PROTEASE PRODUCTION, PHENYLALANINE REMOVING AND PRODUCING BIOACTIVE COMPOUNDS

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ABSTRACT

PROTEASE PRODUCTION, PHENYLALANINE REMOVING AND PRODUCING BIOACTIVE COMPOUNDS

Protease, an enzyme that hydrolyzes polypeptide and protein chains, is commonly used commercially in detergents, food, pharmaceuticals, and leather industries. Among animal, plant or fungal proteases, bacterial proteases appear to be more suitable for usage in industry because of its activity and cost. For this reason, microorganisms producing proteases for commercial interest have been studied by many researchers.

Milk proteins are now a source of various biologically active peptides. These peptides have different biochemical and physiological effects such as inhibition of angiotensin-I converting enzyme (ACE), antimicrobial, antihypertension and antioxidative effect.

In this study, after selection of highly active protease producer bacteria in over 200 species, three different bacteria, *Virgibacillus panthothenticus, Bacillus megaterium* and *Burkholderia gladioli*, were selected in order to generate different fragments from casein, a milk protein. ACE inhibitory effect and antioxidant activity of fragmented peptides was examined and showed promising activity for usage in medical industry.

Phenylketonuria is a hereditary metabolic disorder that causes increased phenylalanine in blood and other body fluids to cause more advanced intelligence disability by destroying the developing brain of the child, since an amino acid called phenylalanine in proteinaceous foods can not be metabolized. In this study pheneylalanine was removed from peptides derived from milk proteins.

ÖZET

PROTEAZ ÜRETİMİ, FENİLALANİN UZAKLAŞTIRILMASI VE BİYOAKTİF PEPTİD ÜRETİMİ

Proteaz, polipeptid ve protein zincirlerini hidroliz eden bir enzimdir ve deterjanlar, gıda, ilaç ve deri endüstrisinde yaygın olarak ticari olarak kullanılır. Hayvan, bitki veya mantar proteazları arasında bakteriyel proteazlar, aktivite ve maliyet açısından, endüstride kullanım için daha uygun gibi görünmektedir. Bu nedenle, ticari ilgi konusu olan proteaz üreten mikroorganizmalar birçok araştırmacı tarafından incelenmiştir.

Süt proteinleri şimdi çeşitli biyolojik olarak aktif peptidlerin bir kaynağıdır. Bu peptitlerin, anjiyotensin-I dönüştürücü enzimin (ACE) inhibisyonu, antimikrobiyal, antihipertansiyon ve antioksidatif etki gibi farklı biyokimyasal ve fizyolojik etkileri vardır.

Bu çalışmada, 200'den fazla yüksek derecede aktif proteaz üreticisi bakteri arasından seçildikten sonra, süt proteinin ve kazeinin farklı parçalarını üretmek için üç farklı bakteri olan *Virgibacillus panthothenticus, Bacillus megaterium* ve *Burkholderia gladioli*'den türetilen proteaz enzimi kullanılmıştır. Parçalanmış peptitlerin ACE engelleyici etkisi ve antioksidan aktivitesi incelendi ve tıp endüstrisinde kullanım için umut verici bir aktivite gösterdi.

Fenilketonüri, proteinli besinlerdeki fenilalanin adı verilen bir amino asidin metabolize edilememesi nedeniyle, kan ve diğer vücut sıvılarında fenilalaninin artmasına neden olan, kalıtsal bir metabolik bozukluktur; çocuğun gelişmekte olan beynini yok ederek daha ileri zekâ özürlülüğüne neden olur. Bu çalışmada, fenilalanin süt proteinlerinden türetilen peptitlerden çıkarılmıştır

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LIST OF SYMBOLS/ABBREVIATIONS

α	Alpha
β	Beta
Da	Dalton
d	Day
γ	Gamma
g	Gram
h	Hour
k	Kilo
L	Liter
M	Molarity
m	Meter
m	Milli
µ	Mu, Micro
N	Normality
nm	Nanometer

ACE	Angiotensin Converting Enzyme
BSA	Bovine Serum Albumin
dH_2O	Distilled water
ddH_2O	Double Distilled water
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
HP	Hydrogen peroxide
NA	Nutrient Agar
NB	Nutrient Broth
SDS	Sodium Dodecyl Sulphate
SMA	Skimmed Milk Agar

STD	Sodium Tetraborate Decahydrate
TCA	Trichloroacetic Acid

1. INTRODUCTION

1.1. PROTEASES

Proteases are enzyme that performs the hydrolysis of peptide bonds in proteins and peptides. Protein, peptide fragments, and free amino acids are products of the proteolysis process [1, 2].

Proteases are found in a wide variation of living organisms and exhibit different physical, physicochemical, chemical and biological functions such as cell growth and differentiation, enzyme modification, gene expression, regulation and transportation of metabolites in homeostasis [3, 4].

There is renewed interest in the examination of proteolytic enzymes, since these enzymes not only play an important role in cellular metabolic processes but also acknowledge the great interest in the industrial community at the same time [5].

1.1.1. Exopeptidases (Peptidases) (E.C. 3.4.11-19)

Exopeptidases are characterized by the cleavage and hydrolysis action of peptide bonds in the internal region of the polypeptide chain. Due to their cleavage sites at *N* or *C* terminus of polypeptide chain, exopeptidases are divided into two groups [3].

1.1.1.1. Aminopeptidases

Aminopeptidases are the exopeptidases that target the free *N*-terminus residue and release tripeptide, dipeptide or an amino acid residue. Aminopeptidases are found in a wide variety of microbial species, including bacteria and fungi. Generally, aminopeptidases are intracellular except that is produced by *Aspergillus oryzae* [3].

1.1.1.2. Carboxypeptidases

Carboxypeptidases are the exopeptidases that that targets the free *C*-terminus residue and release single amino acid or a dipeptide. Carboxypeptidases are grouped into three families, namely, serine carboxypeptidases, metallo carboxypeptidases, and cysteine carboxypeptidases according to the amino acid residue at the active site [3].

1.1.2. Endopeptidases (Proteinases) (E.C. 3.4.21-99)

Endopeptidases are characterized by the cleavage and hydrolysis action of peptide bonds in the internal region of the polypeptide chain. Due to their catalytic mechanisms and the functional group present at the active site, endopeptidases are divided into four subgroups as serine proteases, aspartic proteases, cysteine proteases and metalloproteases [3, 6].

1.1.2.1. Serine Proteases (E.C. 3.4.21)

Serine proteases are endopeptidases whose name come from the serine residues presented in their active sites. Serine proteases are found in numerous and common forms among viruses, bacteria, and eukaryotes. They are known as vital for organisms due to their functional on developmental behaviors. They take role on digesting nutrients, blood clotting for humans while digesting substances for bacteria and infecting of cells for viruses. Because of these roles, endopeptidases is the most studied one of all [3, 7].

Serine proteases are generally showing activities in neutral and alkaline conditions, with a pH range of 7 to 11, while their isoelectric point varies between pH 4 and 6. The molecular weight of serine proteases has changing between 18 to 35 kDa [3].

In the catalytic mechanism of serine proteases, serine residue binds with substrates in the presence of activators or inhibitors. Phenylmethylsulfonyl fluoride (PMSF), and diisopropylfluoro phosphate (DIFP) may be counted as major inhibitors of serine proteases [8].

Serine proteases have twenty families due to their similarities and differences in their amino acid configurations, sequences and also three dimensional structures. Subtilisin and

trypsin families are the most studied and best-known groups of serine proteases. Serine alkaline proteases, largest subgroup of serine proteases, are a subgroup of subtilisin family while chymotrypsin, trypsin, mammalian elastases are subgroups of trypsin family [9-11].

Serine alkaline proteases are produced by many bacteria, yeast, fungi, and mold species and are active at high alkaline conditions. Their function is to cleave the peptide bond with tyrosine, phenylalanine or leucine at the *C*-terminus. Optimum pH value for the activity of serine alkaline protease is about pH 10 while their isoelectric point is about pH 9. Although it is produced by other bacteria, *Bacillus* species are the most studied producers of subtilisins [3].

Serine proteases catalyze the peptide bond by nucleophilic attack on the target carbonyl bond. Thus, a reactive serine residue and acyl-enzyme intermediate form. In the first step, the substrate binds to the active site while performing catalytic reactions of the serine proteases. Substrate specificity is determined by residues located within the active site, and the specific binding determines the proximity between the serine and the carbonyl group of the peptide bond that is interrupted. Here, the cleavage of the peptide bond occurs with the ping-pong mechanism. In this mechanism, the substrate is bound, the product is separated from the *N*-terminus of the peptide, the other substrate is bound to the water, and the *C*-terminus of the peptide is separated as seen in Figure 1.1 [12].

1.1.2.2. Cysteine/Thiol Proteases (E.C. 3.4.22)

Cysteine proteases are generated by both prokaryotes and eukaryotes. Cysteine (SH-) and histidine groups are found in the active site of cysteine proteases. This type of protease becomes activated only in the presence of reducing agents like cysteine and HCN. Cysteine proteases are categorized into four groups according to their side-chain specificity: papain-like, trypsin-like, glutamic acid-like and others. The difference between these groups comes from the order differences of cysteine and histidine residues. Cysteine proteases generally occur in neutral conditions and have neutral pH optima. Their molecular weight is range from 20 to 35 kDa [3, 13, 14].

1.1.2.3. Aspartic Proteases (E.C. 3.4.23)

Aspartic proteases, also known as acidic proteases, are endopeptidases whose names come from the aspartic residues found in their active sites. Aspartic proteases have been classified into three groups as; pepsin, retropepsin, and pararetroviruses' enzymes. Aspartic proteases are produced in acidic conditions and show maximal catalytic activity at acidic pHs. Molecular weights of aspartic proteases range from 30 to 45 kDa, while their isoelectric points vary from pH 3 to pH 4.5. Pepstatin, diazoacetyl norleucine methyl ester (DAN), and 1,2-epoxyp-nitrophenoxy propane (EPNP) may be mentioned as inhibitors of aspartic proteases [3, 15].

1.1.2.4. Metalloprotease (E.C. 3.4.24)

Metalloproteases are the enzymes that characterized by the requirements for divalent metal ion to fulfills its hydrolysis activity. Metalloproteases break down the peptide bonds through the nucleophilic attack of a water molecule corresponding to a divalent metal ion. Due to the specificity of their catalytic mechanism, metalloproteases are classified in 4 groups as, neutral, alkaline, Myxobacter I and Myxobacter II. While the pH range of their activity is between 5 and 9, metalloproteases are generally produced under neutral conditions. Chelating agents like EDTA and 1,10-phenantroline are common inhibitors of metalloproteases [16, 17].

1.1.3. Sources of Proteases

1.1.3.1. Plant Proteases

The use of plants as a source of protease is influenced by different factors such as the climate conditions of the plant, growing rate, and the suitability of the cultivation soil. Plant-derived proteases are derived mostly from tropical plants. The most widely known of plant-derived proteases are papain, bromolein, ficin and keratinase [18].

As plant-based products, it is comprehended as safe and natural content for use in food applications. However, bromelain and papain are generally not cost-effective in

applications where the intensification of soluble solids or viscosity reduction is the main goal. The disadvantage of this production is that the production of protease from plants is a time-consuming process [3].

