinal properties are expanded to include a variety of skin conditions, including atopic dermatitis.

Weight Loss

As mentioned previously, the good lactobacillus bacteria found in kimchi are beneficial to the body's normal processes. This beneficial bacterium lowers

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glucose levels and regulates hunger, which both help with losing weight.

Boosts Immune System

A variety of flavonoids and phenolic components are abundant in the nutrient-dense, nutrient-packed kimchi. The wide range of components used to make kimchee, such as peppers, ginger, and garlic, are known to be immunity system-boosting super defenders.

Anti-aging Properties

Since antioxidants and vitamin C are present in kimchi, it also has anti-aging properties, which is a useful benefit. Research assessing kimchee's anti-aging effects has shown that it aids in controlling and reducing inflammation.

Prevents Cancer

Kimchi is a beneficial meal that appears to lower the chance of developing certain cancers. An investigation on the samples proved its anti-cancer effects. It comprises beneficial flavonoids that are proven to destroy the progression of cancer cells.

Treats Diabetes

Kimchi has anti-diabetic qualities, according to a study done on type-2 diabetics on high-fat diets. The investigation revealed that when the diabetics consumed a diet high in kimchi, their glucose sensitivity improved, and their fasting blood sugar levels decreased.

Reduces Gastric Ulcers

Kimchee is beneficial in treating Helicobacter pylori-caused gastroenteritis

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and ulcers. According to an in-depth investigation, the antagonistic activity of kimchi is ascribed to the profusion of *Lactobacillus* bacteria, which prevents hazardous microorganisms from attaching themselves to human stomach cancerous cells.

Figure 1.2. Various health benefits of kimchi.

(Ref URL: https://www.chefjanapinheiro.com/these-are-the-health-benefits-of-kimchi/)

1.2 Microbial enzymes

Microbial enzymes are found in an extensive range of industrial usage, such as food manufacturing, agriculture, the fermentation process, and chemistry. Recombinant enzymes have been expressed by filamentous fungi, yeasts, and bacteria; the selection of suitable expression systems is crucial for the

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volume of production of enzymes. Because of these species' benefits, the number of biotechnological uses has grown. However, high-level generic enzymatic expression is difficult to accomplish due to physiological effects. The drawbacks of natural enzymes, particularly in industries, include limited activity, stability, and catalytic effectiveness. A staple of Korean cuisine, kimchi is a delicious, sour, and salty concoction of fermented vegetables and condiments. Around 250 varieties of kimchi, and the kind of materials used and how they are prepared greatly affect how the dish tastes. The traditional baechu version calls for Napa cabbage, radishes, green onions, garlic, ginger, and a certain red pepper; however, the distinctive varieties of kimchi are influenced by geography, the seasons, and regional traditions.

1.3. Protease enzymes

Proteins are broken down by enzymes called proteolytic enzymes, or proteases. Plants, bacteria, fungi, and animals all produce these enzymes. Chymotrypsin, bromelain, papain, Serra peptidase, ficin, papain, and trypsin are a few protease enzymes that can be present in supplements.

1.3.1. Sources of protease enzymes

The quest for proteases has greatly expanded due to the enormous demand for them on the global market, even though they are found in all outlines of life, plus bacteria, flora, and wildlife. Plant proteases like ficin, keratinases, and bromelain require a lot of time to produce. Large amounts of purified animal proteases, including renin, pepsin, trypsin, chymotrypsin, and

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pancreatic, are manufactured [1]. Scientists are now researching the manufacture of proteases from microbial sources to meet the macro industrial demand. Microbes produce a very promising number of proteases for of their wide-ranging-spectrum natural variability and accessibility of genetic manipulation [2]. Proteases can be produced by a range of resources, containing bacteria, plants, and mammals, but they are mostly produced by microorganisms. Several genus of fungi, such as *Aspergillus* sp., are well-studied for the manufacture of alkaline protease [3]. *Bacillus sp*. is one of the bacteria that is widely investigated for producing protease on a huge scale, and it is used in many sectors, including leather, soap, medicine, and clothing. Below is a list of bacteria that can produce proteases. The capacity of halophilic enzymes to maintain function under severe stress from organic diluents excluding pyridine, which reduces protease action—and their thermal stability are drawing increased attention in biotechnological applications. For at least 30 minutes, the enzyme activity stayed constant up to 80% at 50, 55, and 60 ◦C [4].

1.3.2. Classifications of protease enzymes

Depending on where they work, proteases can be generally classified into 2 classes: endopeptidases and exopeptidases. Whereas endopeptidases break peptide bonds far from the substrate's terminals, exopeptidases break peptide bonds close to the substrate's amino or carboxy terminals. Aspartic, metallo, serine cysteine are the four main categories into which proteases are further

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divided according to the group of functions that exist at the site of activity.

Exopeptidases: Exopeptidases are enzymes that operate at the extremities of polypeptide chains. These are classed as amino- and carboxypeptidases depending on whether their site of activity is at the N or C terminal.

Aminopeptidases: Aminopeptidases release a specific amino acid residue, when they operate at the polypeptide chain's free N terminus. It is known that they eliminate the N-terminal Met, which is present in some proteins that are expressed heterologous yet not in numerous mature peptides that occur in nature.

Carboxypeptidases: Single amino acids or dipeptides are released by the carboxypeptidases when they act on the C terminals of polypeptide chains. Carboxypeptidases are categorized into three main classes according to the kind of residues of amino acids action Metallo carboxypeptidases, and cysteine carboxy peptidases.

Serine proteases: The existence of a serine unit in the active site of serine proteases is one of their distinguishing features. They appear to be essential to viruses, bacteria, and eukaryotes because they are abundant and distributed throughout these species.

Aspartic proteases: Aspartic acid proteases are endopeptidases whose catalytic activity is reliant on aspartic acid residues. Clan AA is home to the three families of acidic proteases, which are pararetrovirus enzymes (A3), retropepsin (A2), and pepsin (A1) (13) enzymes. While there is some

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evidence of a relationship between members of families A3 and A2, adherents of relations A1 and A2 are known to be linked to one another. The majority of aspartic proteases exhibit their peak activity at low pH levels (between 3 and 4) and have isoelectric values between 3 and 4.5. They range in molecular weight from 30 to 50 kDa.

Cysteine/thiol proteases: Prokaryotes and eukaryotes both have cysteine proteases. There are now about 20 known groups of cysteine proteases. A catalysis dyad made up of cysteine and histidine is necessary for all cysteine proteases activity. Each family has a different sequence for Cys and His residues. Cysteine proteases are often only active when reducers like cysteine or HCN are present. The most well-known cysteine protease is papain.

Metalloproteases: The largest number of proteases that are catalytic are called metalloproteases. Their characteristic of needing a divalent metal ion to function is what makes them unique. Collagenases from greater species, bleeding poisons from snake venom, and thermolysin from bacteria are just a few examples. It is estimated that there are about thirty families of metalloproteases; seventeen of these families hold individual endopeptidases and exopeptidases, and one family (M3) has both endo- and exopeptidases.

1.3.3. Applications of protease enzymes

There are several uses for proteases, mostly in the food and detergent sectors. Proteases are expected to find widespread use in a variety of disciplines, especially in light of the current trend toward the development of

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ecologically benign technology.

Detergents

Proteases are a common component of many soaps, from those used for everyday laundry to those employed as chemicals for washing prosthesis or lenses for contact lenses. About 25% of enzyme sales globally are attributed to the usage of proteases in laundry detergents. To make it easier to remove stains caused by a wide range of substances, including blood, food, and other bodily secretions, the perfect detergent protease should have wide substrate specificity.

Leather Industry

There are various processes involved in preparing leather, including dehairing, soaking, tanning, and bating. The main components of membrane and beard are proteinaceous materials. Hazardous chemicals like sodium sulfide are used in traditional leather manufacturing procedures, which lead to issues with pollution and wastewater disposal. Enzymes have been successfully used in place of chemicals to improve the quality of leather and reduce contamination in the natural world. Proteases are utilized to eliminate nonfibrillar peptides such as globulins and albumins, as well as to selectively hydrolyze noncollagenous components of the skin.

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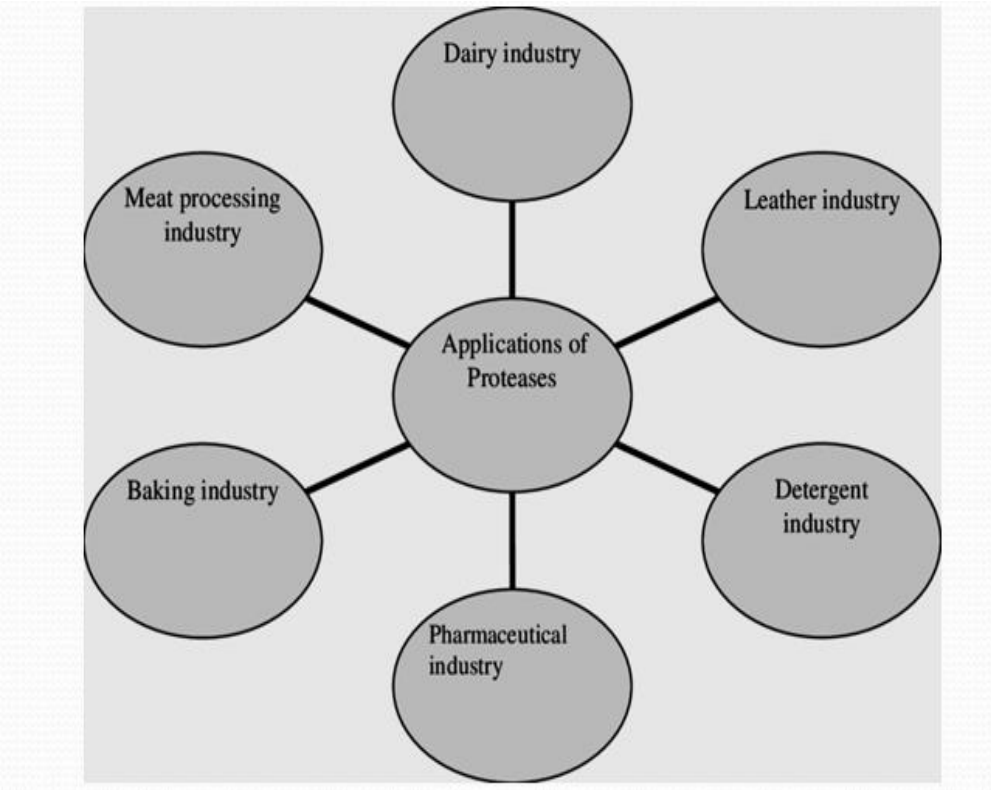

Figure 1.3. Different applications of protease enzymes.

Food Industry

Proteases are frequently utilized in a variety of processes, including baking, cheesemaking, soy hydrolysate manufacturing, and meat tenderization.

Dairy industry: The making of cheese is the primary utilization of proteases in this sector. There are three primary types of milk-coagulating enzymes: animal rennet, bacterial milk coagulators, and heritably reformed chymosin. Milk-coagulating proteases, which have molecular size extending from 30 to 40 kDa, are classified as acid aspartate proteases by both microorganisms and animals.

Baking industry: One important ingredient in baking recipes is wheat flour. It

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has gluten, an insoluble protein that controls the characteristics of bread doughs. Utilizing restricted proteolysis, endo and exoproteinases produced by Aspergillus oryzae are being utilized to alter wheat gluten. The dough can be treated enzymatically to make it easier to handle, machine, and produce a larger variety of goods. Protease inclusion increases loaf volume while cutting down on mixing time. To increase the dough's strength and adaptability, microbial proteases are employed.

Production of soy products: Because soybeans are a great origin of high-quality protein, they are also a rich diet source. Since ancient times, soybean sauce and other soy products have been made using proteases. Soy sauce preparation involves the use of neutral and alkaline proteases that are derived from fungi.

Medical Field

The development of potent medicinal medicines benefits greatly from the broad variety and sensitivity of proteases. Proteases that from *Aspergillus oryzae* (Luizym and Nortase) have been administered orally to treat some lytic enzyme deficient disorders. Burning and infections are treated with a mixture of broad-spectrum antibiotics and clostridial collagenase or subtilisin. In order to remove aspargine from the bloodstream in different kinds of lymphocyte cancer, an asparginase derived from *E. coli* is utilized.

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1.3.4. Physiological roles of protease enzymes

Many complex physiological processes are performed by proteases. Their magnitude in executing the basic physiologic and controlling processes is demonstrated by their presence in the entirety of life. Proteases are essential for numerous processes in biology and medicine, including the breakdown of proteins, coagulation of blood, immigration and growth of cells, tissue organization, morphogenesis through progress, inflammation, metastasis and growth of tumors, activation of zymogens, hormone release, peptides with pharmacological activity derived from precursor proteins and cell membranes proteins.

1.3.5. Future prospects of protease enzymes

Researchers are becoming more interested in the chemical and cellular features of proteolytic networks, including proteases, for a variety of causes. The discovery of this enzyme's biological and economic importance has led scientists and engineers to explore strong and unique bacterial enzymes. Proteases with novel characteristics will be mostly produced in the future through protein engineering. Because of their versatility, alkaline bacterial proteases are essential to many sectors and are expected to see expanded application soon. Scientists are using cutting-edge techniques including molecular biology, biological computation, and protein/genetic manipulation to create better variants of proteases. The main structure of proteins will undergo in vitro evolution to develop varieties of bacteria with desired

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properties. The development of microbial proteases with properties like increased output, modified substrate selectivity, increased heat strength, adjusted ideal pH, and avoided auto-proteolytic deactivation is one of science's main objectives.

1.4. Objective of this study

The increased need for proteases with certain properties has researchers searching for new protease sources. Microbial proteases have the prospective to be medicinal agents for a broad scope of illnesses and infections in addition to being proteolytic enzymes. This could have an important effect on clinical and therapeutic approaches. Numerous microbes produce proteases, which are implicated in the control of consumption and the emergence of a number of infectious illnesses. Antimicrobial resistance has emerged as a major global health problem in recent times, resulting in over ten million deaths annually as treatment failure escalates because of improper utilization of antibiotics. Vegetable fermentation has been widely used to produce bioactive chemicals. Kimchi's strong lactic acid-producing bacterial composition makes it extremely healthy for people. The objective of this study was to investigate the stability and compatibility of protease with different commercial detergents as a potential option for industrial uses as well as evaluate antimicrobial, antioxidant, anti-inflammatory and anticancer properties of protease enzyme by different molecular mechanisms.