1.1.3.2. Animal Proteases

The most known of animal-origin proteases are pancreatic trypsin, chymotrypsin, pepsin and rennin. Pepsin is an acidic protease secreted by the host cells in the margins of almost all vertebrates to break up food proteins into peptides. Pepsin has optimum activity at pH 1-2 while is inactivated above pH 6. This enzyme catalyzes the hydrolysis of peptide bonds between two hydrophobic amino acids [3].

Rennin is a natural, complex enzyme that helps young mammals to digest their mother's milk. Rennin is present as inactive pro-renin the marbles of all milk-bearing mammals. It turns into active rennin by the function of pepsin or by self-catalysis. It is frequently used in the milk industry for the precipitation of milk casein and in the production of curd [3].

These proteases can be purified in excess amounts in large volumes. However, their production is directed by husbandry policies because, it requires the cut-off of the farm animals [19].

1.1.3.3. Microbial Proteases

Due to the lack of suitable climatic conditions in the hands of plant and animal origin proteases and the policies related to animal husbandry, microbial-derived protease is more concentrated that led to the sales venues of microbial proteases as 40% of worldwide enzyme sales [3, 20-22].

Table 1.1.Commerical protease producing *Bacillus* species [5, 20]

***Bacillus* spp.**
Bacillus alcalophilus
B. amyloliquefaciens
B. circulans
B. cereus
B. coagulans
B. firmus
B. intermedius
B. licheniformis
B. megaterium
B. polymyxa
B. proteolitycus
B. pumilus
B. sphaericus
B. subtilis
B. thrungiensis

Because of the demand of proteases, scientific researches have been led to a great deal of interest in microbial proteases via the rapid development of microbial metabolism, low cost production methods and genetically modifying microorganisms to produce more efficient enzymes and obtain highly efficient strains with desirable properties [23, 24].

Today, the most widely used protease sources are microbial proteases derived from bacteria, fungi and viruses. Microbial-derived proteases are preferred over plant- and animal-derived proteases, since microorganisms can be modified for biotechnological applications in the desired direction, proteases can be purified to a lesser extent from microbial sources than plant and animal sources, and microorganisms can be produced in a suitable culture medium [3].

Industrial based microbial proteases are generally extracellular and are directly exported to the fermentation medium by the producer, so that subsequent processing of the enzyme is easier than with animal and plant derived proteases [5].

1.1.3.3.1.Properties of Microbial Proteases

Industrial type bacterial proteases are generally extracellular, stable and active at high or low pH ranges. Because of these advantages, they become suitable for various industrial applications. Most commercial proteases are produced by bacteria of the genus *Bacillus* as neutral and alkaline [20].

Commercial bacterial neutral proteases are active at low temperatures. They show activity between pH 5-8. High affinity to hydrophobic amino acid pairs constitutes the characteristic feature of bacterial neutral proteases [25].

Bacterial alkaline proteases are characterized by high activity in alkaline conditions such as pH 10 and wide substrate specificity. Commonly, optimal temperatures range from 40 to 60°C. At the same time, there are high isoelectric points and they are usually stable between pH 6 and 12. A divalent cation (such as Ca_2^+, Mg_2^+ and Mn_2^+) or combinations of these cations are required to increase the activity of alkaline proteases. Divalent cations have essential role to protect enzyme from thermal denaturation and enhance the thermal stability [26, 27].

1.1.4. Industrial Applications of Proteases

Proteases are one of the most important groups of industrial enzymes. Industrial proteases constitute the broadest category of industrial enzymes account for over 65% of the industrial enzyme market in the world [28].

Proteases have found their use in a wide range of industrial applications such as the leather processing industry, detergent industry, production of protein hydrolysates, pharmaceutical industry, biosynthesis, biomass and biotransformation, and food industry due to their wide substrate specificities [29, 30].

In the production of protease enzymes, the most preferred type of protease is microbial derived proteases. Among the bacteria used, *Bacillus* species are the most commonly used one [30].

Table 1.2. Industrial applications of protease enzyme [31]

Industry	Protease	Application
Baking	Neutral protease	Dough conditioner
Beverage	Papain	Removal of haze in beverages
Dairy	Chymosin, other proteases	Whey protein processing, replacement of calf rennet
Detergent	Alkaline protease, subtilisin	Laundry denetgents for protein removal
Food	Various protease	Modification of protein rich material
Leather	Trypsin	Bating, dehairing
Photography	Various protease	Recovery of silver from photographic films

1.1.4.1. Textile Industry

The textile industry is an industry that closely follows developments in the field of enzymes. Proteases are traditionally being used in processing of wool and raw materials in the field of textiles [32].

The materials required for fabric production are of different fiber structure such as wool, silk, angora and cashmere. The properties of protein based fibers are determined by the type, amount and location of the amino acids. According to these properties, wool based products are treated with papain, pronase, and pepsin to provide flexibility of the fibers, purified from natural dirt, and a whiter color. These enzymatic treatments save both time and energy with lesser need of chemical substances [32, 33].

The silk yarn, which is called silk gum that protects the raw yarn, must be removed before dyeing. The silk yarn is removed by using starch in a controlled manner. This method is quite expensive and, alternatively, the use of enzyme preparations has been suggested prior to dyeing the dye [34].

1.1.4.2. Leather Industry

Hydrogen sulphide and other chemicals used in leather processing are very dangerous in terms of safety and environment. Due to environmental reasons, enzymatic methods are preferred for easy control, rapid processing, and leather processing with the reason of elimination of wastes. Alkaline proteases can be used in leather processing industry due to their elastolytic and keratinolytic activities. Bacterial proteases are used for selective hydrolysis of non-collagenous structures such as globulins and albumin, to remove proteins that are not in the fibrillar structure, to separate and soften the hair [35, 36].

Leather process has different steps as; wetting, tanning, liming, descaling, hair removal and hair washing. Different protease types are used in different stages of the leather industry. Neutral proteases are used during the wetting stage, alkaline proteases are used during the hair removal process, and acid proteases are used during the hair washing stage [37].

Proteases are frequently used in wetting, liming, dehulling, de-waxing and separating stages of animal skins, and exhibit higher activity than chemical substances. The use of enzymes instead of harmful chemicals reduces environmental pollution by 80-90%. When compared with traditional chemical methods, enzymatic processes not only reduce the use of harmful and environmentally polluting chemicals, but also produce high-quality products [38].

In the wetting phase, the hides and the animals are washed and soaked in surfactants and antimicrobials. Alkaline proteases are used to remove non-fibrous proteins such as albumin and globulins. Pancreatin is the main ingredient for skin softening [30].

De-waxing is carried out by applying watering paint to the flesh and keeping it at 20-35°C for 10-20 hours before removing the wool. Deinking paint consists of hydrated lime, sodium chloride, alkaline proteases and water. An alternative method for fine wooly skin requires applying a powder to the fleshy portion and keeping the fleece at 25°C for 24 hours. This powder consists of sodium sulphate and chlorite, ammonium sulphate and chlorite and neutral proteases produced by microorganisms [36].

In the separation step, the hides and animals are processed with enzymes and chemicals to make the hides soft and flexible and be ready for tanning. Today, mainly trypsin, and low amounts of alkaline and neutral proteases are used [30, 36].

1.1.4.3. Detergent Industry

The detergent industry is intensively demanding proteases. The protease enzyme, which is first used enzyme in laundry detergents, is now supported by lipases, amylases and cellulases [30].

There are different parameters in the selection of proteases used in detergent formulations. Ideal detergent proteases should have broad substrate specificity to facilitate removal of most of the stains due to food, blood and other body exudates. One of the important parameters in the selection of detergent proteases is the pI value. It is known that detergent proteases can be used most effectively when the pH of the detergent solution is compatible with the pI value of the enzyme [39, 40].

Detergent components, perfumes, surfactants, whitening chemicals, pH and temperature values during washing and compatibility with the ionic power of the detergent solution, determines the use of the enzyme in detergents as well as the value of pI. It is an advantage that the enzyme is added to detergent formulations which are stable against EDTA. It is preferred that the enzyme to be added to the detergents does not require metal cofactors. Because detergents contain a high amount of chelating agent and it is not possible to remove the chelates bound to metal ions from the detergent solution [5, 41].

The use of bacterial proteases as active ingredients in laundry detergents provides a wide range of applications. Enzymes have become an important compound of detergents formulated for warm and cold water washing temperatures with low phosphate content. The detergents formulated for hot washes include sodium phosphate and sodium perborate, a bleaching agent that is activated at high temperatures (above 60°C). Due to increased environmental pressures to reduce phosphate contamination and increase using of polyester fabrics, sodium phosphate and sodium perborate have been reduced in detergents. The use of bacterial proteases has increased due to environmental safety [42].

Today, the detergent industry is working towards lowering the washing temperature and changing the detergent content, while removing phosphate based detergents and using new alkaline proteases more suitable for detergent applications [41].

1.1.4.4. Food Industry

In the food industry, proteases were often used for a variety of purposes, such as cheese making, baking, preparation of soy hydrolysates, and meat smoothing. Today, alkaline proteases are used to prepare protein hydrolysates with high food value. Protein hydrolysates are used in the regulation of blood pressure, in infant formulas, in specific therapy dietary products and in increasing the shelf life of fruit juices and certain beverage [18, 43].

Endo- and exo-proteinases derived from *Aspergillus oryzae* are used to modify gluten, a water-insoluble protein in wheat that determines the properties of baking pastes. Fungal proteases are used successfully in the making of white breads and pastes, but when used in excessive quantities, they turn the bread into paste like consistence. The enzymatic treatment of dough facilitates its manual and machine production and allows a wider range of products to be produced.

The addition of proteases reduces the mixing time by about 25% in increasing loaf volumes. In particular, *Bacillus subtilis* protease is used in making cakes, biscuits and crackers. The protease is used to delay the softening of pulps and are very important, especially in the production of crackers. Bacterial proteases are used to increase the strength and lengthening capability of the dough [30].

Commercial protein hydrolysates are produced from the buckwheat, wheat and soy protein. It has been reported that *Bacillus subtilis* protease can be used to obtain high protein food protein hydrolysates [17, 44].

1.1.4.5. Other Industrial Applications

Fibrous proteins such as horns, feathers, nails and hair are abundant in nature as waste. These wastes may be converted or destroyed using proteases. Proteases dissolve in protein-containing wastewater, helping to reduce biological oxygen demand in aquatic systems. The proteolytic activity of proteases and the removal of these protein-containing wastes are achieved [45].

The regulation of poultry wastes can be considered as one of the main usage areas of waste treatment of proteases. Alkaline protease of *B. subtilis* was used to remove bird fluids in the poultry. While the waste bird feathers occupy 5% of the total weight of the poultry house, the keratinous structure of these wastes has been totally destroyed and it has become available in the food and feed industry [46, 47].

Proteases are also used in the photography sector. The gelatin layer in photographic films contains 1.5-2% silver. With the burning of the films, the silver on the surface is recovered, but this leads to irreversible environmental pollution. The gelatin-bound silver protein layer can be removed by treatment with proteolytic enzymes. With the enzymatic hydrolysis of gelatin, not only the silver is recovered, but also the polyester-based film becomes usable again [20, 39].

Alkaline proteases are also used in the development of medically important products. Burns, bed wounds, boils and deep abscess' treatment, blood and tissue in the definition of drug substance can be counted. The protease enzyme also has a wide use in the pharmaceutical sector. Enzyme supplements are produced to meet various needs in the body or to meet the need for missing enzymes. Proteins are used in medicines produced to support the digestive system [48, 49].

1.2. MILK AND DAIRY PRODUCTS

Milk is an important food that contains many nutrients necessary for the human health. It is a vital fluid in its true sense and is necessary in every phase of life. Bone health problems especially occurred in childhood, pregnancy, lactation, and aging period, milk is a food that must be regularly included in a child's and mother's diet to ensure that they have adequate and healthy homeostasis. The contribution of milk proteins to the known growth and development in the body, as well as the effect on tissue differentiation, is known to have beneficial effects on calcium absorption and immunological functions, to reduce blood pressure and cancer risk, to protect against body decay, and to protect against tooth decay [50-55].

1.2.1. Properties of Milk

Variables such as acidity, density, fat content, and fat-free dry matter determine the characteristics of raw milk. Milk is freezing at about -0.55°C, lower than distilled water due to the lactose and minerals present in the composition. The lactose and soluble minerals in the milk structure increase the boiling point. Due to these substances, the boiling point is about 100.16°C. The density of the milk varies from 0.93 g/cm^3 to 1.037 g/cm^3, due to the type of milk [56, 57].

Newly harvested fresh and normal milk show acidic reaction. The average acidity of cows' milk is 0.135-0.2%. This acidity first provides casein phosphate and citrates, followed by albumin and molten carbon dioxide. In addition, animal species, race, age, lactation period, diseases and milk composition are effective on the first acidity [58].

1.2.2. Components of Milk

Milk is an important source of many food items such as protein, calcium, phosphorus, vitamins A, B2, and B12. However, many factors such as seasonal change, physiological factors, and diseases influence the content of nutrition food. It was reported that the values between spring and autumn were statistically significant. Protein, fat-free dry matter and ash contents were shown to be higher in the autumn, while the amount of fat was higher in the spring. Also animal species and genetics, lactation stage, and animal nutritional conditions effect content of milk [59, 60].

Approximately 87.2 g of water, 4.9 g of lactose, 3.5 g of fat, 3.5 g of protein, 0.9 g of minerals and trace amounts of vitamins, enzymes, organic acids, preservatives, hormones, hormone-like substances and gases are found in 100 g of milk. Because, the presence of fatty acids, vitamins and minerals, proteins and peptide structures; It has many important features in the life cycle [61, 62].

1.2.2.1. Lipids

The milk fat fraction is present in the globules that are resistant to the lipolysis, unless gastric digestion is predominant. The oil is present in the form of microscopic globules in water emulsion. More than 400 different fatty acids and fatty acid derivatives are included in milk, main composition is commonly as 97-98% triglycerides, 0.2-1.0% phospholipids, 0.22-0.41% free sterols, free fatty acids, vitamins (A, D, E, K). In addition, there are trace hydrocarbons, soluble vitamins in skimmed milk, flavorings, and other ingredients supplied with animal feed. Animal origin, elements that related with feeding, mastitis and ruminal fermentation affect amount and content of milk fatty acids [51, 63].

Low-calorie products are highly demanded; therefore, the creation of new strategies to provide nutrient richness, correct flavor, texture and smell with lower fat and thus lower energy content [51].

1.2.2.2. Carbohydrates

Lactose, basic carbohydrate, is synthesized in breast tissue. Unadulterated cow milk contains an average of 4.7% lactose. The 54% of the dry matter except the oil is lactose. Milk contains glucose, galactose and oligosaccharides in small amounts. Glucose and galactic-powder are formed by the lactase enzyme hydrolyzing lactose. Lactose-reduced or lactose-free milks can be produced using industrial dairy enzymes [64].

1.2.2.3. Proteins

There is an average of 32 g / L protein in high quality protein containing cow's milk. Cow's milk protein is a heterogeneous mixture of enzymes and non-protein compounds containing little nitrogen, and especially casein and whey proteins. Approximately 80% of the total proteins in the milk consist of casein (8% is inorganic molecules, 92% is protein) and 20% is whey protein [51, 62].

Both types of proteins are classified as high-quality proteins, considering human amino acid requirements, digestibility and bioavailability. Based on the amino acid score and

protein digestibility, milk is considered the best protein source in foods. While casein has higher ratio of histidine, methionine, and phenylalanine, whey is rich in leucine, isoleucine, valine, and lysine [65, 66].

In addition to high quality and biological value, several bioactive peptides derived from milk proteins by enzymatic hydrolysis. They have demonstrated multiple biological roles that can play a protective role in human health. These main biological actions include, antihypertensive, antiviral, antioxidant, antimicrobial, antithrombotic, opioid and immunomodulatory roles [67].

1.2.2.3.1 Casein Proteins

Casein is a heterogeneous mixture and has been formed from various components: 38% of αs1-casein, % of 10 αs2-casein, 36% of β-casein, 13% of κ-casein and, 3% of γ–CN. Casein is extremely stable against thermal and mechanical effects, in contrast to the sensitivity to acids and salts in technological conditions [68].

Casein, also known as the most important fraction of milk proteins, is in the form of a colloidal dispersion with dispersed particles ranging in size from 20 to 600 nm. Among the inorganic substances that make up about 7% of the casein are calcium (Ca), phosphate, magnesium, sodium and citrate. Casein forms a complex with these inorganic compounds and this complex is referred to as "Calcium-caseinate-phosphate" or "Calcium-phosphocreatinate." For this reason casein is considered as a phosphoprotein [69, 70].

The size of the casein miscella is dependent on the amount of Ca, and when Ca is removed from the column, the casein is disintegrated into the lower micelles. It can cause collagenous or complex formation with calcium, phosphate, and citrates, which cannot be bound or are present in the medium, causing the growth of casein particles. It also helps to gel the casein flakes that have been shredded with the enzyme during coagulation of the tobacco cheese yeast, which facilitates clotting. When Ca is removed from the medium, coagulation is prevented [69].

1.2.2.3.2 Whey Proteins

Whey proteins, the soluble protein of milk, are consisting of mostly β-lactoglobulin, α-lactoalbumin and also immunoglobulins (Ig), serum albumin, lactoferrin, lactoperoxidase, lysozyme, proteose-peptone, and transferrin proteins. Among them, lactoferrin has ability to prevent formation of free radicals due to its iron-binding property and antimicrobial activity. Together with β-lactoglobulin, lactoferrin can suppress tumor development [71].

Physical and functional properties of whey proteins may be counted as, solubility, viscosity, adhesion and cohesion and absorption of water [72].

Whey is used for animal feeding by feeding directly or adding them to roughage feeds. It is a very convenient raw material to produce beer-like beverages due to some qualities besides producing various soft drinks by adding various fruit flavors to cheese water in different proportions. Cheese juice proteins are used in sugars and many sweet varieties because of their acid stability, structure and moisture control, and their ability to foam and emulsify. The whey protein has also found itself in the cosmetic sector because the low molecular weight components present in the whey proteins have very similar properties to the natural moisturizing factors in the human skin. It is also used in the treatment of diseases such as bowel obstruction, liver disease, poisonings, kidney disorders and obesity because it is a rich protein mixture with broad spectrum chemical, physical and biological properties [73-75].

1.2.2.4. Minerals and Vitamins

Milk contains both liposoluble (liposoluble (A, D, E) and hydrosoluble vitamins (B and C). Milk is generally known as a good vitamin A source with concentration of 172 mg/100 g. As the milk fat decreases, the vitamin content in the oil melt decreases; hence, skim milk and fat-free milk concentrations decrease to 102 mg/100 g and 5 mg/100 g, respectively [76, 77].

Although it is known as a good source, milk does not contain significant amounts of vitamin D. Vitamins D and K are very low in milk. Vitamin D may be fortified to milk, by

this way, vitamin D with a normal concentration of 35 IU/L can be increased to 40-50 IU/L [78].

Calcium is naturally the most abundant mineral present in milk with the average concentration of 1200 mg/L. While in the micellar phase calcium is associated with the phosphoseryl residues of casein, in the aqueous phase, calcium may be bound to the inorganic forms of the whey proteins or salts forming phosphates [79].

Milk is also considered to be a good source of phosphorus in the organic and inorganic forms with average concentration of 950 mg/L. Like calcium, both forms of phosphorus in organic and inorganic forms are in equilibrium and their dispersion is dependent on conditions [72, 80].

Magnesium also is another mineral found in milk with approximate concentration of 120 mg/L. The factors affecting the dynamic balance of magnesium and calcium are also passed on to magnesium. Zinc and selenium are microelements in milk with concentration of 4 mg/L and 30 mg/L respectively [80].

1.2.3. Milk as Bioactive Peptide Source

Amino acid chains and peptides which have significant physiological roles with specific properties are defined as functional peptides or bioactive peptides. They are not active in the structural protein, they require enzymatic activity to become active. It was noticed many years ago that the milk contains almost all kinds of functional peptides [81, 82].

The amino acid sequence of a particular peptide determines the function of the peptide. Bioactive peptides contain about 3-20 amino acids per molecule. From a nutritional perspective, milk and milk products contain important biologically active ingredients. The heads of these, bioactive peptides, folic acid, B6 (pyridoxine) and B12 (cyanocobalamin) and conjugated linoleic acid (CLA) [83, 84].

In recent years, it has been shown that α-lactalbumin and β-lactoglobulin, known as fundamental whey proteins, contain bioactive amino acid chains. Bioactive peptides derived from milk proteins have different physiological bioactivity such as antihypertension, antimicrobial, antioxidative, antithrombotic, immunomodulatory, mineral binding and opioid effect (sedative effect) [85-88].

1.2.3.1. Antioxidative Peptides

When the formation of free radicals, which are the result of oxidation, is excessive, proteolytic enzymes such as catalase and peroxidase are released. These enzymes are also destructive to the organ, causing great destruction in the cell and DNA. It has been reported that antioxidative peptides have many advantages over antioxidant enzymes such as being more stable under different conditions and not participating in dangerous immuno reactions.

Some dairy proteins and protein hydrolysates have been reported to have antioxidant capacity. Milk proteins such as lactoferrin, ß-lactoglobulin, and casein are known to be bioactive peptides with antioxidant capacity. The antioxidant activity of the proteins is due to the amino acids in the structures and the bioactive peptides obtained by the enzymatic hydrolysis of the protease. Antioxidative peptides obtained from milk are used for cell protection and repair.

1.2.3.2. Angiotensin-I Converting Enzyme Inhibitory Peptides

The angiotensin I-converting enzyme (ACE) is exopeptidase and elicits dipeptides from the C-terminal ends of various peptide substrates. ACE (EC 3.4.15.1) is traditionally associated with the rennin-angiotensin system that regulates peripheral blood pressure. ACE enhances blood pressure by converting angiotensin I, released from angiotensinogen, into rennin into strong vasoconstrictor angiotensin II. ACE-inhibitor peptides regulate blood pressure by blocking these effects. Several ACE-inhibitor peptides have been identified in specific peptides of ACE-inhibitors, enzymatic hydrolysates and casein proteins, and are now accounted for by casein and serum protein [89-91].

Pepsin, trypsin and combination those with chymotrypsin were used for generation of fragments from α-lactalbumin as 50^{th} – 53th, 99^{th} – 108^{th}, 50^{th} – 52th, respectively, showed ACE inhibitory effect [84].