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CHAPTER TWO

SCREENING, ISOLATION, PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF PROTEASE SH21

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2.1. INTRODUCTION

There are various kinds of industrial enzymes in the marketplace. However, proteases embody one of the most valuable groups, containing around 40% of the manufacturing enzyme market share worldwide [5]. In a few industries, they are employed for a variety of uses, including laundry detergent, foodstuff, pharmaceuticals, rawhide, peptide combination, and retrieval of silver from applied X-ray films [6]. The most frequently used commercial enzymes are microbial proteases, particularly those from *Bacillus sp.*, which find widespread use in cleaner components and further commercial uses. Applications in bioengineering and biotechnology are interested in proteases with a higher activity and stability in higher alkaline ranges, because laundry detergents typically have a pH between 9.0 and 12.0, where they are mostly used. The pH of the cleansing mixture, laundry temperature, and soap constitution are only a few of the variables that affect how well detergent proteases work. Consequently, proteolytic enzymes used in detergent compositions need to have a substantial amount of activity.

Numerous investigations have been carried out on alkaline proteases from strains that produce high yields, but bleach-stable enzymes are rarely found, excluding limited studies [7,8]. Furthermore, several alkali proteases isolated from various bacilli strains have been purified and characterized [9,10]. Due to their broad specificity, a few commercial proteases, such as subtilisin isolated from Bacillus sp., have been chosen for detergent formulation

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[11,12]. This enzyme displayed its highest activity at pH levels of 8.0 to 10.0. Nowadays, researchers are looking into additional resources of proteases due to the rising necessity for proteases including specific characteristics. Unlike those isolated from plants and animals, microbial proteases often exist extracellularly and are directly released by the producer into fermentation media that ease the subsequent processing, purification, and enzyme recovery. Finally, we explore the strength and compatibility with diverse industrial detergents of protease SH21 as a possible entrant for business applications, mainly in the detergent formulation.

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2.2. MATERIALS AND METHODS

2.2.1. Materials and bacterial strains

The strain CSB55 was mostly derived from fermented Korean food kimchi. The columns sepharose CL-6B and Sephadex G-75 column were purchased from Pharmacia. Commercial media (MRS) De Man, Rogosa and Sharpe, and Muller-Hinton (MH) were bought from Becton-Dickinson.

2.2.2. Screening, isolation, and phylogenic tree analysis of Protease enzyme

Samples of kimchi were collected from various South Korean provinces. For isolating bacteria, one g of kimchi was combined with 0.85% sodium chloride solution and then keep alive at 37 ◦C for 24 h. For adjusting the CFU, the mixed samples were streaked on (MHA) plate composed of 0.2% beef extract, 1.76% acid digest of casein, 0.20% lactose, and 0.17% agar powder (*w*/*v*) after being serially diluted up to 10-7. Appropriately diluted samples were preserved in glycerol (20%) at -80 ◦C. Eighty strains were streaked in casein-agar plates containing 1% casein mixed with 0.5% peptone, 0.5% tryptone, 0.02% MgSO4.7H2O, 0.03% CaCl2, 0.03% KH2HPO4, and 1.5% agar for primary screening, and plates were developed at 37 ◦C for 24 h. Congo red (0.5%) was used and then washed with distilled water. Afterwards, the plates were filled through 1M sodium chloride solution for 20 min and cleaned with filtered water to detect a clear zone of

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casein hydrolysis. Primarily selected strains were grown in a protease production medium at 37 ◦C for 96 h, and the protease activity was observed at every 4 h interval. The strain CSB55 exhibited the utmost protease action and was preferred for the current study. The identification of his strain was done based on morphologic features and 16S rRNA gene sequence assessment.

2.2.3. Media and Culture Conditions

Protease production was performed by a *Bacillus siamensis* CSB55 strain in a medium involving (g/L): casein, 11; peptone, 6; tryptone, 6; $MgSO_4.7H_2O$, 0.1; KH_2HPO_4, 0.2; CaCl2, 0.1; K_2HPO_4, 0.1; pH 8.5. To make the seed culture inoculum, Luria-Bertani (LB) broth medium was used, made of (g/L): peptone, 12; yeast extract, 6; NaCl, 0.5; pH 7.0, and then it heated at 121 ◦C for 20 min. A 1% seed culture was added to a 2L Erlenmeyer baffle flask containing 300 mL protease production media and incubated for 64 h at 37 ◦C with constant shaking at 160 rpm. The cultured medium was centrifuged at 10,000 rpm for 30 min at 4 ◦C, and the supernatant was utilized as a crude protease.

2.2.4. Protease Assay

The activity of the protease enzyme was estimated corresponding to Benmebarek method. [53] with slight changes. In short, 150 µL of the substrate solution (azocasein 0.5%) was combined with 350 µL of the enzyme sample. The mixer was developed at 55 ◦C for 30 min. Then, 500 µL

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of TCA (10% *v*/*v*) was inserted to stop the reaction and at normal conditions for 30 min. Then, the centrifuge was carried out at 12,000 g for 10 min to eliminate undigested sample. Later, 200 µL of supernatant was mixed with 800 µL of 1 N NaOH solution.

2.2.5. Purification of Protease SH21

After *Bacillus siamensis* CSB55 strain was grown for 64 h, the cell-free broth was stored by centrifugation at 12,000 g for 30 min. The protease-containing supernatant was exposed to the subsequent purification procedures. At first, ammonium sulfate (40–80%) was added to the supernatant by softly stirring and was kept overnight at 4 ◦C. Then, it was re-centrifuged and precipitation pellets were suspended in a required volume of (20 mM, pH 9.0) Tris-HCl buffer. The enzyme sample was subjected to the Sepharose CL-6B column (80 cm × 1.8 cm), which was earlier equilibrized with a Tris-HCl buffer. Fractions were collected with an elution rate of 0.30 mL/min. Then, lyophilization was done to concentrate the active fractions. The thick protein was loaded into a Sephadex G-75 column (20 cm × 2.0 cm), and protein elution was performed utilizing the same buffer. After that, active protease fractions were collected, they were lyophilized, and their purity was analyzed. The purified protease was used for other characteristics and applications.

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2.2.6. Protein Measurements, SDS-PAGE, and Zymogram Analysis

The Bradford method was used to estimate the protein concentrations [54], and the BSA standard was used. SDS-PAGE was done operating 12% (*w*/*v*) separating and 5% (*w*/*v*) stacking gel, as reported by Laemmli et al. [55]. Coomassie Brilliant Blue R-250 (BioRad Laboratories, Inc., Hercules, CA, USA) was used to color the gel to visualize the protein bands. PageRuler Prestained protein ladder (10–170 kDa) was used as a standard protein marker. As described earlier, zymography staining was done by using casein as a substrate [56]. After electrophoresis, the gel was rinsed twice for 1 h with Triton X-100 (2.5% *v*/*v*) to substitute the separation buffer and SDS in the gel. Then the gel was hatched at 45 ◦C for 1 h in Tris-HCl buffer containing casein 1% (*w*/*v*) to produce a cleared area at the site of the proteolytic band. Afterwards, the gel was fixed by using 20% (*w*/*v*) ice-cold TCA for 1 h surveyed by tint with Coomassie Brilliant Blue R-250 (0.1%, *w*/*v*) in a solution containing acetic acid/methanol/ water (10:40:50), and distaining was done with the similar solution in the absence of the dye.

2.2.7. Determination of Optimum pH and Stability

To investigate the influence of pH on the enzyme activity, azocasein (0.5%) was used as a substrate, and an assay was performed by a conducting enzyme (0.01 mg) with twelve buffers extending from pH 2 to 13 at 55 ◦C for 2 h. The pH constancy was also observed by brewing the enzyme with different pH buffer solutions at 40 ◦C for 24 h.

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2.2.8. Determination of Optimum Temperature and Thermal Stability

To evaluate the temperature impact on the enzymatic activity, purified protease (0.01 mg) was incubated at numerous temperatures between 30 and 80 ◦C with an interlude of 5 ◦C for 1 h. The thermostability of the protease was analyzed by incubating the solution at several temperatures for 24 h. Proteolytic activity was estimated by applying azocasein as a substrate. The activity of the non-heated sample was assumed as 100%.

2.2.9. Effect of Inhibitors, Metals, Surfactants, and Bleaching Agents on Protease Activity

To check the effect of protease inhibitors on the enzyme activity, purified SH21 (0.01 mg) was incubated with PMSF, DFP, TLCK, TPCK, SBTI, benzamidine, iodoacetamide, 2-mercaptoethanol, leupeptin, pepstatin A, DTNB, EDTA, and EGDA for 2 h at 55 ◦C. The enzyme activity was employed under specific conditions and considered 100% in the absence of inhibitors. The effects of a certain number of monovalent and divalent metallic ions (3 mM), surfactants such as SDS (1% *w*/*v*), Triton X-100, Tween 80, Tween 20 (1% *v*/*v*), and bleaching agents (1% *v*/*v*) H_2O_2, and NaClO, were investigated by preincubating with 0.01 mg of the protease enzyme at 55 ◦C for 1 h. Protease activity was determined under standard environments. Lacking any reagents, the activity was assumed as 100%.

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2.2.10. Effect of Organic Solvents on Protease Activity

Different concentrations (25%, 50%, and 100% *v*/*v*) of various organic solvents, including DMSO, ethanol, butanol, acetone, methanol, , acetonitrile, chloroform, cyclohexane, hexane, decane, and toluene were incubated with the enzyme (0.01 mg) at normal temperature for 24 h. The remaining activity was calculated under a normal assay environment. The enzyme activity in the lack of any reagent was considered 100%.

2.2.11. Substrate Specificity and Kinetics Parameters of Protease SH21

To evaluate the hydrolytic activity of protease SH21, the enzyme solution (0.01 mg) was incubated with various substrates (1% *w*/*v*) such as casein, azocasein, gelatin, egg albumin, and collagen. To analyze K_m and V_{max} were computed using a Lineweaver–Burk plot. To perform the kinetic test, different azocasein substrate concentrations (0.6–10.0 mg/mL) were applied.

2.2.12. Stability and Compatibility of Protease with Commercial Laundry Detergents

Purified protease was investigated for stability and compatibility with currently commercialized laundry detergents. The detergents utilized in this experiment included Wheel, Surf Excel, Rin (Unilever, Mumbai, India), Chaka (Square Toiletries, Dhaka, Bangladesh), Fast wash (Kohinoor Chemical, Dhaka, Bangladesh), Bright (Mukunghwa, Seoul, Republic of Korea), and Super magic (Green Tech, Gwangju, Republic of Korea). The desired intensity of 7 mg/mL was achieved to make rinsing situation by

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diluting the mentioned detergents with tap water. Before adding the enzyme, diluted detergents were boiled at 70 ◦C for 1 h to deactivate the endogenous protease of laundry detergents. Then, the enzyme was incubated with detergents at 40 ◦C for 2 h, and the remaining activity was examined under normal assay environment. The activity with no detergent was assumed as 100%.

2.3. RESULT AND DISCUSSIONS

2.3.1. Screening Identification and phylogenetic tree of protease-producing strain.

In the extant exploration, about eighty new microscopic sorts were isolated from kimchi, and they were found as protease producers on casein agar media at pH 9 based on the clear area developed upon the hydrolysis of casein. The proportion of the clear area to colony diameter was used to choose strains with significant protease-producing capabilities. Among those isolates, 17 strains exhibited a clear hydrolytic zone on casein agar media after 20 h of development at 37 ◦C. Among them, a strain called CSB55 produced the highest clear hydrolytic zone and exhibited a high protease activity, thereby being chosen for further study. The identification of the strain CSB55 was labeled allowing to Systemic Biology based on morphologic and biochemical properties. A phylogenic tree was then constructed from a 16S rRNA gene sequence that exhibited 99.99%

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similarity to *Bacillus siamensis* KCTC 13613 (Figure 2.1.).

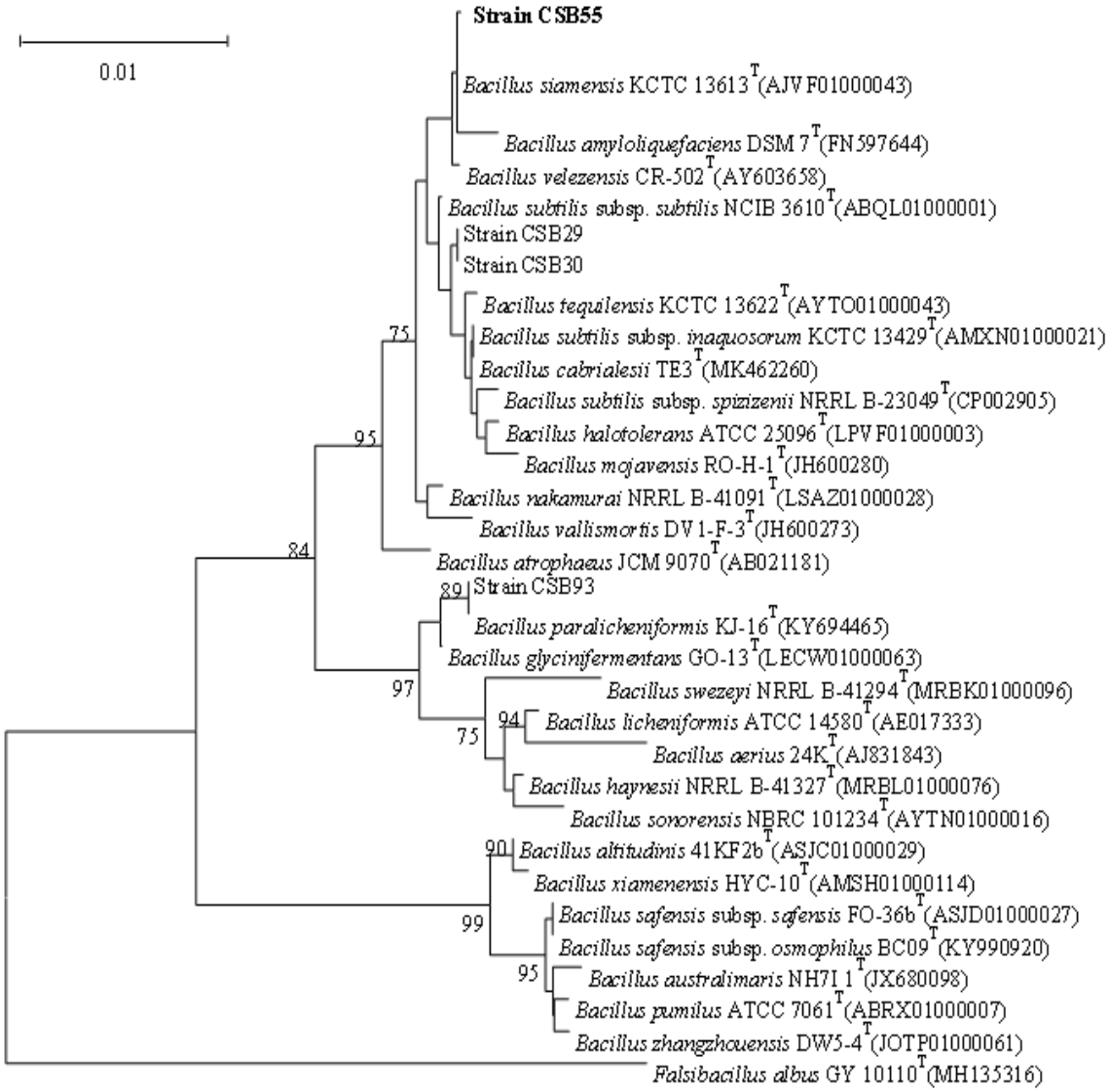

Figure 2.1. Neighbor-joining phylogenetic tree reconstructed based on 16s rRNA gene sequences displaying relationships between CSB55 and several nearly *Bacillus sp.*