2. AIM OF STUDY

Milk proteins have various benefits for health issues to reduce or prevent the risk of developing nutritional diseases, especially cardiovascular disorders. Many animal or plant derived peptides with bioactive potential have been found and these peptides have been isolated to date from mostly milk derived products.

In this study, as the first goal, protease enzymes obtained from different microorganisms were used to break down the protein in different fractions and consequently to obtain peptides with different bioactivity. Subsequently, the obtained peptides were aimed to be used in medical industrial applications suitable with their Angiotensin I-converting enzyme (ACE) inhibitory activity and antioxidant activity.

Phenylketonuria is a hereditary metabolic disease. Children born with this disease cannot metabolize phenylalanine found in foods containing protein. As a result, phenylalanine and its residues, which have increased in blood and other body fluids, cause damage to the developing brain of the child, leading to further mental retardation and many other symptoms involving the nervous system. In addition to goals mentioned above, phenylalanine was wanted to remove from milk products to use in milk products for the patients with phenylketonuria.

3. MATERIAL AND METHOD

3.1. MATERIAL

3.1.1. Microorganisms

Zymomonas mobilis (NRRL – B14023), *Alcaligenes faecalis* (NRRL – B-170), *Pseudomonas oleovorans* (NRRL – B14683) *Bacillus pumilus* (SAFR – 032) were purchased from Culture Collections. *Entercoccus faecalis, Paenibacillus polymxia, Paenibacillus lentimorbus, Bacillus megaterium, Bacillus cereus, Acinetobacter calcoaceticus, Burkholderia gladioli, Staphylococcus aureus, Escherichia coli, Virgibacillus pantothenticus, Bacillus subtilis, Brevibacillus parabrevis, Bacillus flexus, Gluconacetobacter xylinus, Micrococcus luteus, and, Exiguobacterium indicum* bacteria were all isolated from 20lbümin20ate locations.

3.1.2. Chemicals

L-Tyrosine (Sigma-Aldrich – T3754), glycine (Sigma-Aldrich – G7126), sodium hydroxide (Sigma-Aldrich – 06203), Folin&Ciocateu's phenol reagent (Sigma-Aldrich – F9252), Trichloroacetic acid (Sigma-Aldrich – 27242), Casein from bovine milk (Sigma-Aldrich – C7078), sodium carbonate (Riedel de Haen – 13418), Nutrient broth (MERCK – 1.05443.0500), nutrient agar (MERCK – 1.05450.0500), skim milk powder (Sigma-Aldrich – 70166), Agar (Sigma-Aldrich – A1296) ACE Kit (Dojindo Molecular Technologies – A502-10), 2,2-Diphenyl-1-picrylhydrazyl(Sigma Aldrich – D9132), Ammonium persulfate (Sigma Aldrich – A3678), N,N,N′,N′-Tetramethylethylenediamine (Sigma Aldrich – T9281), Acrylamide/Bis-acrylamide 30% solution (Sigma Aldrich – A43574), Sodium dodecyl sulfate (Sigma Aldrich – 436143), Trizma base (Sigma Aldrich –T1503), Hydrochloric acid (Sigma Aldrich – 07102), Amonium sulfate (Sigma Aldrich – 31119), Extra Light Milk (Pınar Süt Mamülleri San. A.Ş.)

3.1.3. Equipments

Water Bath (Stuart – SB540), Shaker (Sartorius – innova 44), Centrifuge (Beckman Coulter – Avanti J-E), Centrifuge (Eppendorf 5810 R), Spectrophotometer (Thermo Lab Systems – 5118500), Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane (Merck – UFC901008), Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane (Merck – UFC903008), Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 membrane (Merck – UFC900308), Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (BioRad), Mini-PROTEAN® Tetra Handcast Systems (BioRad), Chemi-Doc XRS Image System (BioRad). Dialysis tubing cellulose membrane (Sigma Aldrich – D9402)

3.2. METHOD

3.2.1. Selection of Microorganisms

Zymomonas mobilis, Alcaligenes faecalis, Pseudomonas oleovorans, Bacillus pumilus, Entercoccus faecalis, Paenibacillus polymxia, Paenibacillus lentimorbus, Bacillus megaterium, Bacillus cereus, Acinetobacter calcoaceticus, Burkholderia gladioli, Staphylococcus aureus, Escherichia coli, Virgibacillus pantothenticus, Bacillus subtilis, Brevibacillus parabrevis, Bacillus flexus, Gluconacetobacter xylinus, Micrococcus luteus, Exiguobacterium indicum types of bacteria were thawed and 10 µl from stock culture was dropped on a nutrient broth medium and lest for incubation at 30^{o}C for 1 day.. After 1 day of incubation, bacteria were seeded on skimmed milk agar plate to screen protease activity. A blank disk placed in the middle of the skimmed milk agar (SMA), which was prepared by mixing 5 g of skim milk and 1 g of agar in 100 ml distilled water then autoclaved at 121^{o}C for 5 minutes and 10 µl of inoculant were dropped on blank disk. SMA plates were incubated at 30^{o}C for 1 day. Protease activity was obtained by the zone diameter appeared around the disk in the middle of the plate, due to cleavage of casein in skimmed milk by the protease enzyme that bacteria produced. Bacteria species were categorized by wide zone, narrow zone and no zone that indicated as high protease activity, low protease activity and no protease activity respectively.

3.2.2. Cultivation of Microorganism

Nutrient agar (NA) and nutrient broth (NB) was prepared according to instructions. Selected microorganisms were seeded to nutrient agar from stock culture. Inoculation was done for 2 days at 30˚C. Inoculant was prepared by mixing nutrient broth (30 ml) and a loop of bacteria from stock solution in a sterile falcon tubes then were placed in shaker which was set to 150 rpm at 30˚C for 1 day for incubation.

3.2.3. Inoculation

Erlenmeyer flasks and nutrient broth was sterilized at 121ºC for 15 minutes. For this purpose erlenmeyer flasks were covered with cotton and aluminum foil. After cooling of erlenmeyer flasks and NB, NB (294 ml) and bacteria from previously prepared inoculants (6 ml) were mixed in sterile erlenmeyer flasks to produce 2% (v/v) inoculant. Flasks were placed in shaker which was set to 150 rpm at 30˚C for 18 hours. Inoculant medium was centrifuged at 16,800 g for 45 minutes at 4ºC. Supernatant was taken and centrifuged again. Supernatant of second centrifuge process was taken and filtered through syringe filter then was collected. Supernatant was stored at +4ºC.

3.2.4. Determination of Protease Activity

L-tyrosine was used for standard curve of protease activity assay and 1,993 mg of it was mixed with 10 distilled water to prepare 1.1 Mm L-tyrosine standard. Standard samples were prepared as in Table-1. Samples were mixed and incubated at 37ºC for 30 minutes. Each sample (300 µl) was transferred to 96-well plate in three replicates. Absorbance was recorded at 660 nm.

Table 3.1.Reagent amounts required for calculating standard curve of protease activity assay

Reagent	Standard Blank (µl)	Standard Sample 1 (µl)	Standard Sample 2 (µl)	Standard Sample 3 (µl)	Standard Sample 4 (µl)	Standard Sample 5 (µl)	Standard Sample 6 (µl)
Tyrosine Standard	0.0	5.0	10.0	20.0	30.0	40.0	50.0
Water	250	245	240	230	220	210	200
Sodium Carbonate	625	625	625	625	625	625	625
Diluted FC Reagent	125	125	125	125	125	125	125
µmoles of Tyrosine	0	0.0055	0.011	0.022	0.033	0.044	0.055

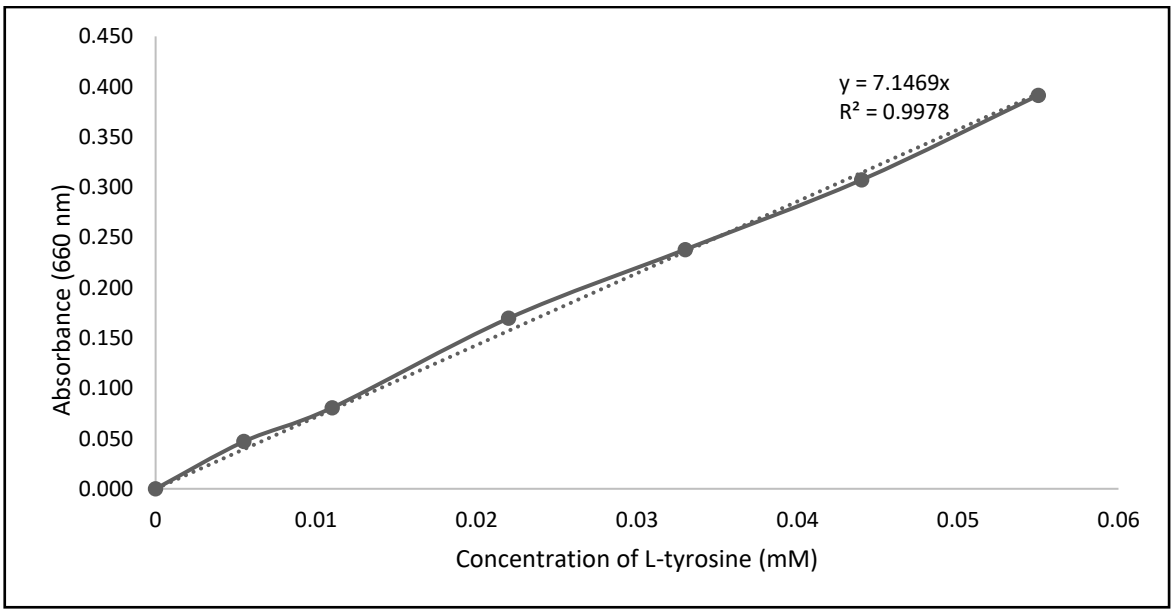

Figure 3.1. Standard curve of L-tyrosine standard solution

To prepare buffer solution of protease activity assay, 1.501 g of glycine was dissolved in 100 ml distilled water while 0.8 g of NaOH was also dissolved in 100 ml of distilled water, and then 17.6 ml of 0.2 M NaOH were mixed with 100 ml of glycine solution. Another

glycine-NaOH solution was prepared as solvent of substrate, casein. Casein solution (0.65% (w/v)) was prepared by mixing 65 mg of casein with 100 ml glycine – NaOH buffer. Then trichloroacetic acid solution (6.1N, 100mM) was prepared by dissolving 99,66 g TCA in 100 ml distilled water.. TCA solution was diluted in ratio of 1:55 before use. Folin Ciocalteu's reagent was also diluted in ratio of 1:4 with distilled water and 24 g of sodium carbonate was dissolved in 100 ml of distilled water to prepare 0.4M sodium carbonate solution.