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2.3.2 Enzyme Production and Purification of Protease SH21

Protease production media was used to produce the protease enzyme from *Bacillus siamensis* CSB55, and casein was used as a protease inducer in the medium. Enzyme production began at 16 h and increased to its highest level at 37 °C for 64 h. The crude enzyme preparation of protease from *B. siamensis* CSB55 was obtained using 1500 mL of culture supernatant. The SH21 was purified by three-step procedures with ammonium sulfate precipitation (40–80%) and using two size exclusion chromatography including Sepharose CL-6B and Sephadex G-75. All steps of purification are brief in Table 2.1. The elution outlines of Sepharose CL-6B and Sephadex G-75 are presented in Figure 2.2. As a result of the last purification stage, the enzyme SH21 had a yield of 16.23%, a specific activity of 2926.67 U/mg, and was 23.09-fold pure.

Table 2.1. Purification steps of protease SH21 from *Bacillus siamensis* CSB55

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Cell free	256	32454	126.77	1	100
Ammonium	132	24475	185.42	1.46	75.41
Sepharose	24	16562	690.08	5.44	51.03
Sephadex G-75	1.8	5268	2926.67	23.09	16.23

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2.3.3. Molecular weight determination and N- terminal amino acid sequence

The purified protease exhibited a single band on SDS-PAGE, indicating its homogeneousness. SH21 had a molecular size of nearly 25 kDa (Fig. 2.2.). In comparison with other *Bacillus* proteases, purified SH21 had a lower molecular weight than *Bacillus stearothermophilus* (28 kDa) [32], *Bacillus amyloliquefaciens* (43 kDa) [31], but higher than compared with those produced by *Bacillus subtillis* (17.1 kDa) [34], *Bacillus licheniformis* (19.7 kDa) [39]. For the purified sample, zymogram activity specified a caseinolytic zone with a projected molecular weight of 25 kDa was seen co-migrating with a protein. (Fig. 2.2). These findings strongly recommend that SH21 is a monomeric protein, like those found in other bacillus proteases. The N-terminal sequences of purified SH21 were determined to be QTGGSFFEPFNSYNSGLWQKANGYS. The arrangement analysis of the amino acid sequences of SH21 with other similar proteins from various *Bacillus* species is shown in Table 2.2.

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Table 2.2. Alignment of the N-terminal acid sequence of purified protease SH21 with theoretical proteins from various *Bacillus sp.*

SN	Microorganisms	N- terminal amino acid sequences	Identity (%)	NCBI References
1	*Bacillus siamensis*	QTGGSFFEPFNSYNSGLWQKANGYS	100	Current study
2	*Bacillus halotolerans*	QTGGSFFDPFNSYNSGLWQKANGYS	96	WP_059335710.1
3	*Paenibacillus macerans*	QTGGSFFEPFNSYNSGTWEKADGYS	88	1U0A_A
4	*Bacillus licheniformis*	QTGGSFYEPFNNYNTGLWQKADGYS	84	1GBG_A
5	*Bacillus subtilis*	QTGGSFFDPFNGYNSGFWQKADGYS	84	3O5S_A
6	*Bacillus subtilis*	GSVFWEP-KSYFNPSTWEKADGYS	58.33	1AXK_A

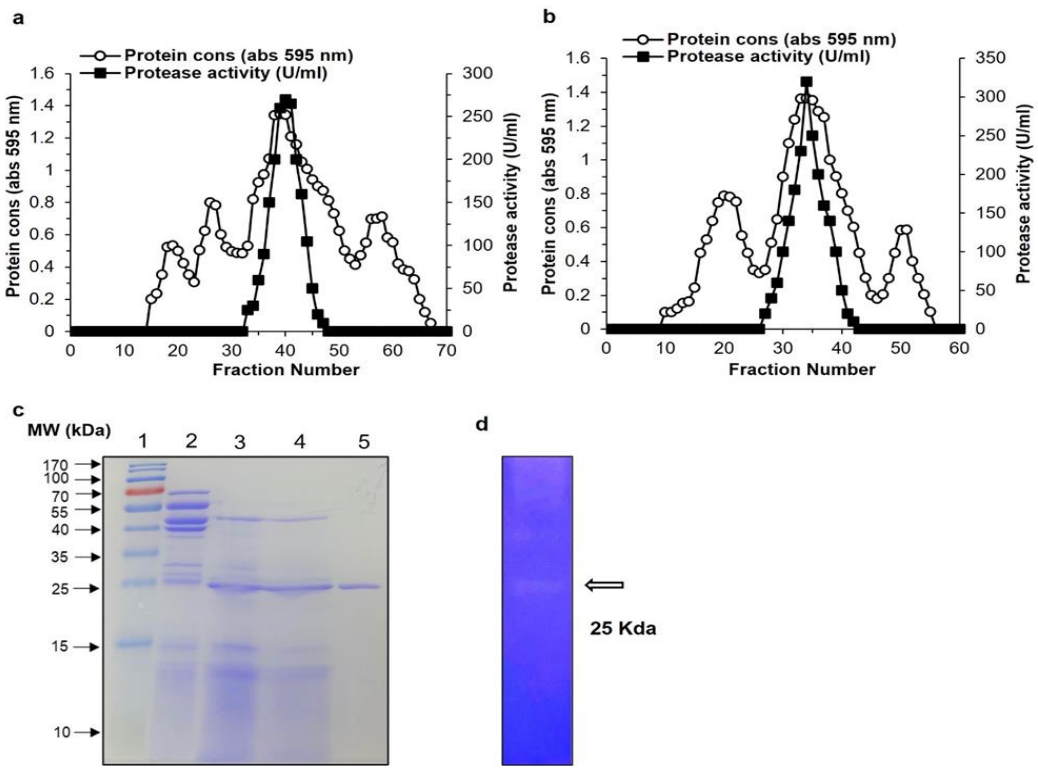

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Figure 2.2. Purification of protease SH21 from *Bacillus siamensis* strain CSB55. Chromatography profile of SH21 on gel filtration using (a) Sepharose CL-6B (80 cm × 1.8 cm) and (b) Sephadex G-75 column (1.5× 20 cm). (c) SDS-PAGE (12%) of purified protease SH21. Lane 1, Molecular weight marker standard from 10 to 170 kDa. Lane 2, crude extract. Lane 3, ammonium sulfate precipitation (40-80%). Lane 4, sample after Sepharose CL-6B chromatography. Lane 5, purified protease SH21 after Sephadex G-75 column. (d) zymography of purified SH21.

2.3.4. Effect of pH on the activity and stability of protease SH21

Bacillus sp. proteases are well known for their ability to tolerate acidic, alkaline conditions [36]. These are suitable criteria for the industrial use of protease enzymes. The enzyme SH21 revealed activity a wide range of pH (3.0-13.0) and was highest at pH 9.0 (Fig. 2.3a). The remaining activities were 52% and 50% at pH 6 and 12, respectively. However, protease SH21 displayed excellent activity from pH 7 to 11, and it was around 72 to 100%. The stability of protease was also good at a large variety of pH from 7 to 12 (Fig. 2.3b). SH21 is more effective at alkaline pH in comparison to commercially available detergent enzymes like Alcalase, which is produced by *Bacillus licheniformis* and has a maximum activity range of 8 to 9 [37]. The extreme activity and stability displayed by SH21 at alkaline conditions is an incredibly crucial property given the potential strength of future industrial

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applications, especially in detergent preparations, since they are mostly formulated at pH 7-11 [38].SH21 protease exhibits higher pH stability than currently commercialized detergent proteases on the market.

2.3.5. Effect of temperature on the activity and stability of protease SH21

Thermostability is a crucial factor when determining their possibility for purpose in an industrial process that demands high temperatures. Usually, thermostable proteases are used in industrial processes that are not possible at normal temperatures. The protease activity was remarked at pH 9.0 over a broad sort of temperatures from 30 to 80 °C with the maximum at 55 °C (Fig. 2.3c) which is higher than those protease enzymes *Bacillus pumilus* (40 °C), *B. subtilis* (50 °C) [39] and lower than *B. subtilis* (60 °C) [34], *Bacillus sp*. (70 °C) [40]. The thermostability profile of SH21 is presented in Fig. 2.3d. Protease SH21 showed good activity (≥80%) after being incubated at 40, 50, 60, 70, and 80 °C for 16,14, 10, 6, and 4 h, respectively. The enzyme activity remained around 50% when it was incubated at 80 and 90 °C for 4 and 2 hours, separately. So, this attribute indicated that protease SH21 had excellent thermal stability. Higher temperature is critical in chemical reactions because of the more excellent solubility of substrates, superior mixing, quicker reactions, decreased stickiness, and reduced possibility of bacterial contamination [37].

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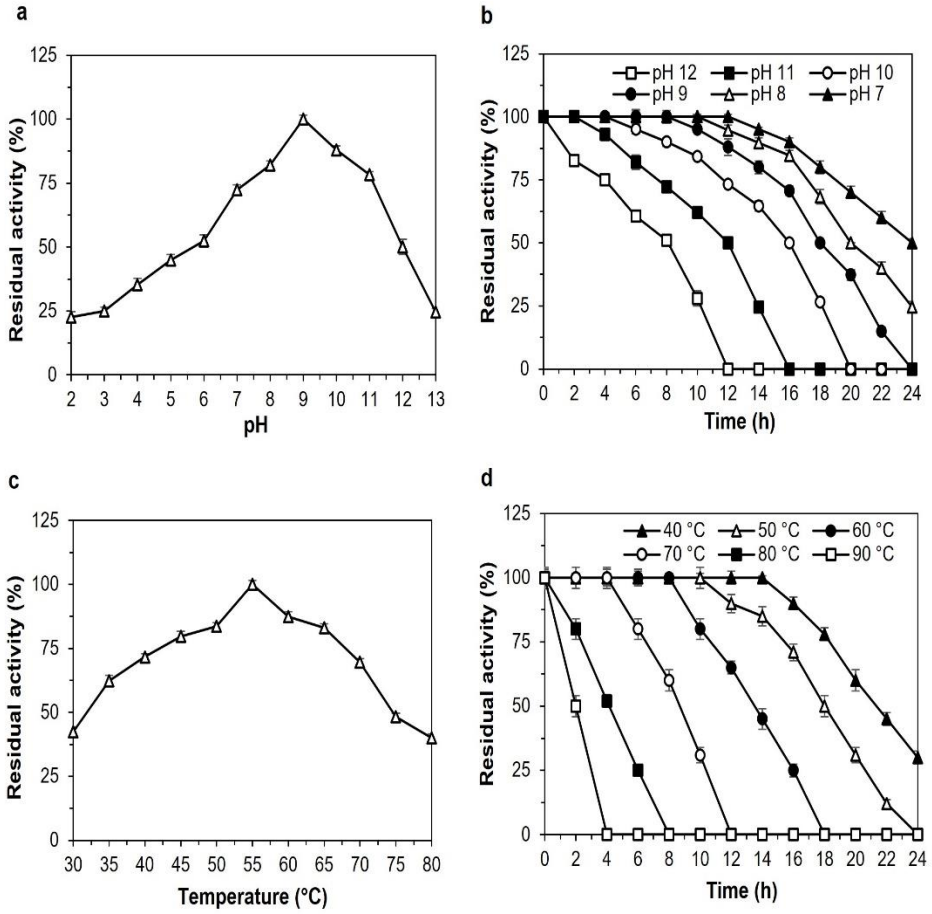

Figure 2.3. Effect of pH on the (a) activity and (b) stability of purified protease SH21 from *Bacillus siamensis* strain CSB55. To check the effect of pH on purified SH21, an enzyme assay was carried out using different pH ranges (2-13.0) at 55 °C. The enzyme activity at pH 9.0 was taken as 100%. Effects of (c) thermoactivated and (d) thermostability of purified SH21. To determine thermoactivated, enzyme SH21 was incubated at different temperatures ranging from 30 to 80 °C with optimum pH (9.0) while, thermostability was evaluated at various temperatures ranging from 40 to 90 °C for 24 h at 2 h intervals.