Glycine – NaOH buffer (100 µl), Casein solution (100 µl, 0.65 %; w/v), and enzyme solution (100 µl) were mixed in different Eppendorf tubes while blank sample only contains glycine – NaOH buffer (100 µl), casein solution (100 µl 0.65 %; w/v). Tubes were inoculated at 40˚C in water bath for 30 minutes. After incubation trichloroacetic acid (400 µl) was poured to each tube. Blank samples were completed with glycine – NaOH (100 µl) buffer then all of the samples and blank sample were centrifuged at 10.000 rpm for 10 minutes. After the centrifugation, sample (500 µl), Na_2CO_3 (500 µl) and FC reagent (200 µl) were poured in new tubes. Absorbance was measured at 660 nm by spectrophotometer. Protease activity was calculated as in formula below.

$$Protease\ activity\ \left(\frac{Units}{ml}\right) = \frac{\left(total\ volume\ of\ assay\ (ml) x\ amount\ of\ tyrosine\ equivalents\ released\ (\mu mole)\right)}{\left(time\ of\ assay\ (min) x\ volume\ of\ enzyme\ used\ (ml) x\ volume\ used\ in\ colorimetric\ determination\ (ml)\right)} \quad (3.1)$$

3.2.5. Characterization of Protease Enzyme

The effects on temperature, pH, inhibitor, metal ion, substrate, detergent and salt protease activity were investigated. In these experiments, in the sets in which metal ion, inhibitor, detergent and salt effects were investigated, the pre-selected materials were dissolved in the Gly.NaOH buffer solution in the desired concentration and new buffer solutions were formed. Standard protease activity measurement procedure was performed one by one using fresh buffer solutions prepared instead of Gly.NaOH buffer solution.

3.2.5.1. Substrate Effect on Protease Activity

For the substrate effect experiments, the determined substrates which are bovine serum albumin (BSA) were dissolved in Gly.NaOH buffer at a concentration of 0.65% (w/v) and these solutions were used instead of the casein substrate solution used in the standard procedure. The substrates were dissolved in Gly.NaOH (100 ml) buffer solution.

3.2.5.2. Temperature Effect on Protease Activity

Temperature effect was examined by changing the incubation temperature to 10, 20, 30, 50, 60, 70 and 80^{o}C. Protease enzymes were incubated with casein substrate for 30 minutes. Activity measurements were done as mentioned above except incubation temperature.

3.2.5.3. pH Effect on Protease Activity

The effect of pH values on protease activity was checked using Gly.NaOH (9.2), potassium phosphate buffer (7.2) and succinate (5.4) buffer solutions. First, 65 mg of casein was dissolved in 100 ml of these solutions to prepare substrate solution. Then, these prepared substrate solutions and buffer solutions were used in activity assay instead of standard solutions.

3.2.5.4. Detergent Effect on Protease Activity

When the detergent effect was examined, sodium tetraborate decahydrate(STD), sodium dodecyl sulfate (SDS) and hydrogen peroxide were used. SDS and Sodium tetraborate decahydrate were dissolved in Gly.NaOH buffer solution at 5 mM and hydrogen peroxide at 1%(v/v) concentration. Solutions were prepared as in the table below. Then, these prepared buffer solutions were used in activity assay instead of standard Gly.NaOH buffer solution.

3.2.5.5. Inhibitor Effect on Protease Enzyme Activity

EDTA, iodoacetamide and urea were used to investigate the inhibitor effect. The inhibitors were dissolved separately in 10 ml of Gly.NaOH with final concentration of 5 mM buffer solution and these solutions were used as the buffer solution. Inhibitors were prepared as in the table below. Standard assay procedure was performed with these prepared buffer solutions instead of standard Gly.NaOH buffer solution.

3.2.6. Degradation of Casein

For proteolysis of the proteins, 130 mg of casein was mixed with 200 ml of distilled water to prepare 0.65% (w/v) casein solution and placed in 4 different glass bottles with 50 ml of solution in each. Three different protease enzymes were added onto the solutions, as their activities were 0.05 U/ml and the remaining final casein solution was used as a control. Solutions were placed in water bath at 40^{o}C for 1 hour. At the end of incubation solutions were taken and placed on ice.

3.2.7. Fractination of protein

Degraded casein solutions of all three bacteria and 0.65% (w/v) casein solution, as control, were loaded into centrifugal filtration tubes (100 kDa) and centrifuge was performed at 5.000 x g for 15minutes. The filtrate was then filled into other centrifugal filtration tubes (50 kDa) and centrifuged in the same manner. The fractions passing through this filtrate were taken and filled into centrifugal filtration tubes (30 kDa) and centrifuged again in the same settings. This centrifuge process was followed with 10 kDa and 3 kDa filtration tubes. After each centrifugation, samples (200 µl) were taken from the sections passing and not passing through the filter.

3.2.8. Determination of Protein Amount

Bovine serum albumin (BSA) was measured as 14 mg and then dissolved in 10 ml of distilled water to get 1.4 mg/ml BSA stock solution for standard curve. Stock BSA solution

was diluted as its concentration will be 0.1, 0.25, 0.5 and 1 mg/ml. 4 µl of these each BSA solution with different concentration was taken and added 96-well plate and filled with 196 µl of ready to use Bradford Reagent. 96-well plate was incubated at room temperature for 5 minutes and prevented from light. Spectrophotometric measurement was done at 595 nm.

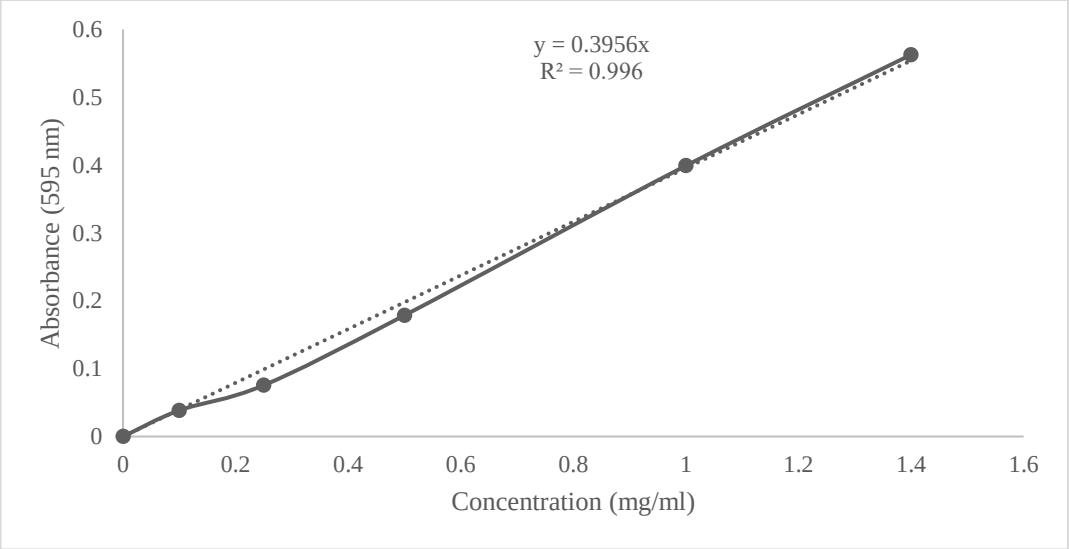

Figure 3.2. Standard curve of Bradford protein determination method

3.2.9. Removal of Phenylalanine

The 0.80 g of active carbon was added to 100 ml of 0.65% casein, 0.65% whey and milk. Also same amount of active carbon was added to degraded 0.65% casein, degraded 0.65% whey and degraded milk and stirred for 30 minutes at 25°C. After mixing, the solutions were centrifuged at 14,000 g for 10 minutes. Measurements of the samples were taken between 250 and 280 nm [93].

To obtain a standard curve, 82 mg of the phenylalanine was dissolved in 100 ml of glycine-NaOH buffer to a final concentration of 5 mM and diluted to 0.5, 1, 2, 3, 4 mM concentrations. The same procedure was repeated for phenylalanine solutions.

3.2.10. Second Derivative Spectra Analysis

The 82 mg of phenylalanine was dissolved in 100 ml of glycine-NaOH buffer to a final concentration of 5 mM and diluted to concentrations of 0.1, 0.5, 1, 2, 3 and 4 mM.

Spectrophotometric measurements were taken between 250 and 280 nm, and the second derivative of the samples was used to obtain an absorbance graph. The negative peak area remained at 260 and 275 nm was measured. Area-Phenylalanine concentration graph was plotted as a standard graph. Gnuplot 5.0 software was used for the calculation of graphs and equations.

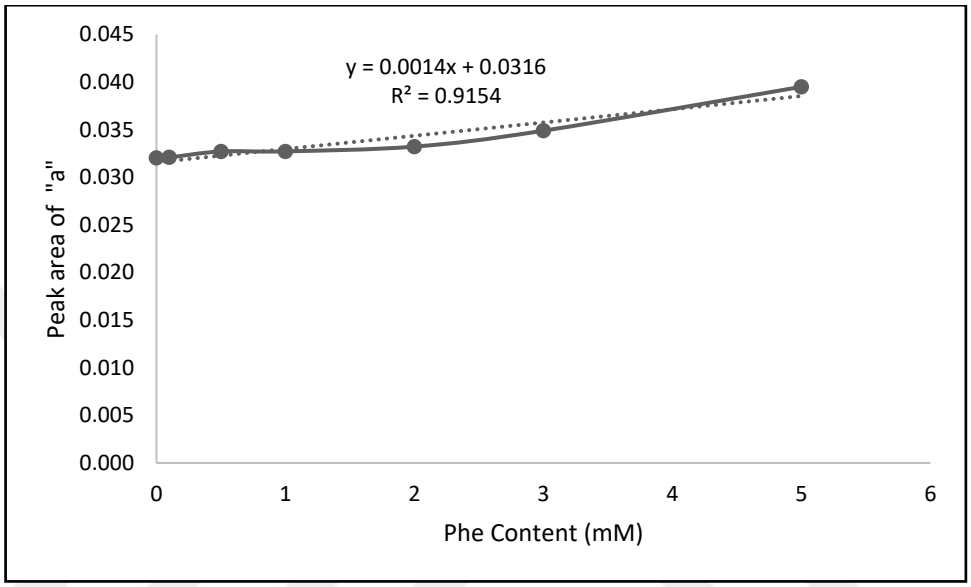

Figure 3.3. Standard curve of Phe. Peak ‘‘a’’ is the negative peak in the second derivative spectrum of Phe between 260 and 275 nm

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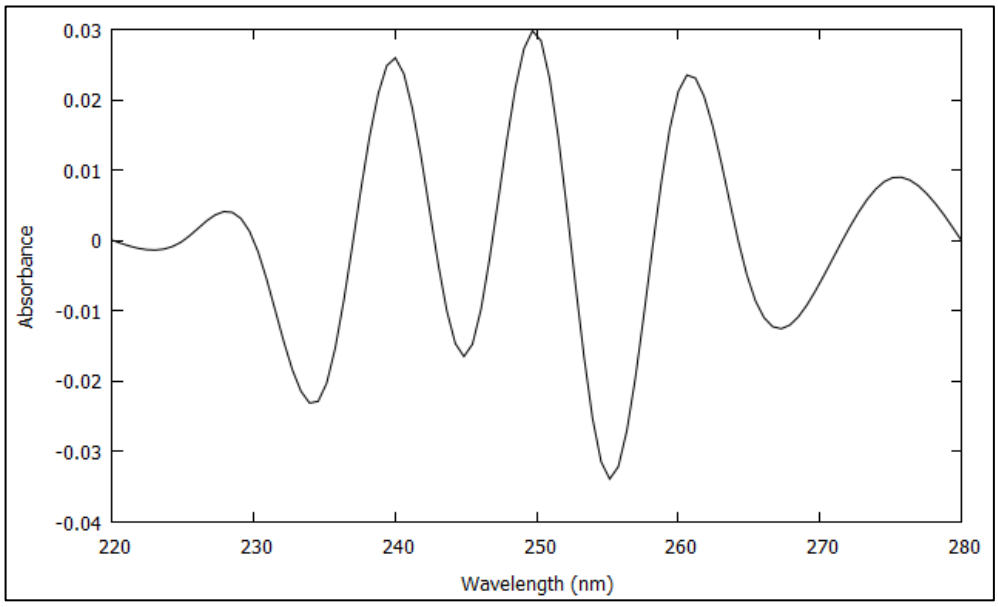

Figure 3.4. Absorbance and second derivative spectra of Phe between 220 and 280 nm

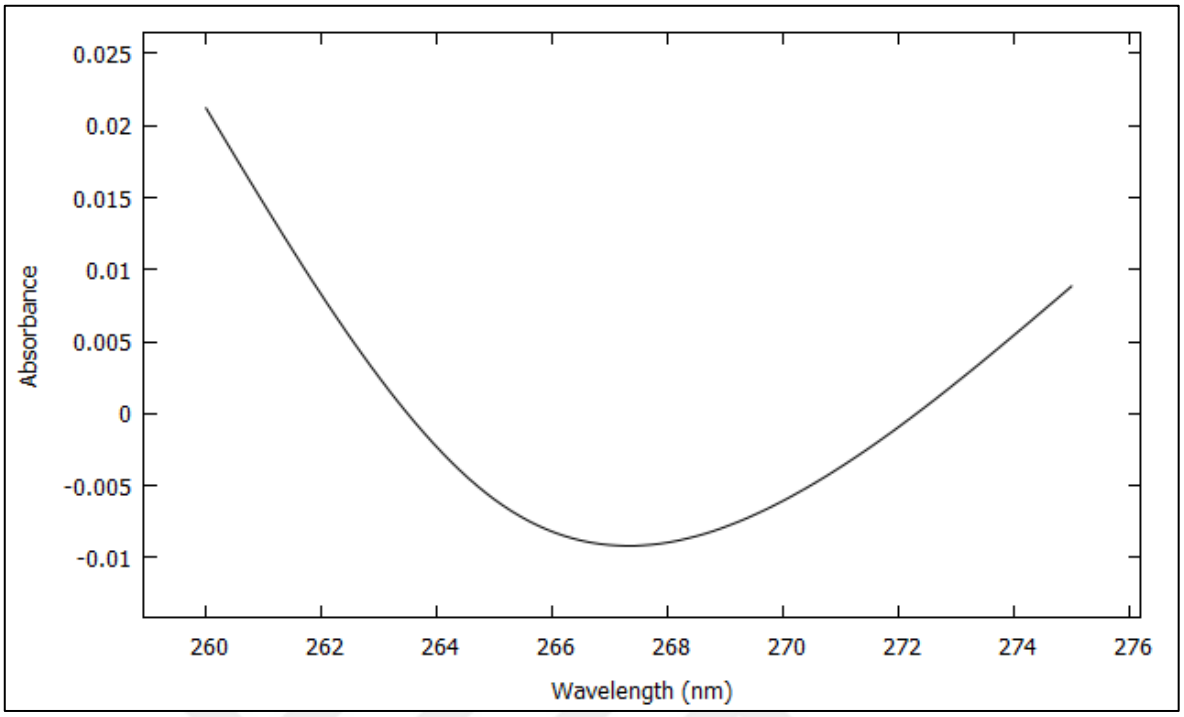

Figure 3.5. Absorbance and second derivative spectra of Phe between 260 and 275 nm

0.80 g of active carbon was added to 100 ml of 0.65% casein, 0.65% whey and skim milk samples which were hydrolased with protease enzymes derived from selected bacteria and also to 0.65% casein, whey and skim milk as a control. Samples stirred for 30 minutes at 25°C. After mixing, the solutions were centrifuged at 10,000 rpm for 10 minutes. Spectrophotometric measurements of the samples were taken from 250 to 280 nm.

3.2.11. ACE-Inhibitory Activity

Angiotensin-converting enzyme (ACE) activity assay was done by using a special kit. Enzyme B was dissolved with 2 ml of deionized water to prepare Enzyme B solution through septum with a syringe. Then 1.5 ml of Enzyme B solution was added to Enzyme A to prepare Enzyme working solution. Indicator working solution was prepared by dissolving Enzyme C and Coenzyme with 3 ml of deionized water each. 2.8 ml of Enzyme C solution and 2.8 ml of Coenzyme solution were added to Indicator solution to prepare Indicator working solution.

20 µl of sample solution was added to 96-well plate and 20 µl of deionized water to blank 1 and blank 2 wells and then, 20 µl of Substrate buffer was added to each well. 20 µl of deionized water was added to blank 2 well. 20 µl of Enzyme working solution was added to each sample well and blank 1 well and left to Incubate at 37°C for 1 hour. 200 µl of Indicator working solution was added to each well and Incubated at room temperature for 10 minutes. Absorbance measurement was done at 450 nm with a spectrophotometer. ACE inhibitory activity was calculated by the equation below.

$$\text{ACE inhibitory activity (inhibition rate \%)} = \left[\frac{(\text{Ablank 1} - \text{Asample})}{(\text{Ablank 1} - \text{Ablank 2})}\right] \text{X } 100 \qquad (3.2)$$

3.2.12. Antiradical Activity

A 23.66 mg of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in 30 ml of ethanol and kept at -20°C. until use. The samples were diluted 1:10, 1:25, 1:50 and 1:100 ratios. At the same time ascorbic acid was diluted in the same volume as positive control while distilled water was used as negative control. 600 µl from DPPH was added to each sample. 200 µl of the new mixture were added in 96-well plates and incubation was allowed for 30 minutes at room temperature and experiment was performed in dark conditions. At the end of the incubation, the spectrophotometric measurement was performed at 540 nanometers. Antioxidant activity measurements were calculated according to the following formula:

$$\text{Antioxidant Activity (\%)} = 100 - \{[(A_{Sample} - A_{Blank}) \text{ x } 100]/A_{Control}\} \qquad (3.3)$$

4. RESULTS AND DISCUSSION

4.1. SELECTION OF MICROORGANISMS

According to results shown in Figure 4.1. *Entercoccus faecalis, Paenibacillus lentimorbus, Staphylococcus aureus* and *Brevibacillus parabrevis* bacteria have lower protease activity then *Bacillus subtilis, Bacillus megaterium, Virgibacillus pantothenticus* and *Burkholderia gladioli* while *Zymomonas mobilis, Gluconacetobacter xylinus*, and *Micrococcus luteus* bacteria have no protease activity.

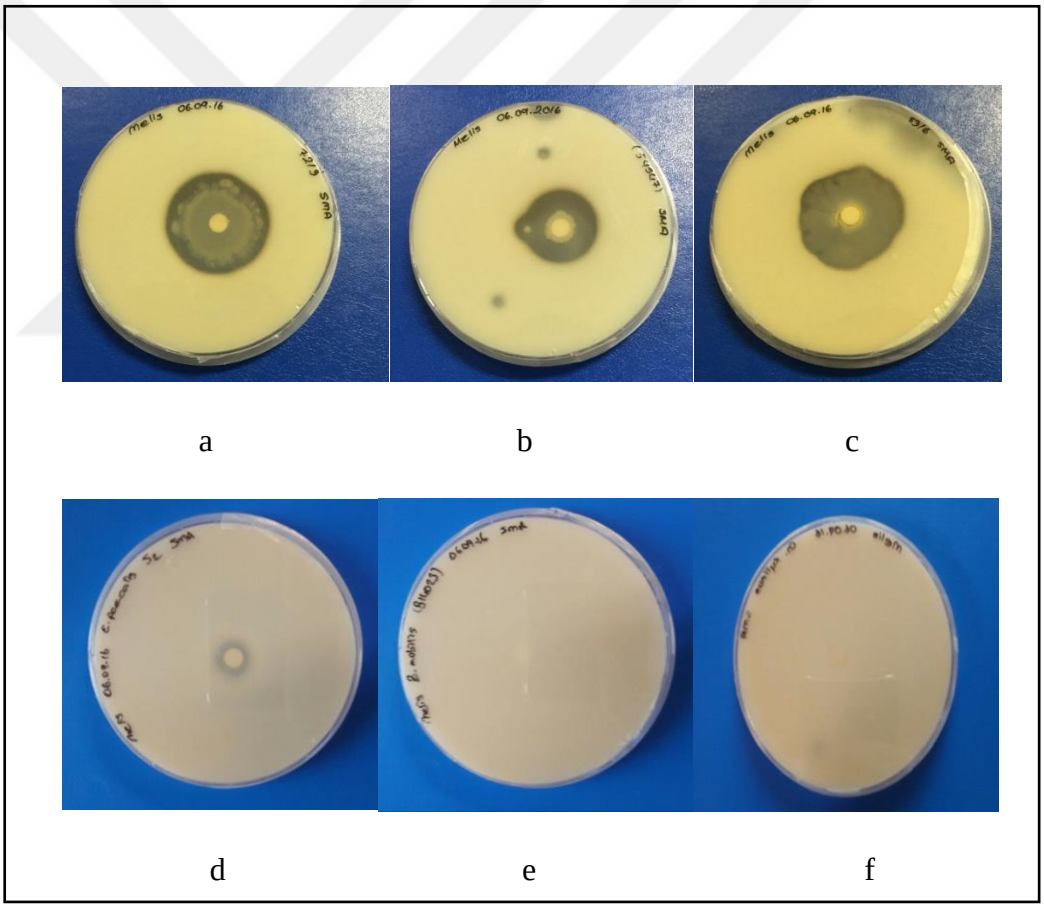

Figure 4.1. Protease activity screening of *Virgibacillus pantothenticus, Bacillus subtilis* and *Burkholderia gladioli, Entercoccus faecalis, Zymomonas mobilis*, and *Gluconacetobacter xylinus* as a,b,c,d,e and f, respectively.

Virgibacillus pantothenticus, Bacillus subtilis and *Burkholderia gladioli* were selected for further use of protease enzymes according to their high protease activity.

V.panthothenticus cells are long rods that form chains or filaments. Cells are mobile and Gram-positive. After 2 days of incubation, the colonies are 1-4 mm in diameter, low convex, circular and slightly irregular, grey and almost soft, egg shell or shiny profile. Organisms are facultative anaerobes. Growth of *V.panthothenticus* can be between 15 and 50°C and at an optimum level of about 37°C.

Burkholderia gladioli are motile, Gram-negative rod shape bacteria which are flat or curved. These are aerobic and non-spore former cells. *B. gladioli* has been known as plant pathogens, and it has been recently stated to cause infections in humans, too.

Bacillus megaterium is rod shaped, Gram-positive, aerobic spore-forming bacteria. *B. megaterium* is the best known bacteria with a cell length of up to 4 μm and a diameter of 1.5 μm. Cells are often found in pairs and chains, where cells are bound to cell walls by polysaccharides. *B. megaterium* is grown at temperatures between 3°C and 45°C and optimum at 30°C.