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2.3.6. Effect of inhibitors, metal ions, surfactants, and bleaches on protease stability

Enzyme inhibitors are elements that interact with enzymes (temporary or permanent) to inhibit the rate of an enzyme-catalyzed reaction or prevent enzymes from functioning correctly. Proteases are categorized according to how sensitive they are to specific inhibitors [42]. The influences of different protease inhibitors and other chemicals are brief in Table 2.3. Inhibition of protease activity by PMSF and DFP led to the conclusion that the co-produced protease goes to the serine protease family. DFP and PMSF act as covalent modifiers of the enzyme's functional position, inhibiting each serine protease [43]. According to the literature, serine proteases account for nearly one-third of all proteases [44]. Further inhibitors, such as TLCK, TPCK, SBTI, and Benzamidine did not exhibit any repressive activity. Furthermore, Pepstatin A (aspartyl protease inhibitor). thiol reagent (2-mercaptoethanol, Iodoacetamide, DTNB, Leupeptin) and gave nearly no substantial impact on SH21 action. EDTA (10 mM) and EGTA (1 mM), both metalloprotease chelating inhibitors, had reduced SH21 activity to 87% and 85%, respectively, when they were added to purified protease, where they may serve as cofactors. Chelators' insensitivity to the enzyme would be a desirable attribute because they used detergent compositions as water softeners and stain removers [4]. The consequence of numerous metallic ions and other chemicals on SH21 activity was also examined. So, the SH21

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action stayed lifted in a remarkable manner by 135%, 120%, 125%, and 145% following the addition of Mg^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} separately at a ultimate dose of 3 mM. This product suggested that the enzyme necessary magnesium, manganese, zinc, and iron to function optimally. The activity was unchanged by Ca^{2+} Cu^{2+} and Na^{+} and was almost entirely prevented by Ni^{2+}, Co^{2+}, Cd^{2+}, and Hg^{2+} at the same concentration as other metallic ions. Increasing the activity of protease with Mn^{2+} and Mg^{2+} showed that the metallic ions had a defensive attitude against denaturation, therefore allowing the enzyme to retain its activity at higher temperatures [6, 45]. The poisonous metallic ions caused the denaturation of proteins, including enzymes, by attaching to a series of chemical ligands. Heavy metallic ions have an inhibiting effect that has been widely documented in the literature [46]. In addition, SH21 was greatly lasting with the addition of several anionic and non-ionic surfactants at 1% concentrations, and retained its activity around 85%, 90%, 88%, and 92% accordingly. The enzyme stability was also examined by incubating H_2O_2 as well as NaClO (1% v/v) as bleaching agents, and it maintained 80% and 83% activity, respectively. However, there are limited studies on alkaline proteases in the reaction with bleaching agents and surfactants [47]. In general, proteases tend to be constant in many soap factors, but they are not fixed in bleaching agents. Protease SH21 showed significant strength and activity with wetting agent, bleaching agents, and other chemicals, which are crucial properties for use in detergent

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preparations and additional engineering purposes.

Table 2.3. Effects of various inhibitors, metal ions, surfactants, and bleaching agents on purified protease SH21.

Inhibitors, metals, surfactants, and bleaching agents	Concentrations	Residual activity (%)
None	-	100 ± 2.2
PMSF	5 mM	10 ± 1.8
DFP	5 mM	12 ± 1.5
TLCK	1 mM	102 ± 1.2
TPCK	1 mM	101 ± 1.5
SBTI	3 mg/ml	103 ± 1.7
Benzamidine	5 mM	104 ± 1.3
Iodoacetamide	5 mM	95 ± 2.3
2-mercaptoethanol	5 mM	90 ± 1.8
Leupeptin	50 μg/ml	96 ± 1.9
Pepstatin A	10 μg/ml	93 ± 1.4
DTNB	10 mM	92 ± 1.6
EDTA	10 mM	87 ± 1.8
EGTA	1 mM	85 ± 1.4
Mg^{2+}	3 mM	135 ± 1.3
Zn^{2+}	3 mM	125 ± 1.7
Mn^{2+}	3 mM	120 ± 2.4
Fe^{2+}	3 mM	145 ± 2.0
Na^{+}, Ca^{2+}, Cu^{2+}	3 mM	100 ± 1.5
Ni^{2+}, Co^{2+}, Cd^{2+}, Hg^{2+}	3 mM	10 ± 1.2
SDS	1 % (w/v)	85 ± 1.3
Tween 20	1 % (v/v)	90 ± 1.6
Tween 80	1 % (v/v)	88 ± 1.8
Triton X-100	1 % (v/v)	92 ± 1.5
H_2O_2	1 % (v/v)	80 ± 1.2
NaClO	1 % (v/v)	83 ± 1.6

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2.3.7. Effect of organic solvents on the activity and stability of protease SH21

The influence of several diluents was examined on protease constancy at concentrations of 25, 50, and 100 % (v/v) for 24 hours (Fig. 2.4a). In most cases, connections involving an enzyme and a solvent substantially impact the secondary and tertiary structures of the enzyme, as well as its action and strength. Surprisingly the SH21 activity was marginally enriched in the existence of acetonitrile, acetone, and hexane. There was evidence that the enzyme activity could be increased due to some water molecules may be replaced by solvent molecules, thereby changing the structure of the enzyme in the existence of diluents [36]. The enzyme preserved (70-100) % of the original activity with the addition of dimethyl sulfoxide (DMSO), chloroform, cyclohexane, decane, and toluene at all concentrations. Also, protease maintained its activity more than 50% after added to the enzyme solution of 100% of ethanol, methanol, and butanol, while it showed over 65% activity with the same solvents at 25 and 50% concentrations. The enzyme SH21 was recovered to be significantly extra constant in organic diluents than other microbial proteases from bacillus sp. [48,49]. These results revealed that protease SH21 is highly stable in various organic solvents, which is vital for industrial purposes. The most useful application of solvent-resistant protease is the synthesis of peptide and ester, which is done under non-aqueous environments in industries.

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2.3.8. Substrate specificity and enzyme kinetics of protease SH21

The substrate specificity of alkaline proteases is generally wide, and they can hydrolyze a broad range of substrates. Various substrates were investigated to find out the best substrate for proteolytic reaction including azocasein, casein, skimmed milk, gelatin, egg albumin, and collagen (Fig. 2.4b). Azocasein was a very suitable substrate for the purified protease SH21, and residual activity was 100%. Moreover, the enzyme was competent to resolve other substrates such as casein, skim milk, and gelatin with residual activity of 95%, 60%, and 77%, accordingly, but egg albumin and collagen showed poor hydrolysis. This result indicated that protease SH21 has broad substrate specificity. The K_m and V_{max} were established for purified enzyme SH21 employing azocasein as substrate. The K_m value of an enzyme suggests the affinity of the enzyme to its substrate. The minor Km and higher V_{max} represent the greater attraction and catalytic efficiency of the enzyme for its substrates. The purified protease showed K_m and V_{max} values of 0.197 mg/mL and 1.22×10^3 U/mg, correspondingly, and it was analyzed by Lineweaver–Burk plot. Protease SH21 from *B. siamensis* CSB55 had a lower Km value than other proteases from *B. subtilis* (1.17 mg/mL) [52], *Bacillus megaterium* (0.722 mg/mL) [51], and *Bacillus licheniformis* (1.60 mg/mL) [53]. However, this result indicated that the enzyme SH21 showed a very high affinity to its substrate since its Km value is much lower than other proteases obtained from another *Bacillus* sp.

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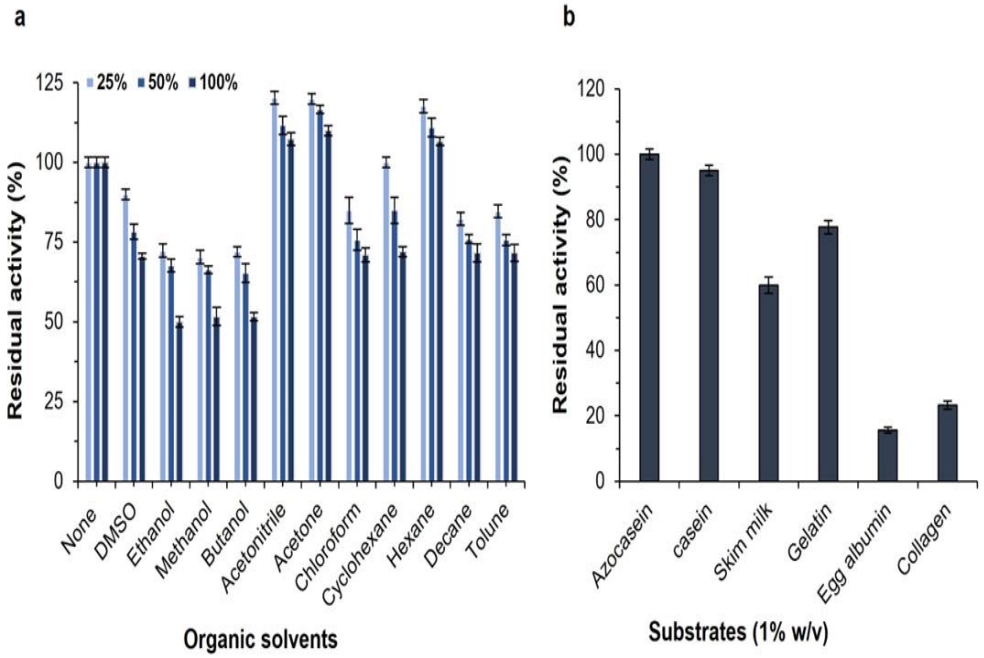

Figure 2.4. (a) Effect of organic solvents on the activity of purified protease SH21 from *Bacillus siamensis* CSB55. The enzyme activity was determined by incubating purified SH21 with numerous solvents for 24 hours. (b) Effect of different substrates on purified protease SH21 from *Bacillus siamensis* CSB55.

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2.4. CONCLUSION

Finally, we found a unique alkaline protease SH21 generated by the *Bacillus siamensis* CSB55 strain with some prominent characteristics. Purified protease showed homogeneity and the molecular mass was 25 kDa. The results demonstrated that it was greatly steady and effective at a large scale of pH and temperature with the highest of 9.0 and 55 °C. It also displayed excellent tolerability and stability even upon exposure to several organic solvents, surfactants, and commercial laundry detergents. Considering these characteristics, it may be used in laundry detergent formulation as an additive.

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CHAPTER THREE

ANTIMICROBIAL AND ANTIBIOFILM ACTIVITY OF PROTEASE SH21

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3.1. INTRODUCTION

The opportunity for proteases, not only as proteolytic enzymes but also as antimicrobial agents for many diseases and infections, could have an enormous impact on medical and various clinical treatments. Protease enzymes are produced by several microorganisms and are implicated in metabolic regulation and the development of numerous infectious maladies. Recently, antimicrobial fighting has become a concern problem, leading to ten million deaths yearly as treatment failure increases due to the ineffective use of antibiotics [13]. The proteases from *Bacillus subtilis*, *Bacillus sp*., and *Artocarpus heterophyllus* have been reported to be antimicrobial in nature against diverse pathogenic bacteria [16].

Microbial protease has also been reported to inhibit biofilm formation due to its anti-biofilm properties. It is considered an eco-friendly and cost-effective method because of its non-toxic and biodegradable nature. Biofilms are external-involved microbial cells enclosed inside an extracellular medium and arranged in complex communal tertiary structures. Bacteria produce and secrete proteins, lipids, polysaccharides, and nucleic acids, called extracellular polymeric materials, which make up approximately 96% of the biofilm matrix. Some opportunistic bacteria, such as *E. coli*, *Staphylococcus*, *Pseudomonas*, etc., form biofilms that impair healing in chronic and acute dermal wounds and result in reinfections and sepsis [18]. Therefore, it is crucial to treat infectious diseases to prevent biofilm formation and disrupt

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existing biofilms. Some heavy metals, including silver, mercury, copper, nickel, zinc, and other chlorinated compounds, have been used for the dispersion of biofilms. However, these chemicals are no longer used because of their severe health risk [23]. It is necessary to replace chemicals with enzymes to remove biofilms due to the diverse restrictions of such elements. Antibiofilm potential enzymes such as protease, glycosidase, and DNases have the capacity to damage the extracellular matrix, releasing planktonic cells and their constituents that can be more simply accessed by antimicrobial agents [21]. Proteases are considered the best enzymes for removing biofilms since proteins are significant components of biofilms [22]. Various microorganisms produce proteases that degrade biofilms, such as staphopain A and B, aureolysin, spl protease, and proteinase K [21]. This report aimed to explore the steadiness and compatibility of using different industrial cleansers of protease SH21 as a possible candidate for manufacturing purposes, mainly in the detergent formulation and the medical sections.

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3.2. MATERIALS AND METHODS

3.2.1. Determination of Minimum inhibitory concentration (MIC) of protease SH21

The antibacterial activity of protease SH21 was evaluated by determining minimal inhibitory concentration (MIC) against different pathogenic bacteria. The Microdilution technique was used to define the MIC value using Mueller Hinton broth (MHB) which was reported before [26] . In short, a two-fold serial dilution of SH21 was supplementary to the microtiter plate and then diluted cultured bacterial suspension (2×10^6 CFU/mL) was added subsequently. The experiment was done three times for each sample and bacterium. The analyzed microorganisms were *Escherichia coli* KCTC 1923, *Pseudomonas aeruginosa* KCTC 1637, *Salmonella typhimurium* KCTC 1925, *Staphylococcus aureus* KCTC 1928, *Micrococcus luteus* ATCC 9341, *Bacillus subtilis* ATCC 6633, and *Micrococcus luteus* ATCC 9341. MIC was defined as bacterial growth that is entirely inhibited at the lowest concentration of antimicrobial agents with a change in absorption of less than 0.05.

3.2.2. Inhibition of biofilm formation (MBIC assay)

The impact of SH21 on biofilm development was assessed by MBIC using crystal violet staining in 96-well plates, as reported earlier [29]. Briefly, 100 μL of the three bacterial cells (*P. aeruginosa*, *S. aureus*, and *E. coli*) were placed in varying concentrations of the enzyme SH21 from 1/8× MIC to 2×

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MIC and incubated at 37 °C for 48 hours. After then, planktonic cells were softly withdrawn, and plates were cleaned thrice with PBS (10 mM) buffer. To stain the biofilms, crystal violet (0.1%) blend was applied to each well for 30 mins. Afterward, deionized water was used twice to rinse each well of the unbound dye and air dried. For dissolving the remaining crystal violet, 100 µL of ethanol was added. Biofilm biomass was defined as 100% for the control group (without SH21 treatment). The MBIC is defined as the lowest antimicrobial concentration, which inhibits the growth of biofilms (99.99%) or an increase of 10% in optical density over the initial measurement [30].

3.2.3. Eradication of preformed biofilm (MBEC assay)

MBEC assay was used to eradicate the performed biofilm, as studied earlier. Microtiter 96-well plates were seeded with suspended bacteria (1×10^8 CFU/mL) and cultured at 37 °C to generate biofilms. 48 h later, planktonic microbes were separated, disappearing deposited biofilms at the end side. Then, SH21 at different concentrations (from 1/2× MIC to 8× MIC) was added. After that, the supernatant was eliminated carefully and stained the remaining biofilms with crystal violet 0.1%. The subsequent steps were the same as above. The MBEC was defined as the lowest antimicrobial agent concentration that eradicates 99.9% of preformed biofilm cells [31]. All experiments were performed three times.

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3.2.4. Confocal Laser Scanning Microscopy Analysis.