4.2. MICROBIAL GROWTH

Selected microorganisms were inoculated in nutrient broth medium and OD measurements were done in every 2 hours to detect their stationary phase, the state that protease enzyme is produced. It was determined that all three bacteria entered the stationary phase at about 16^{th} hour. Bacteria were collected at 18^{th} hour and centrifuged to get supernatant which includes extracellular protease enzyme besides other components; lipids, carbohydrates, proteins.

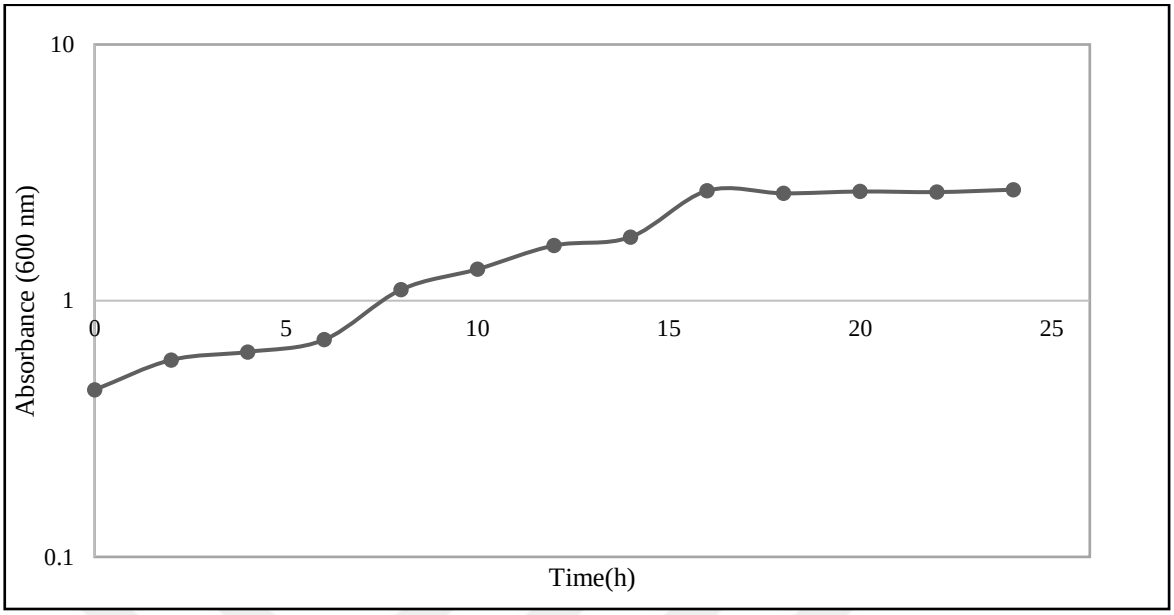

Figure 4.2. Growth curve of *Virgibacillus panthothenticus*

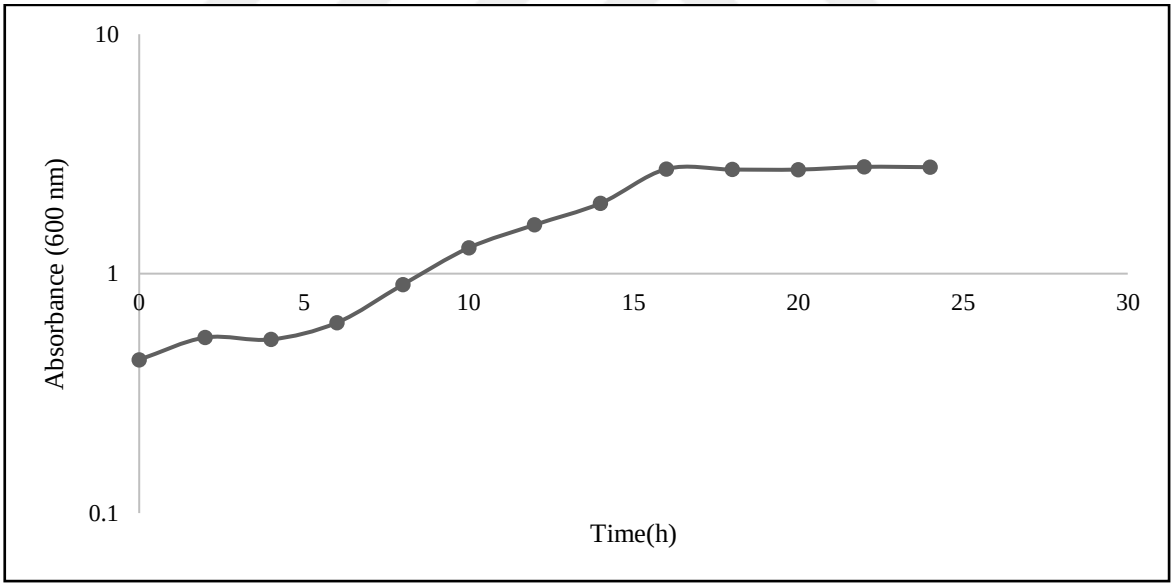

Figure 4.3. Growth curve of *Burkholderia gladioli*

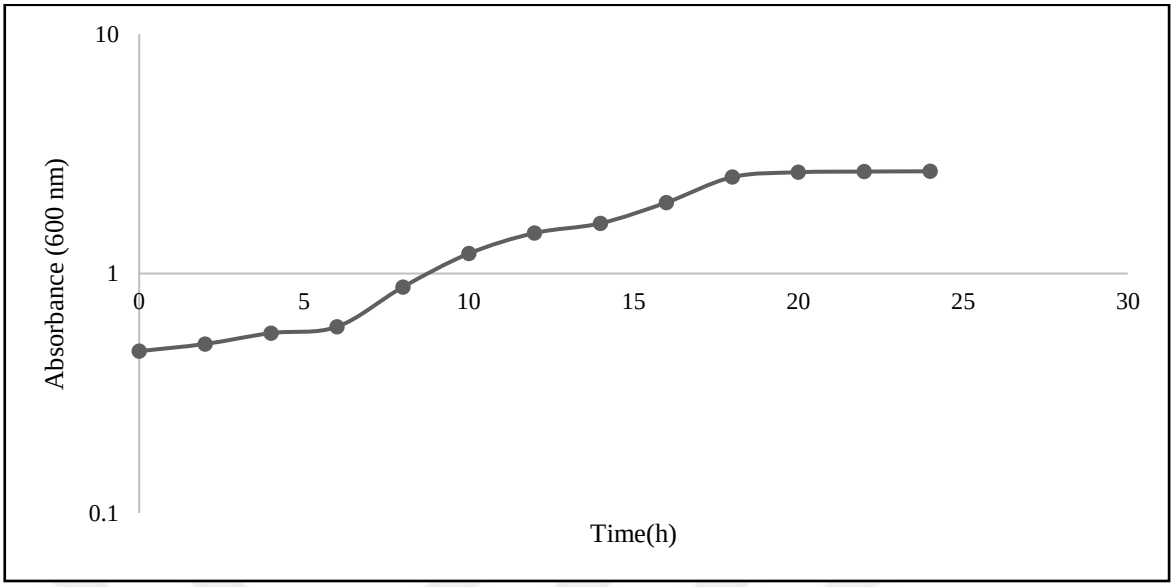

Figure 4.4. Growth curve of *Bacillus megaterium*

4.3. ACTIVITY OF PROTEASE ENZYME

According to Figure 2.1. the equation that is used to calculate L-tyrosine amount of samples was found as y=7.1469x with R^2 value of 0.9978 which shows this standard curve is acceptable.

Because this study was aimed used on the industrial area. Separation and purification processes that required excessive cost were refrained and the separation was limited to cell distinction only.

4.3.1. Substrate Effect on Protease Activity

Casein, a milk-derived protein, and BSA, an animal protein, were selected for the investigation of the substrate effect on protease activity. *V.panthothenticus* showed almost the same protease activity as 0.0435 U/ml and 0.0436 U/ml for casein and BSA respectively. *B.gladioli* and *B.megaterium* showed higher protease activity with casein substrate. Protease activities of *B.gladioli* were calculated as 0.0402 U/ml with casein and 0.0388 U/ml with BSA while for *B.megaterium* they were 0.0420 U/ml and 0.0368 U/ml.

The type and value of the structure, charge, polarity or hydrophobicity of the amino acids in the substrate binding site highly effect to substrate specificity. Therefore, the specificity may vary from enzyme to enzyme. For the substrate specificities of *V.panthothenticus* and *B.gladioli*, they were nearly the same for casein and BSA. On the other hand there was a significant difference between the casein and BSA specificities of *B.megaterium* protease. This distinction can be described as a disadvantage for the detergent industry. It is desirable that the detergent industry has different type of stains that can be removed. Only a casein degrading enzyme is insufficient to remove other substrate-derived stains. Because of this, the protease of *B.megaterium* is not suitable for the detergent industry.

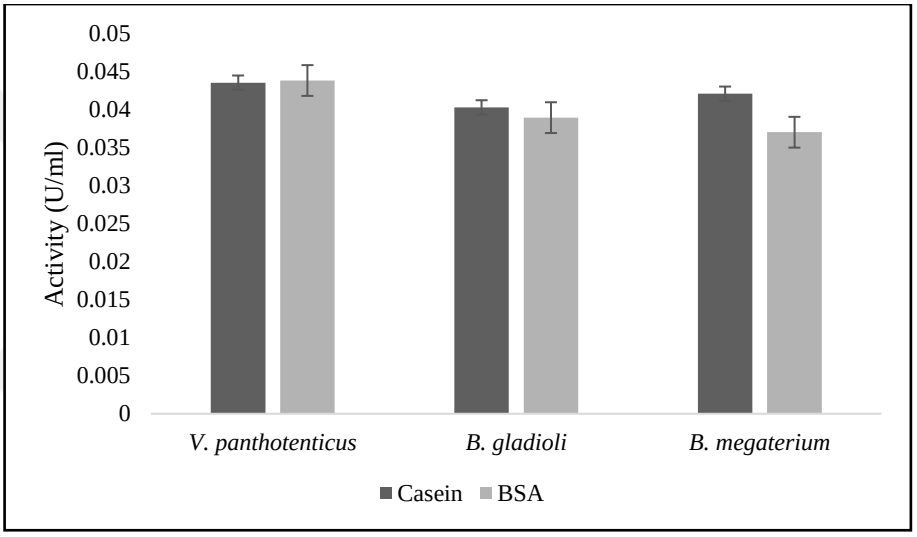

Figure 4.5. Substrate effect on protease activity

4.3.2. Temperature Effect on Protease Activity

It is known that generally proteases have maximum activity at about 40ºC. (3) Protease activity assay whose incubation temperature was 40ºC was used as control group and relative activities were calculated. The activity at this temperature was assumed to be 100%.

All three bacteria showed almost same pattern for protease activity based on different temperature. At 30°C, protease activities decreased as 7 – 10%, while at 20ºC, activities decreased as 38%. At high temperatures, such as 50°C, the changings of activities were

almost same as cold temperature as 20°C. Relative activities of proteases were around 30% at 10, 60, 70, and 80°C.

These enzymes have chance to use in industrial area within the temperatures of 20 to 50°C.