A multidrug resistance pathogenic bacterium, *P. aeruginosa* were cultured in 24 well plates in MHB glucose medium containing glass coverslips to form biofilms for 48 hours. After biofilms were grown on glass coverslips, planktonic bacteria were eradicated, and biofilms stood washed with PBS buffer three times. Then, Glass coverslips were transferred to fresh 24 well plates containing three different concentrations (2× MIC, 4× MIC, and 8× MIC) of SH21 and incubated for 6 hours. After incubation, coverslips were washed two times with PBS buffer, and added LIVE/ DEAD BacLight Bacterial kit (6.7 µM SYTO 9 and 40 µM PI) was worked to stain the cells. After incubation in a darkness lay at 37 °C for 20-30 mins, images were taken using (63X) CLSM (Carl Zeiss LSM510 microscope, Jena, Germany).

3.3. RESULT AND DISCUSSIONS

3.3.1. Antimicrobial activity of protease SH21

The antimicrobial activity of SH21 was investigated in terms of MIC against several gram-positive and gram-negative bacteria. In present investigations, we evaluated the antimicrobic activity of protease SH21 with two reference antibiotics (Bacitracin and Vancomycin) (Table 3.1). Researchers in the pharmaceutical industry face challenges due to the occurrence of multidrug challenges. Nonetheless, there are attempts to forward the current antibiotic crisis, and microbial proteases gained attention as an optimistic resource to explore new antimicrobial agents [57]. Some gram-positive bacteria,

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especially *S. aureus* are known to cause skin diseases, respiratory complications, and food poisoning. Protease SH21 showed a strong antagonistic effect against some pathogenic bacteria like other microbial proteases from *bacillus sp*. were previously reported with antimicrobial potential [14]. Protease SH21 displayed intense antimicrobic action alongside *S. aureus* and *B. subtilis* with a MIC value of 16 µg/mL, which is significantly lower than the two reference antibiotics. The effect of SH21 was better than bacitracin against *E. coli*, *M. luteus*, and *M. smegmatis*. Also, SH21 showed a similar effect of bacitracin against *P. aeruginosa* and *S. typhimurium*. Gram-negative bacteria showed higher MIC than Gram-positive bacteria, indicating that Gram-positive bacteria are vastly vulnerable to SH21. The antibacterial properties of SH21 were better in comparison with other previously reported proteases [59]. These results indicated that protease SH21 has potent antimicrobial activity.

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Table 3.1. Antibacterial and antibiofilm activity of protease SH21

Microorganisms	MIC (μg/mL)			MBIC (μg/mL)	MBEC (μg/mL)
	Protease SH21	Bacitracin	Vancomycin	Protease SH21	Protease SH21
Gram-					
Escherichia	16	128	128	32 (2× MIC)	128(8× MIC)
Pseudomonas aeruginosa	64	64	2	128(2×MIC)	512(8× MIC)
Salmonella typhimurium KCTC	32	32	2		
Gram-					
Staphylococcus aureus KCTC	16	128	64	32 (2× MIC)	128(8× MIC)
Micrococcus luteus	32	128	128		
Bacillus subtilis	16	64	32		
Mycobacterium smegmatis	16	32	2		

MIC, Minimum inhibitory concentration. MBIC, Minimum biofilm inhibitory concentration. MBEC, Minimum biofilm eradication concentration.

3.3.2. Antibiofilm activity of protease SH21

Biofilm is made up of several microbes implanted in independently created external polymer materials. Due to the extracellular polymeric substances of biofilm, it plays a significant role, acting as a barrier or inhibiting the interaction of antimicrobial agents with bacterial cells. Generally, it needs a higher concentration of antimicrobial agents to inhibit biofilm than planktonic bacteria cells [61]. We explored the antibiofilm action of SH21 to

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determine MBIC and MBEC against three pathogenic bacteria using a crystal violet assay. SH21 exhibited good antibiofilm activity against *P. aeruginosa*, *S. aureus*, and *E. coli*. Biofilm formation rates were around 20% for all examined bacteria at 1× MIC concentration compared to the control (Fig. 3.1a). SH21 inhibits 99.9% of biofilm formation against was also observed on *S. aureus* and *E. coli* with the equal concentration. SH21 exhibited MBIC of 128 µg/mL against *P. aeruginosa*, and 32 µg/mL for both *S. aureus* and *E. coli* (Table 3.1).

In another experiment, we found that SH21 could eradicate preformed biofilms (Fig. 3.2b). Removing preformed biofilms is much more difficult than preventing biofilm formation because many intricate elements exist in the solid-designed structures. SH21 eradicated more than 80% (retained less than 20 %) of preformed biofilm at 4× MIC concentration, and it retained only 0.01% biofilm when treated with 8× MIC concentration against all tested bacteria. MBEC value of protease SH21 was 512 µg/mL for *P. aeruginosa* while 128 µg/mL against *S. aureus* and *E. coli* (Table 3.1). In addition, we explored the effect of SH21 on preformed biofilm by confocal microscopy analysis in a dose-dependent manner (Fig. 3.1c). *P. aeruginosa* biofilms were cultured for 48 hours and treated with three concentrations of SH21 (2× MIC, 4× MIC, and 8× MIC) followed by incubation for 1 hour. As a result, the control group of bacteria was aggregated into a large quantity of biofilms with the highest number of living bacterial cells producing green

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fluorescence and did not display red fluorescence due to the intact living cells. This result suggested that the bacteria incubated in an untreated condition were unable to pass through the PI, and gradually increasing the concentration of SH21 increased the number of dead cells (red) relative to the green-stained living cells of the biofilm. Overall, these observations indicated that SH21 destroys the bacterial biofilm and substantially removes the biofilm cells implying it would be a potential therapeutic approach for wound treatment.

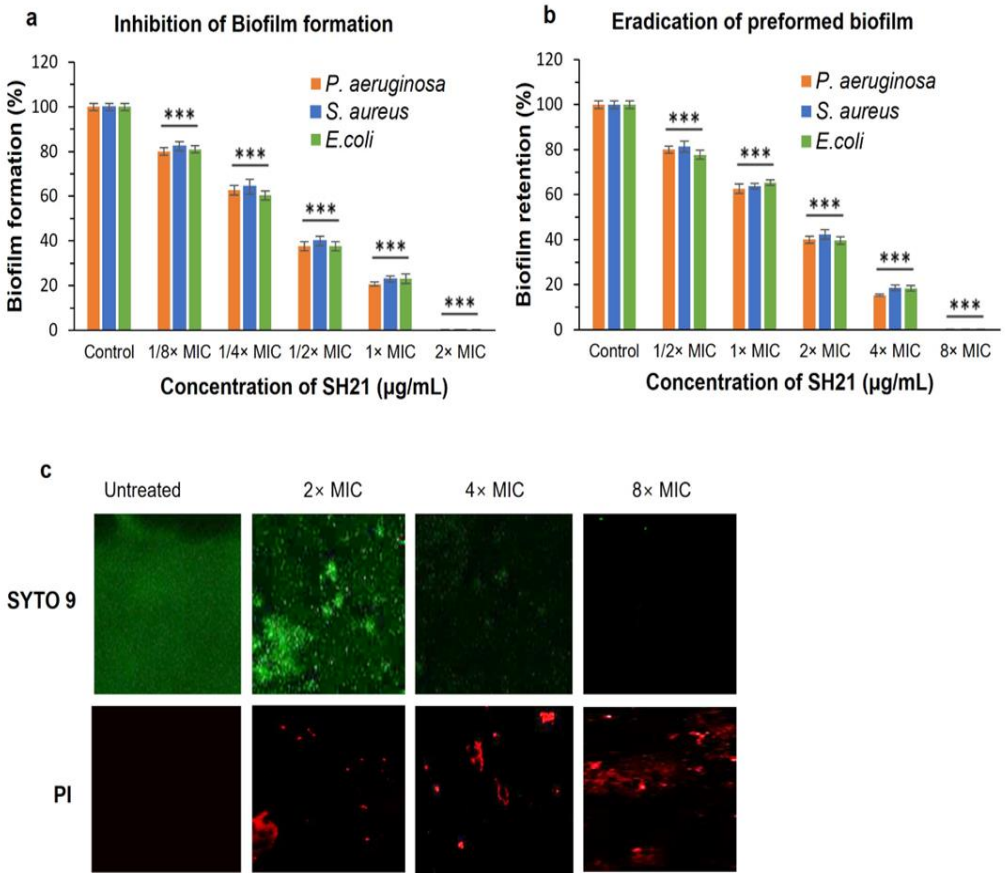

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Figure 3.1. Antibiofilm effect of protease SH21. (a) Inhibition of biofilm formation and (b) Eradication of preformed biofilms against *P. aeruginosa*, *S. aureus*, and *E. coli*. (c) Effects of protease SH21 on *P. aeruginosa* 48 h old biofilms evaluated by applying CLSM. *P. aeruginosa* was incubated alone or with three different concentrations (2× MIC, 4× MIC, and 8× MIC) of SH21. Live–dead viability staining (SYTO 9/PI) was used to visualize biofilms.

3.4. CONCLUSION

SH21 showed good antibacterial and antibiofilm activity against some pathogenic bacteria. Based on these findings, SH21 may serve as a promising candidate for bacterial infections and antibiofilm treatments. Future research is needed to understand how protease SH21 functions against pathogenic bacteria and how it can be used in other bio-industrial applications.

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CHAPTER FOUR

ANTIOXIDANT, ANTIINFLAMMATORY AND ANTICANCER MECHANISM STUDY OF PROTEASE SH21

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4.1. INTRODUCTION

Reactive oxygen species (ROS) can be formed when oxygen is partially reduced during normal metabolism. Damage to essential cellular components can result from oxidative stress occurring when antioxidant immunity is overloaded by ROS or loses its ability to respond [62]. It has been demonstrated that excessive ROS contributes to the etiology of a few human chronic illnesses involving inflammation, cardiovascular and neurological disorders, and cancer [63]. Depending on their mechanism, antioxidants can defend cells counter to oxidative emphasis, whether through indirect or direct routes. Antioxidants remove nitrogen species and reactive oxygen by utilization or organic transformation in the case of direct pathways. Indirect pathways are interested in the upregulation of antioxidant enzymes and Phase II detoxifying enzymes. Microorganisms have robust antioxidant defense systems that include primary enzymes such as CAT, SOD, GPx-1, and induced such as HO-1 and NQO1, which are triggered by Nrf2 [3]. Many studies suggest that the Nrf2/HO-1 showing pathways activate antioxidant enzymes that neutralize free radicals and prevent oxidative damage to cells. In normal conditions, the Keap1/Nrf2 complex controls the degradation of Nrf2 through the proteasome. Among many other things, the Nrf2/Keap1 pathway also performances a crucial function in regulating oxidative underline conditions in a way that contributes to inflammation, endothelial dysfunction, and cancer development [65]. The use of antioxidants has

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gained more attention for maintaining human health and the restraint and management of specific disorders. Synthetically produced antioxidants are commonly applied to treat oxidative damage. However, synthetic antioxidants have limited use due to their carcinogenicity, and the screening of natural biomaterial is currently an important consideration for several medical circumstances [66]. Chronic inflammation is implicated with a greater risk of tumor growth and cancer, as reported by experimental, medical, and epidemiological data, which have shown that it contributes to the onset of 15–20% of global malignancies [70]. Macrophages, which act as invisible cells, release a small amount of highly bioactive immune mediators, such as nitric oxide, that play a critical function in tissue healing besides are complicated in a diversity of disorders, including atherosclerosis, inflammation, carcinogenesis, hypertension, obesity, and diabetes. Furthermore, ROS promotes the release of these mediators, increasing the presence of more macrophages in inflammatory sites, thereby spreading inflammation. Tumor invasion and metastasis are believed to be caused by persistent inflammation-induced cell recruitment, ROS release, and genetic instability. [71]. Cancer progression tends to be facilitated by oxidative stress and the inflammatory response [72]. Antioxidant and anti-inflammatory characteristics could be effective indicators for detecting anticancer agents. Therefore, experts have kept looking into screening new biological substances. Several reports stated that kimchi has antioxidant, anti-

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inflammatory, anticancer, antimicrobial, anti-atherosclerotic, anti-diabetic, and anti-obesity activities. SH21 is the first protease with potential multifunctional biological activity which has been separated from the incited food, kimchi.

In the present study, we evaluate antioxidant, anti-inflammatory and anticancer properties as evaluated by numerous in vitro tests. We also revealed the antioxidant mechanisms of SH21 by determining antioxidant enzyme expression and inducing HO-1 by the triggering of Nrf2 in RAW 264.7 cells, and subsequently inhibited ROS generation and oxidative damage. These results suggest that SH21 reduces ROS free radicals and oxidation via the inauguration of the Nrf2/HO-1 pathway.

4.2. MATERIALS AND METHODS

4.2.1. Materials

To perform expeimental tests, all analytical grade reagents were employed. 2 DPPH, ABTS, Neocaprione, TPTZ, MTT, and DCFH-DA were bought from Sigma Aldrich. DMEM, RPMI-1640, and FBS were purchased from Gibco, Antibodies, including anti-Superoxide dismutase1 (SOD1), anti-Glutathione peroxidase 1 (GPx-1), and anti-Catalase (CAT), anti-HO-1, aNrf2, and β-actin and lamin B were obtained from Santa Cruz Biotechnology.

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4.2.2. Cell Culture and Maintenance

All cells were grown and preserved in a suitable medium. RAW 264.7 (Murine macrophage cell) and Hela cells (Human cervical cell) were grown in DMEM. HL-60 and A549 cells were grown in RPMI-1640 medium combined with FBS (10%, *v*/*v*) and streptomycin- penicillin (100 µg/mL each) at 37 ◦C with 5% CO2. RAW 264.7 cells were obtained from ATCC, and all three tested cancer cells were collected from Korean Cell Line Bank.

4.2.3. DPPH Assay

The capacity to remove free radicals of SH21 was evaluated using the DPPH radical-scavenging assay, and the process was operated as reported earlier [83]. In brief, 190 µL of DPPH mixture (0.2 mM, in 95% ethanol) was added to several concentrations (2.5–20 µg/mL) of 10 µL SH21. The mixture was then vortexed and kept for 30 min at normal temperature in a dark place. The resulting mixture absorbance was taken at 517 nm.

4.2.4. ABTS Assay

ABTS radical decolorization test was carried out following the technique reported by Ye et al. [84]. The combination of ABTS (7 mM) and potassium persulfate (2.45 mM) resulted in ABTS •+ cation radicals, which were then kept in darkness location for 15 h. Further dilution of the reaction mixture in methanol allowed for the measurement of an optical density of 0.706 ± 0.001 at 734 nm. The consequence between several concentrations of 10 µL SH21 and 190 µL of the ABTS •+ liquid was permitted to proceed for 5 min, and

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the absorbance at 734 nm was taken.

4.2.5. Superoxide Radical Scavenging Activity Assay

The test was conducted to examine the potential of SH21 to inhibit the development of formazan by sifting O2- produced in the riboflavin–light–NBT approach [85]. First, 1 mL of solution containing 0.1 mg NBT, 20 mg riboflavin, and EDTA (12 mM, pH 7.6) was combined up with 0.5 mL of the sample. A fluorescence lamp was employed to illuminate the reaction mixture.

4.2.6. Hydroxyl Radical Scavenging Activity Assay

To investigate the ability of SH21 as a scavenger of (•OH), a Fenton reaction was conducted using Fe^{3+} ascorbate-EDTA-H_2O_2 as the source of hydroxyl ions [86]. The reaction solution consists of 2.8 mM deoxyribose (400 µL) in KH_2PO_4-KOH buffer (pH 7.4), 0.1 M $FeCl_3$ and 0.1 M EDTA (150 µL, 1:1 *v*/*v*), and 0.75 µL of 0.2 M H2O2 mixed with or without SH21. Solution mixing started by mixing 0.75 µL of 0.3 M ascorbate and then put for 1 h at 37 ◦C. TCA (1 ml) (2.8% *w*/*v*) was merged with 500 uL of the reaction mixture before being combined with 1 mL of TBA (1% *w*/*v*). Finally, the resulting solution was kept 20 min at 100 ◦C. This test is based on the amount of the 2-deoxy-2-ribose sugar degradation product via reduction with TBA to generate a pinkish colour. The color was observed at 532 nm.

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4.2.7. FRAP Assay

A FRAP experiment was conducted to determine the reducing power activity of SH21 with minor modifications, as previously described [87]. The FRAP reagent consists of 10 mM of TPTZ mixture in HCL (40 mM), 300 mM acetate buffer (pH 3.5), and 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) solution in the following ratio: 8:88:8. Different concentrations of 10 µL SH21 and 190 µL of FRAP component were merged and 595 nm was used to taken abs. An ascorbic acid standard curve was employed to calculate the FRAP value.

4.2.8. CUPRAC Assay

The CUPRAC analysis was conducted by applying as reported before [88]. Several concentrations of SH21 were then mixed with a solution containing CuCl2 (10 mM), neocuproine (7.5 mM), and (1 M, pH 7.0) ammonium acetate buffer. Absorbance measurement was performed at 450 nm after 1 h of maturation at normal heat. Finally, the ascorbic acid standard curve was employed to estimate the CUPRAC value.

4.2.9. Cell Viability and Intracellular ROS Generation Inhibition in Raw 264.7 Cell

The viability of RAW 264.7 cells was assessed utilizing the colorimetric (MTT) assay [89]. Prior to being treated with various concentrations (2.5 to 100 µg/mL) of SH21, RAW 264.7 cells were grown in 96-well plates at a density of 2×10^4 cells/well for 24 h. The plates were incubated at 37 ◦C for 1 h afterward, and MTT reagent was mixed to every well after 24 h of

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incubation. After removing the medium, PBS (pH 7.4) was used to wash the plates twice. DMSO was utilized for dissolving the intracellularly insoluble formazan. Cell viability percentage (%) was then estimated by taking absorbance at 570 nm with a microplate reader. Oxidative stress is caused by the ROS produced by LPS, which was quantified spectrofluorometrically employing the dichloro-dihydrofluorescein diacetate (DCFH-DA) technique [89]. Macrophage cells were first grown at a intensity of 2×10^5 Raw 264.7 cells/well using DMEM in 96-well plates for 24 h. After treating them for 1 h with (2.5 to 50 µg/mL) of SH21, cells were stimulated with LPS and kept for 24 h. Then, cells were treated with DCF-DA (25 µM) at 37 ◦C for 30 min after being cleansed twice with PBS (pH 7.4) The fluorescence intensity was checked at excitation for 485 nm and emission for 528 wavelengths.

4.2.10. Cell Lysates Preparation and Western Blot Analysis

Cell lysates were made employing a normal procedure, combined with sample buffer consisting of Tris-HCl (250 mM, pH 6.8), dithiothreitol 0.5 M (DTT), 0.5 % bromophenol blue, glycerol 50%, 10 % SDS, and 2-mercaptoethanol (5%), and denaturized for 5 min at 100 ◦C. Protein extraction was done by anuclear/cytosolic fractionation kit. SDS-PAGE (10%) was employed to separate the sample proteins (20 µg), and the primary antibody was incubated on the membranes for an entire night in skim milk. (5%, *w*/*v*) after electrotransfer on nitrocellulose membranes (Whatman, Dassel, Germany). Primary antibodies (1:1000), including

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antiSOD1 (sc-101523), anti-CAT (sc-515782), anti-GPx-1 (sc-133152), anti-HO-1 (sc-136256), antiNrf2 (sc-81342), β-actin (sc-47778), and lamin B (sc-374015) (Santa Cruz, CA, USA), were applied. Anti-Mouse IgG-HRP (Santa Cruz) was employed as a secondary antibody. An ECL solution method was used to identify the antigen–antibody reaction. The density of the protein bands was normalized using the same samples on the β-actin.

4.2.11. RT-PCR Analysis

TRI-zol (Life Technologies, Gaithersburg, MD, USA) was applied to take out total RNA from Raw 264.7 cells according to the manufacturer's protocols. RT-&GO Mastermix was employed to transcribe the RNA (2 μg) into first-strand cDNA, and the ensuing outcome was applied as the PCR model. The following primer (HO-1, CAT, SOD1, GPx-1, IL-6, IL1β, iNOS, TNF-α and GAPDH) sequences (Table 4.1) were employed in RT-PCR applying a Takara PCR thermal cycler. After electrophoresis, staining was performed with ethidium bromide to visualize the PCR results. Analyzing the bands was performed using Image Lab Software (version 5.2).

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Table 4.1. List of primer sequences used in RT-PCR investigation.

Gene	Forward primer sequences (5′-3′)	Reverse primer sequences (5′-3′)
SOD1	AGGGCGTCATTCACTTCGAG	TCCTTTCCAGCAGCCACATT
CAT	AGGCTCAGCTGACACAGT TC	GCCATTCATGTGCCGATGTC
GPx-1	GCTCACCCGCTCTTTACC TT	GATGTCGATGGTGCGAAAGC
HO-1	TGAGAGGAACCAAGTGTTTGAG	CAGGGGGACTTTAGCTTTAGAA
Nrf2	CTTTAGTCAGCGACAGAAGGAC	TCCAGAGAGCTATTGAGGGACT
iNOS	CCCTTCCGAAGTTTCTGGCAGCAGC	GGCTGTCAGAGCCTCGTGGCTTTGG
TNF-α	TCTCATCAGTTCTATGGCCC	GGGAGTAGACAAGGTACAAC
IL-6	GTTCTCTGGGAAATCGTGGA	TGTACTCCAGGTAGCTATGG
IL-1β	GGACGGACCCCAAAAGATG	AGAAGGTGCTCATGTCCTCA
GAPDH	GCGAGATCCCGCTAACATCA	AGTGATGGCATGGACTGTGG

4.2.12. Nitric Oxide (NO) Generation Inhibition and Cytokines Assay

The Griess reaction was used to determine NO production by assessing the amounts of nitrite allowed to the method of Zhou [29]. Before LPS stimulation, Raw 264.7cells were pretreated with numerous dilutions (20 to 100 µg/mL) of SH21 for 3 h. After LPS (1 µg/mL) stimulation for 24 h, equivalent amounts (100 µL) of cultured supernatant and Griess reagent (in 2.5% phosphoric acid) were added to 96-well plates and kept at normal environment for 10 min in a wicked place. 540 nm was used to take abs. For

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the cytokines assay, RAW 264.7 cells were cultured in 48-well plates at a density of 1 × 10^4 cells/well. Several concentrations (20 to 100 μg/mL) of SH21 for 2 h and then promoted for 24 h with LPS (1 μg/mL). The amounts of cytokines such as TNF-α (560478, BD Biosciences) IL-6 (550950, BD Biosciences), and IL-1β (569603, BD Biosciences) were measured in the supernatant by employing ELISA kits procedures. All tests were performed in triplicate.

4.2.13. MTT Assay

Colorimetric MTT test [91] was employed to establish the cytotoxicity of SH21 counter to 3 cancer cells (HL-60, A549, and Hela). 100 μL of cell suspension was seeded at a density of 1 × 10^4 cells/well and incubated with 5% (*v*/*v*) CO_2 at 37 ◦C for 24 h. Then, the medium in the wells was removed and various concentrations (50 to 500 μg/mL) of 100 μL SH21 solution were mixed. After wards, media was taken out and 50 μL of MTT dye solution (5 mg/mL) was applied to each well of the plate and keep alive at 37 ◦C for 3 h. After withdrawing the MTT solution, 100 μL of DMSO was used to disappear the formazan mineral. Absorbance taken at 540. IC_{50} was defined as the concentration that reduced cell viability by 50%.

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4.2.14. The Lactate Dehydrogenase (LDH) Release Assay

LDH leakage analysis is a simple way of estimating and assessing lactate dehydrogenase enzyme release from lysed cancer cells. This assay was performed by using the LDH assay kit (Cat. No.: 601170). Briefly, all three cancer cells (HL-60, A549, and Hela) were plated in 96-well plates at a extent of 4×10^3 cells/well for 24 h and then incubated with different concentrations (50–500 µg/mL) of SH21 for 1 h. 100 µL of culture supernatant from moved to original sheets and then combined with 110 µL of LDH reaction liquid. Absorbance was measured at 490 nm after plates had been gently 30 min shaken at 37 ◦C. 1% Triton X-100 and phosphate buffer saline (PBS) in media were utilized. LDH release was compared to cells treated with Triton X-100. All experiments were performed three times.

4.2.15. Live/Dead Staining Assay

To investigate the cell membrane integrity, three cancer cells were planted at a denseness of 3×10^4 cells/well in 24-well plates. After incubation, the culture media was wipe out and again incubated with clear medium surrounding SH21 at a intensity of 500 µg/mL for 24 h. Then, adhering cells were gently splashed with PBS and cells were stained with 2 µM Calcein-AM and 5 µM Ehtidium homodimer-1 and placed in a dark place for 30 min. Finally, images were taken by using CLSM (Carl Zeiss LSM510 microscope, Jena, Germany).

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4.3. RESULT AND DISCUSSIONS

4.3.1 Antioxidant Activity of SH21

Antioxidants provide protection against oxidation damage directly and indirectly to cells according to the mechanism that they use [96]. The capacity to hunt free radicals, nitrogen and reactive oxygen by donation of hydrogen or electrons may be characterized as direct antioxidant capacity. On the other hand, the indirect ability of antioxidants is involved in reducing oxidative stress through expressing antioxidant and detoxifying genes. Investigation of whether SH21 possesses direct antioxidant activity relative to radicals of DPPH, ABTS $^{\bullet+}$, superoxide (O^{2-}), and hydroxyl (•OH) was performed in terms of scavenging activities. To assess hydrogen donors and free radical scavengers' ability of a compound, DPPH radicals were extensively applied. The resulting mixture's brightness of color is correlated with its concentration. and efficiency of antioxidants, as observed by the change in color. A high level of free radicals leads to increased oxidation, which has a detrimental effect on biological systems [97]. The ABTS •+ radical scavenging assay generates blue/green chromophore radical cations released by the oxidation of ABTS •+ in the presence of potassium persulfate, which decline in the existence of hydrogen-contributing antioxidants.

Interestingly, SH21 displayed extreme scavenging activity for both DPPH and ABTS •+ radicals in a concentration-dependent approach (Figure 4.1

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A,B). Superoxide radical (O2-) is a highly toxic substance created by specific biological reactions. These radical anions, such as hydroxyl radicals and scavenging radicals, may play a role in the generation of highly reactive species even though they cannot initiate direct lipid oxidation, which is of great interest [98]. Hydroxyl radicals are highly reactive free radicals produced in the body and can damage almost any element that exists in living cells. Notably, SH21 substantially inhibited superoxide and hydroxyl radicals through the transfer of hydrogen atoms in a sequence's dosages. (Figure 4.1C ,D). In addition, assays were carried out to evaluate the electron donating capacity of SH21 CUPRAC) and (FRAP). SH21 displayed strong reducing power potentiality (Figure 4.1 E, F). Based on these results, we predicted that SH21 has a high potential to scavenge different free radicals via hydrogen atom transfer/electron contribution.

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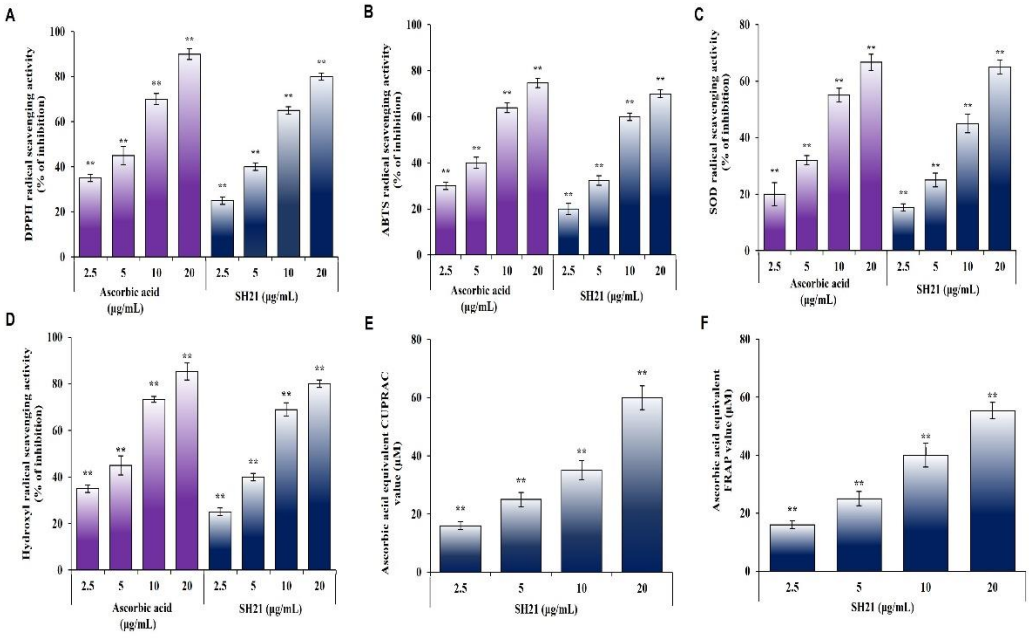

Figure 4.1. Antioxidant activity of protease SH21. The (A) DPPH assay, (B) ABTS assay, (C) Superoxide scavenging assay, (D) Hydroxyl scavenging assay, (E) CUPRAC assay, and (F) FRAP assay were conducted with different concentrations of SH21.

4.3.2. Cell Cytotoxicity Effect and ROS Generation Inhibition of SH21

The toxic effects on RAW 264.7 cells with numerous strengths (2.5 to 100 μg/mL) of SH21 were examined prior to investigating LPS-induced ROS scavenging activity. Reactive oxygen species (ROS) are dangerous to cellular function as they induce changes in nucleic acids, proteins, and lipids, ultimately leading to the development of inflammation, cancer, and neuron-related diseases. The results showed that SH21 treatment inhibited ROS creation in LPS-encouraged RAW 264.7 cells in a quantity-reliant approach

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without showing cytotoxicity.

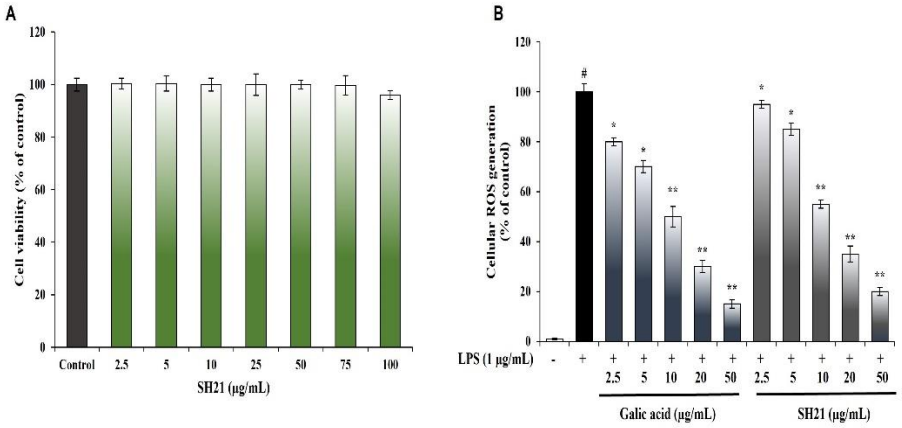

Figure 4.2. Investigation of cell viability and ROS generation inhibition. (A) RAW 264.7 cells were planted at a bulk of 2 × 10^4 cells per well and underwent an MTT assay. (B) Intracellular ROS generation. Each experiment was carried out three (n = 3) times (±) standard deviation.

4.3.3. Antioxidant Enzymes Expression by SH21 in Raw 264.7 Cells

The expression of phase II-detoxifying and antioxidant enzymes protects against oxidative stress and facilitates ROS scavenging capability in sustaining cellular homeostasis during cell proliferation. Free radicals within cells are removed by enzymes, including SOD, CAT, and GPx-1. Damage to these enzymes is a contributing factor in some chronic diseases [100]. In general, one electron donation of oxygen (O2) on the mitochondrial electron transfer sequence is responsible for the production of cytosolic superoxide (O_2). It is widely acknowledged that SOD helps superoxide (O_2) convert into H_2O_2. In addition, other scavenging enzymes such as CAT and GPx-1

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detoxified H_2O_2 into H2O. As part of the free radical metabolism pathway, all these enzymes work together [40]. To examine the protein levels and mRNA expression of antioxidant enzymes, SH21 was applied at concentrations of 5, 10, and 20 g/mL to Raw 264.7 cells for 24 h and significantly increased the mRNA and protein levels in a dose-dependent manner (Figure 4.3A,B). In addition, RT-PCR assessment explained that the mRNA quantities of phase II detoxification enzyme (HO-1) and transcription factor Nrf2 prominently increased after SH21 treatment (Figure 4.3C). Nrf2 and Keap1 are usually linked in normal conditions, and the activation can only occur if Nrf2 is released from the Keap1 protein and nucleus translocation occurred. [101]. Furthermore, to examine the protein levels of HO-1 and Nrf2, western blot analysis was performed in both concentration and time-dependent (Figure 4.3D–G) approaches. SH21 increased protein expression of Nrf2, showing a maximum at 10 g/mL at 12 h, whereas HO-1 peaked at 20 g/mL at 24 h. Interestingly, protein levels of Nrf2 were slightly decreased after 12 h of treatment, but HO-1 protein levels were gradually improved until 24 h. HO-1 helps by changing heme into a strong pro-oxidant called biliverdin which has potential anti-inflammatory, antioxidant, and antiproliferative effects; it also converts to another powerful antioxidant called bilirubin [102]. Some nutrients such as caffeic acid ester, hydroxytyrosol, eckol, and curcumin have been found to help with oxidative stress by increasing HO-1 expression [104]. Thus, the instrument of

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communication between SH21 and Nrf2 is hypothesized to imitate the activity of other Nrf2 stimulants such as 5-O-caffoylquinic acid, which affects translocation of Nrf2 into nucleus and expression of ARE-dependent gene as in GST, NQO-1, HO-1, in HT29 cells [44]. Based on this finding, SH21 indicates the upregulating of antioxidant enzymes by triggering the Nrf2/HO-1 indicating pathway.

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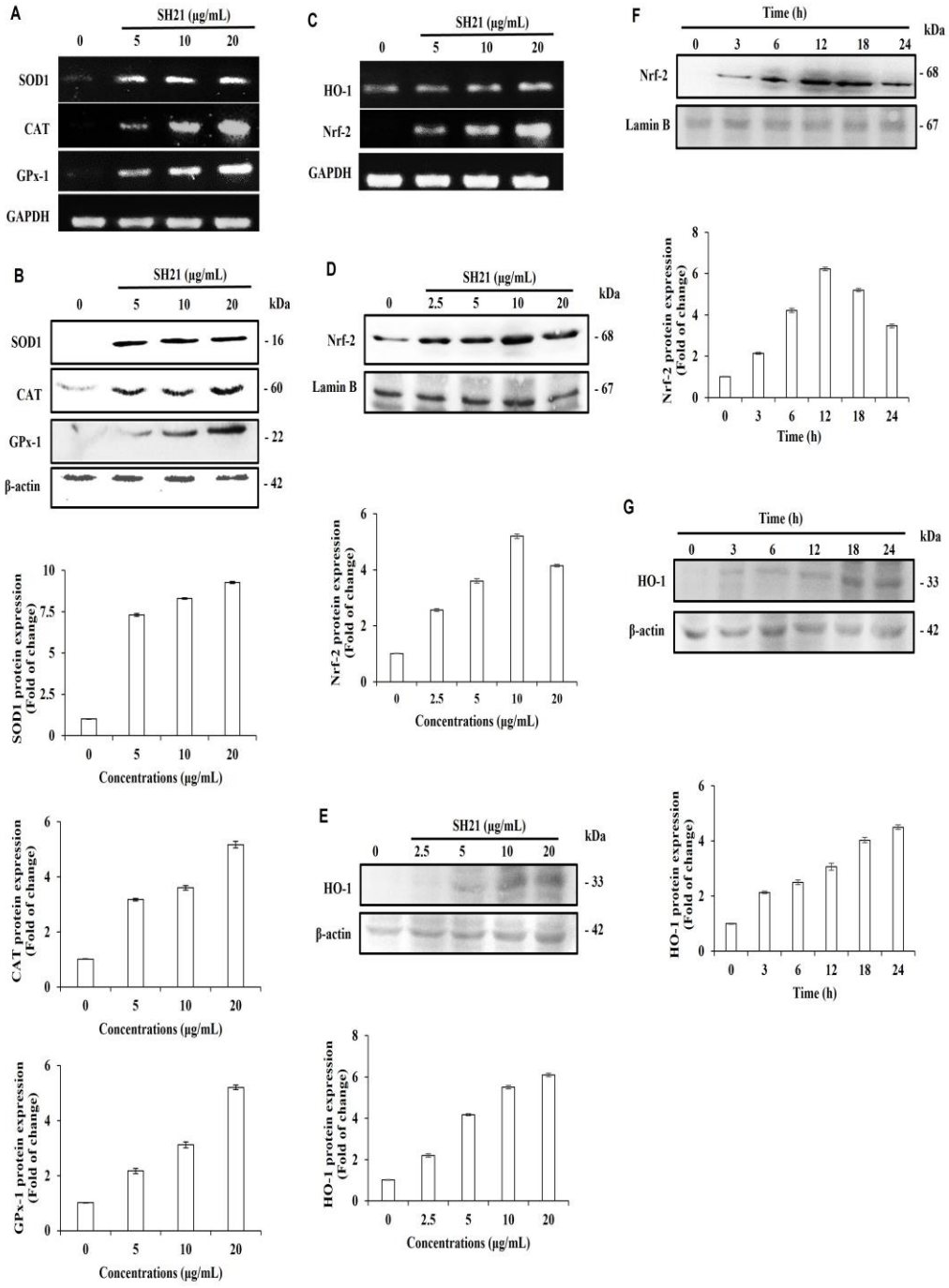

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Figure 4.3. Evaluation of primary and antioxidant enzymes. RAW 264.7 cells were pretreated for 24 h with various concentrations of SH21. (A) The mRNA expressions SOD1, CAT, GPx-1 were quantified by RT-PCR and (B) western blot was carried out to estimate protein levels by using the same concentrations. (C) The mRNA levels of detoxifying enzyme HO-1 and nuclear factor Nrf2 were measured by RT-PCR in a dose-dependent manner. The protein expressions of Nrf2 and HO-1 were measured in (D,E) dose-dependent and (F,G) time-dependent manners by western blot analysis.

4.3.4. SH21 Exerts Anti-Inflammatory Activity

Macrophages are critical exempt cells that can be initiated by LPS (lipopolysaccharide). LPS is an endotoxin that exists in the cell exterior of Gram-negative bacteria, which induces Raw 264.7 cells to produce mediators such as nitric oxide and cytokines (IL-6, TNF-alpha IL-1), leading to inflammation [106]. These cytokines may contribute to various pathophysiological conditions by further activating macrophage cells and stimulating the generation of other cytokines. The suppression of these cytokines is an important phase in the anti-inflammatory response. The inhibitory effects of SH21 on chemical mediators and cytokines were checked to evaluate the anti-inflammatory function. In the presence of LPS, the levels of NO and cytokines were remarkably increased in raw cells.

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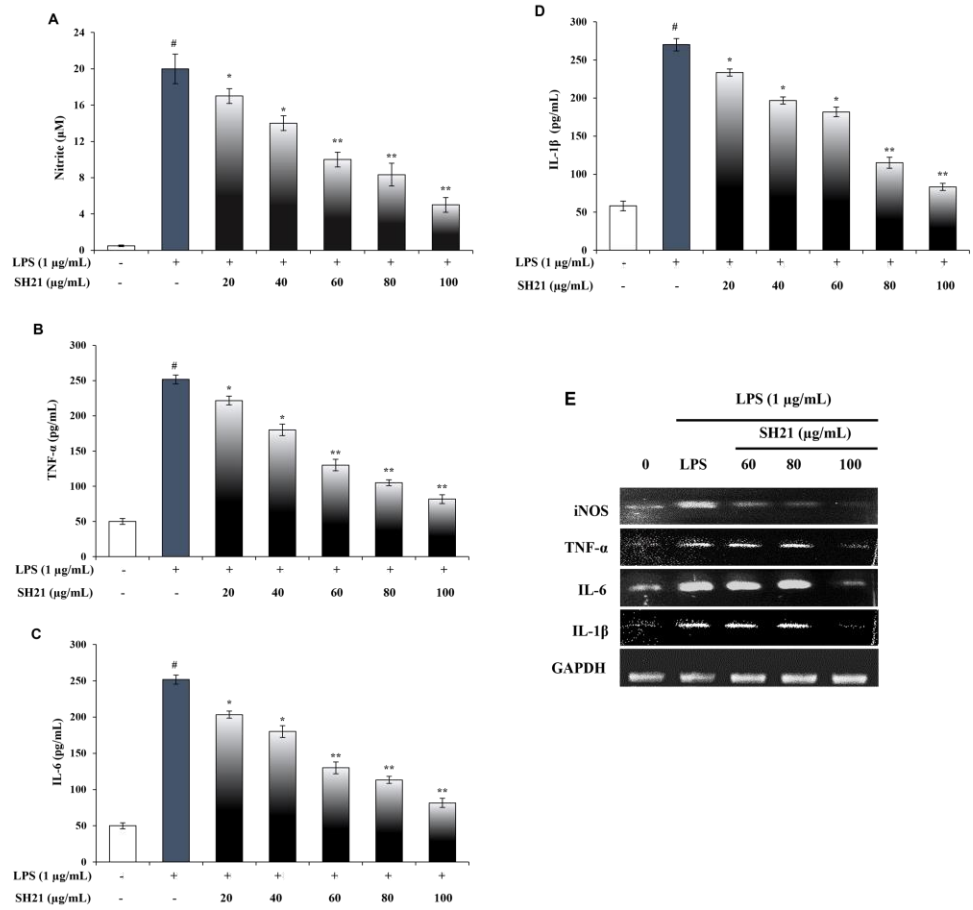

Figure 4.4. Anti-inflammatory activity of protease SH21. Effect of SH21 on the production of (A) NO, (B) TNF-α, (C) IL-6, and (D) IL-1β in LPS-induced RAW 264.7 cells. (E) Effects of SH21 on LPS-induced mRNA expression of iNOS, TNF-α, IL-6 and IL-1β.

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SH21 showed significantly stronger repressing impacts on the mediator and cytokines in a dose-dependent approach (Figure 4.4A–D). These results revealed that SH21 has potent anti-inflammatory activity. Furthermore, to evaluate whether the above repressing impacts of SH21 on NO production and proinflammatory cytokines was related to changes in gene expressions, we examined the mRNA levels of mediators and cytokines by RT-PCR analysis. LPS treatment enhanced mRNA levels of, IL-1, TNF-, iNOS, and IL-6, and treatment of cells with SH21 dose-dependently suppressed LPS-enhanced expression (Figure 4.4E). The attenuation of gene expressions indicates a positive effect of SH21 on anti-inflammatory properties.

4.3.5. Anticancer Activity of SH21

The anticancer effect of SH21 was assayed against three cancer cells, namely, HL-60, A549, and Hela cells. The anticancer activity was measured in terms of investigating cell viability and IC50 values. Cell development was determined by MTT test and was used to detect cells that were metabolically live. The three cancer cells were treated with various doses (50–500 μg/mL) of SH21. SH21 inhibited different percentages of cell progression in a concentration reliant approach (Figure 4.5A-C). Furthermore, IC50 values of 310.64 ± 0.24, 300.27 ± 45, and 317.14 ± 21 μg/mL against HL-60, A549, and Hela cells, respectively, were found after SH21 treatment. (Table 4.2). The lowest IC50 value of SH21 was observed against A549, whereas the highest IC50 value was against Hela cells.

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Doxorubicin (0.25–10 μg/mL) was considered as a standard and showed IC_{50} values of 2.4 ± 36, 2 ± 38, and 2.8 ± 74 μg/mL against HL-60, A549, and Hela cells separately (Table 4.2). The IC_{50} amount refers to the intensity of the test sample that can prevent the progression of cells by 50%. This result suggests that SH21 has considerable anticancer activity.

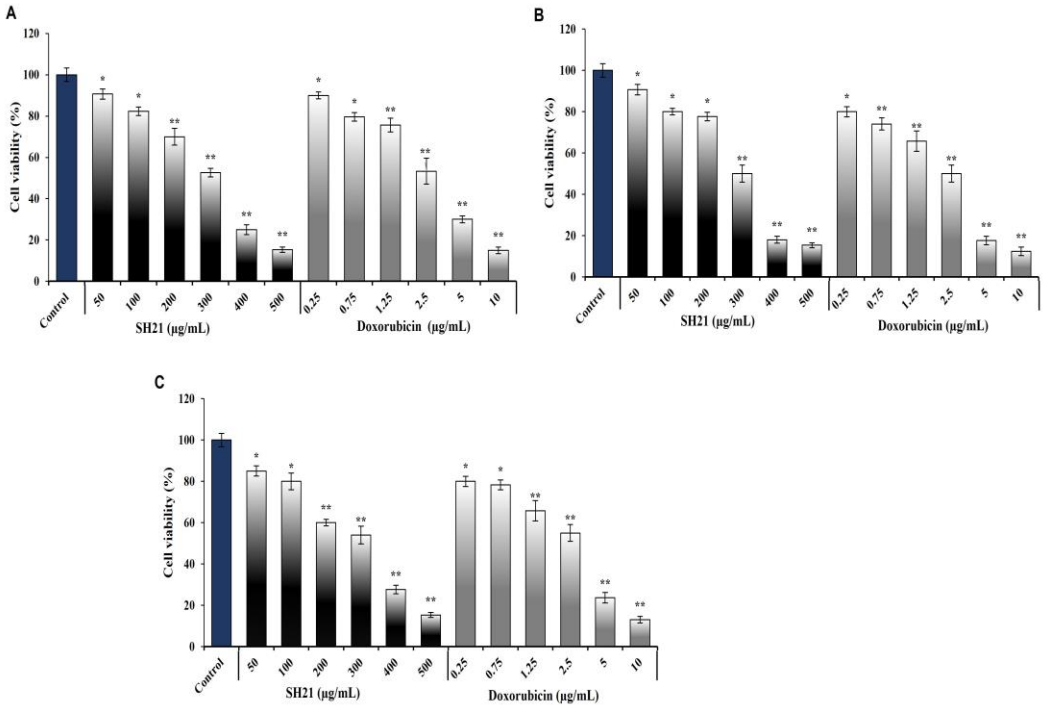

Figure 4.5. Anticancer activity of protease SH21 against three different cancer cell lines: (A) HL-60, (B) A549, and (C) Hela cells.. Each experiment was performed three times (±) standard deviation.

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Table 4.2. Cytotoxicity assessment of SH21 against three cancer cell lines.

SH21 (µg/mL)	**Cell type**					
	HL-60		**A549**		**Hela**	
	% of inhibition	**IC_{50}**	**% of Inhibition**	**IC_{50}**	**% of inhibition**	**IC_{50}**
50	9.33 ± 24	310.64 ± 24	9.31 ± 47	300.27 ± 45	15.15 ± 51	317.14 ± 21
100	17.67 ± 14		20.27 ± 51		20.51 ± 42	
200	30.14 ± 72		22.33 ± 57		40.38 ± 37	
300	47.33 ± 34		50.07 ± 42		46.36 ± 26	
400	75.28 ± 18		82.34 ± 63		72.33 ± 30	
500	84.67 ± 57		54.67 ± 17		84.67 ± 52	
Doxorubicin (µg/mL)	**% of inhibition**	**IC_{50}**	**% of inhibition**	**IC_{50}**	**% of inhibition**	**IC_{50}**
0.25	10.13 ± 38	2.4 ± 36	20.32 ± 23	2.5 ± 38	20.18 ± 37	2.8 ± 74
0.75	20.21 ± 56		26.29 ± 29		21.67 ± 25	
1.25	24.33 ± 41		34.33 ± 83		34.33 ± 37	
2.50	46.67 ± 37		50.04 ± 25		45.34 ± 46	
5.0	70.15 ± 18		82.33 ± 54		76.33 ± 74	
10	85.17 ± 39		87.67 ± 32		87.61 ± 48	

Values are the means of triplicates ± standard deviation (SD).

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Lactate dehydrogenase (LDH) is a constant cytoplasmatic enzyme that is found in all cells, and it plays a role in the conversion of pyruvate to lactate. The effect of SH21 on LDH release from tested cancer cells was investigated. Our result demonstrated that LDH was noticeably released from all tested cancer cells in a dose-dependent approach (Figure 4.6A–C), which indicates the disruption of the cell membranes. In another experiment, we further verify the capability of SH21 to disrupt the cancer cell membrane. Calcein-AM, a cell-permeant nonfluorescent dye, is transformed to calcein by the esterase activity of living cells, suggesting an intact plasma membrane with green fluorescence. On the other hand, Ethidium homodimer-1 interacts with DNA and generates red signals when it penetrates the cell membrane. As shown in Figure 4.6D, SH21 produced red fluorescence, which indicates damaged cell membranes in all the tested cancer cells. These results indicated SH21 has the potential ability to damage the cancer cell membrane. Oxidation and ROS are key contributors to the cancer signature, including angiogenesis, invasion, stem cell function, and metastases. Therefore, it has been shown that reduction in oxidation and ROS free radicals with potent antioxidants would be an effective strategy for the prevention of cancer.

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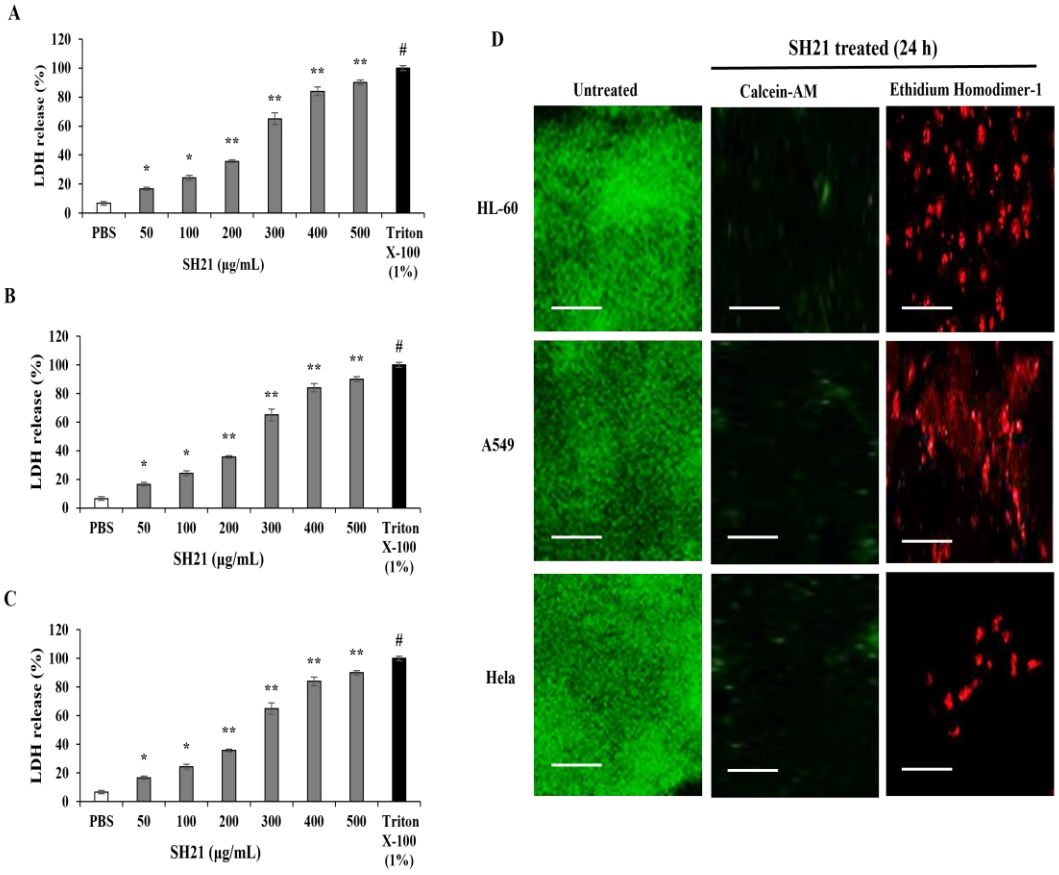

Figure 4.6. The membrane disruption ability of SH21 was assessed by observing LDH release and confocal microscopy assay. LDH leakage was monitored in three cancer cells, namely, (A) HL-60, (B) A549, and (C) Hela cells. All cancer cells were treated with different concentrations (50–500 μg/mL) of SH21, and absorbance were taken at 490 nm. PBS and Triton X-100 (1%, v/v) employed negative and positive controls, respectively. Values were the means of triplicates (±) standard deviation (SD). (D) Live/dead staining assay of SH21 against cancer cells (HL-60, A549, and Hela). Membrane damage was evaluated by employing CLSM. All tested cancer

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cells were incubated with 500 μg/mL of SH21 for 24 h. Green fluorescence (Calcein-AM) and red fluorescence (Ethidium homodimer-1) indicated live and dead cells, respectively. Scale Bars = 100 μm.

While antioxidant compounds are implicated in numerous molecular processes, the possible antioxidant process of SH21 may be regulated by nuclear translocation of Nrf2 into the nucleus, which activates HO-1 expression (Figure 8). This subsequently causes expression of antioxidant enzymes that inhibit ROS free radicals, oxidative damage, and proinflammatory cytokine production, which helps to decrease the chance of inflammation-related diseases and cancers. Since oxidative stress and inflammation perform influential functions in a variety of degenerative diseases, preventing them is one of the most effective ways to prevent disease. SH21 has been protect cells from oxidative stress and inflammation by significantly increasing both the protein levels and mRNA of antioxidant enzymes such as SOD1, GPx-1, and CAT, in RAW264.7 cells and inhibiting ROS generation and proinflammatory cytokines. Therefore, it is interesting that a novel biological agent, such as SH21, can treat oxidative stress-connected pathophysiological circumstances. Furthermore, our study demonstrated that SH21 has direct anti-inflammatory and anticancer properties. As far we know, no other antioxidative protease has ever been reported that reveals prominent anti-inflammatory and anticancer activity that was isolated and purified from kimchi.

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4.4. CONCLUSION

In the present study, SH21 revealed intense antioxidant activity, which was evaluated by various dose-dependent antioxidant assays. Additionally, SH21 increased the expression of antioxidants and detoxifying enzymes by activating the Nrf2/HO-1 pathway and prevented oxidative stress by inhibiting ROS generation in Raw 264.7 cells. In addition, SH21 exhibited substantial anti-inflammatory activity by downregulating NO and proinflammatory cytokines. Therefore, suppressing mRNA levels of iNOS and cytokines may help better our understanding of anti-inflammatory effectiveness. Subsequently, SH21 strongly inhibited the proliferation and disrupted the membrane of three tested cancer cells. Together, these results suggest that SH21 could be a potential therapeutic candidate for preventing oxidative damage and treating inflammation-related diseases.

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5. FUTURE DIRECTIONS

Proteases are a wide class of very important industrial enzymes that are engaged in many different physiologic and cellular functions. Proteases are discovered in all surviving things, including microorganisms, plants, and animals, because they are physiologically required. However, due to their quick growth, small cultivation area requirements, and easy accessibility to genetic modification, microorganisms are a jackpot of proteases and a favorite supplier of enzymes. Since ancient times, the food, dairy, and detergent industries have made substantial use of microbial proteases. Proteases are gaining attention again as potential targets for the development of therapeutic interventions to stop the unchecked spread of deadly illnesses including cancer and inflammatory diseases. Over the past 20 years, proteases have seen a sharp increase in both industrial and medicinal applications. The markets for commercial proteases will continue to grow thanks to innovative protein engineering tactics and methods. The recent success of using synthetic small-molecule-activated proteases to activate apoptotic caspase is encouraging as it offers a novel approach to precisely regulate human protease activity for therapeutic purposes. Furthermore, utilizing the proteolytic activities of proteases in ill tissues may present a novel approach for tumor imaging and site-specific medication targeting. Furthermore, research is required to demonstrate the protease's in vivo effectiveness in animal models.

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7. LIST OF PUBLICATIONS

1. **Tarek, H**.; Cho, S.S.; Hossain, M.S.; Yoo, J.C. Attenuation of Oxidative Damage via Upregulating Nrf2/HO-1 Signaling Pathway by Protease SH21 with Exerting Anti-Inflammatory and Anticancer Properties In Vitro. *Cells* 2023, *12*, 2190.
2. **Tarek, H**., Nam, K.B., Kim, Y.K., Suchi, S.A. and Yoo, J.C., 2023. Biochemical Characterization and Application of a Detergent Stable, Antimicrobial, and Antibiofilm Potential Protease from *Bacillus siamensis.* International Journal of Molecular Sciences, 24(6),p.57
3. Suchi, S.A., Nam, K.B., Kim, Y.K., **Tarek, H**. and Yoo, J.C., 2023. A novel antimicrobial peptide YS12 isolated from *Bacillus velezensis* CBSYS12 exerts anti-biofilm properties against drug resistant bacteria. Bioprocess and Biosystems Engineering, pp.1-16.

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