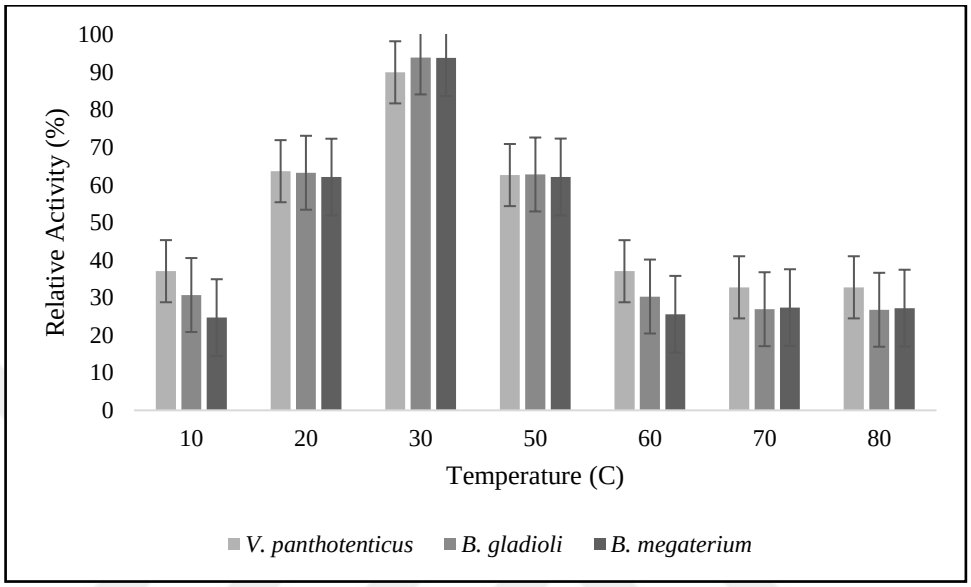

Figure 4.6. Temperature effect on protease activity

The protease enzymes of the three selected microorganisms are not suitable for the production of detergents intended to be used at high temperatures due to their low activity at high temperatures.

4.3.3. pH effect on Protease Activity

Activity assay which was done with Gly.NaOH buffer with pH of 9.2 was used as control group. Potassium phosphate buffer with pH of 7.2 was selected as neutral buffer while and succinate buffer with pH of 5.4 was selected as acidic buffer. For the characterization of protease enzymes of selected bacteria, effect of pH was examined.

V.panthothenticus showed higher relative activity at acidic conditions with 85.72% over 82.15%. For other two bacteria, neutral condition was more suitable. As acidity increased, activity of proteases derived from both *B.gladioli* and *B.megaterium* decreased. *B.gladioli* showed 96.73% at pH 7.2 and 82.80% at pH 5.4 while *B.megaterium* showed 92.84% and 78.20% respectively.

From the results, it has been observed that the use of the proteases obtained from these bacteria may be suitable for use in the medium with the aforementioned pH value since high activity declines are not observed.

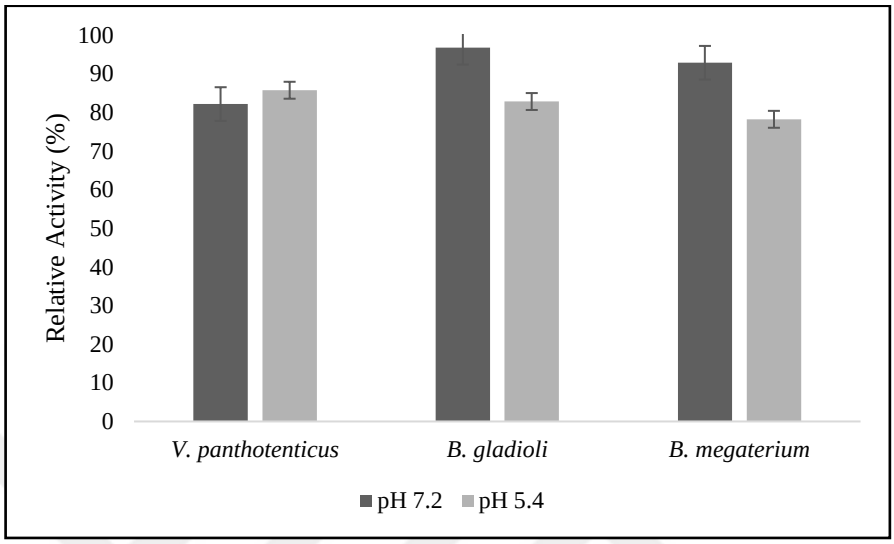

Figure 4.7. Effect of pH on protease activity

4.3.4. Inhibitor Effect on Protease Activity

Iodoacetamide was added in buffer to observe the inhibitory effect on protease activity but results showed that iodoacetamide has no effect on activities of proteases from all three bacteria. On the other hand, EDTA decreased the activities as around 20% of all proteases.

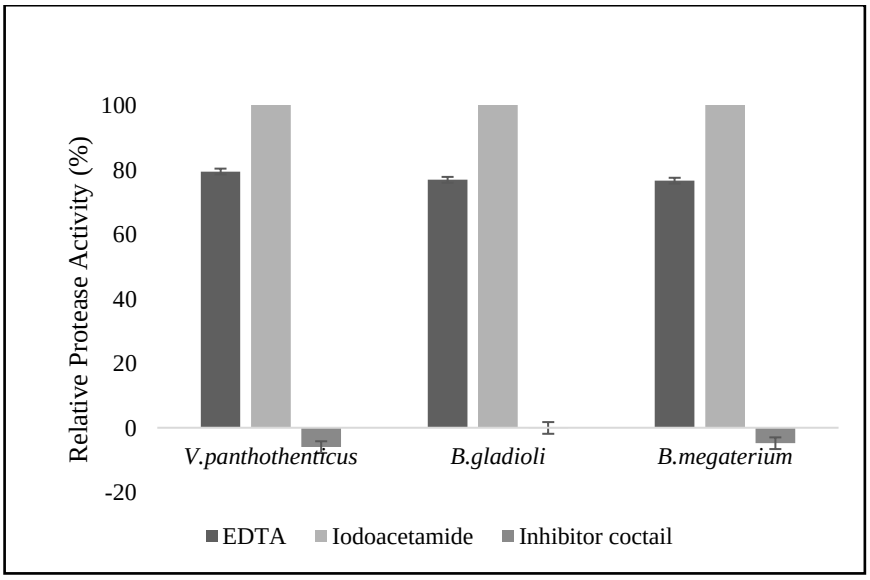

Figure 4.8. Effect of inhibitor on protease activity

4.4. PROTEIN FRACTIONATION

The amount of proteins separated into fractions after centrifugal filtration and the amount of undisrupted casein protein were found to be correlated with the amount of protein at the beginning.

The highest total protein ratio, ranging from 53-44%, was seen in size of above 100 kDa proteins. The fractions obtained with *B.megaterium* and *B.gladioli* enzymes between 100 and 50 kDa contain 16% of the total protein, while the fraction obtained with the *V.panthothenticus* enzyme contains approximately 11% of the total protein. Between 50 and 30 kDa, the total protein content of fractions obtained by enzymes of *B.gladioli* be *B.megaterium* decreased to 13%, while *V.panthotenticus* contained 11% of total protein again in this range.

In the range of 30 to 10 kDa, the *V.panthothenticus*, *B.gladioli*, and *B.megaterium* fractions have 9%, 8% and 6% of the total protein, respectively. In fractions with sizes between 10 and 3 kDa, the highest total protein content is attributed to 8% for *B.gladioli*, while for *B.megaterium* and *V.panthothenticus* this value is approximately 7%.

For fractions smaller than 3 kDa, the total protein ratios increased when compared to fractions between 10 and 3 kDa. An increase of about 3% was observed for the fractions obtained with the enzymes of all three bacterial species.

After degradation of casein by proteases, protein contents were higher in degraded casein than control below 100 kDa. Degradation results showed that proteases of *B.gladioli* and *B.megaterium* prepared high and low molecular weight peptides, while there was no significant distribution in proteases of *V.panthothenticus*.

Table 4.1. Protein amount of casein fractions in percentage

kDa Range	**Casein**	***V.panthothenticus***	***B.gladioli***	***B.megaterium***
>100	55.53	53.43	44.00	46.45
100-50	11.99	10.68	16.05	16.52
50-30	17.74	11.18	13.19	13.44
30-10	4.59	8.70	7.70	5.72
10-3	4.13	6.60	8.03	7.44
<3	6.02	9.42	11.02	10.43

4.5. REMOVAL OF PHENYLALANINE

Phenylalanine was removed from degraded products using active carbon. The removal efficiency was calculated by second derivative spectra method. Second derivative of standard solutions' absorbance values were calculated. By Gnuplot 5.0 software standard curve was drawn and equation of line was calculated as y=0.0014x + 0.0316 and R^2 value was 0.9154 which was acceptable.

Peak area between 260 and 275 nm was calculated and phenylalanine concentrations were calculated according to y=0.0014x + 0.0316. After treatment with active carbon, removal of phenylalanine was calculated as in the Table 4.1. below.

Table 4.2. Removal of Phe from whey, casein and milk hydrolysates in percentage.

Protease source	**Whey**	**Casein**	**Milk**
V.panthothenticus	78.198	72.398	69.759
B.gladioli	64.619	54.009	79.490
B.megaterium	57.898	51.184	53.356
Control group	0.709	1.316	3.295

Removing the phenylalanine from the milk was one of the main purposes of this study. It is aimed at milk and dairy products to be able to find their place in the diet of patients with phenylketonuria, supported by future studies. According to the results, phenylalanine was successfully removed in degraded products by the proteases of *V.panthothenticus*. The least disturbance was seen in products degraded by the enzyme obtained from the *B.megaterium* protease.

Phenylalanine was removed from the whey, casein and milk digested by the *B.gladioli* enzyme by 64.61%, 54.01% and 79.49%, respectively. Although the most successful removal of the phenylalanine in the study was obtained by degradation with the enzyme obtained from *B.gladioli*; it was in the second place for removal of phenylalanine from whey and casein.

B.megaterium protease was found to have the lowest removal rate of phenylalanine compared to the other two bacteria. Phenylalanine was removed at rates of 57.90, 51.18, and 53.35% from the whey, casein, and milk, respectively.

Finally, the highest yield was obtained in the removal of phenylalanine from substrates degraded by enzyme of *V.panthothenticus*. However, in the removal of the phenylalanine from the milk, the yield of 69.76% was obtained, remaining behind the *B.gladioli*.

Table 4.3. Removal of total protein from whey, casein and milk hydrolysates in percentage.

Protease source	**Whey**	**Casein**	**Milk**
V.panthothenticus	10.737	12.622	13.461
B.gladioli	11.668	13.498	18.559
B.megaterium	13.749	14.217	16.485
Control group	5.768	5.168	2.219

During the phenylalanine removal, total protein loss was also determined. The highest loss was observed in the milk for all three bacteria. This value for *B.gladioli* was the highest among the three bacteria with value of 18.56%, while for *B.megaterium* and *V.panthothenticus* the loss was calculated to be 16.49% and 13.46% respectively.

Protein lost in casein and whey was different from milk. Protein lost was around 12 – 14% in casein and after whey degradation; protein lost from whey was 2% lower than casein.

Total protein removal was higher in *B.megaterium* protease while *B.gladioli* protease showed higher protein removal in milk than others.

Active carbon removes aromatic amino acids such as tyrosine and tryptophan as well as phenylalanine. The total losses there can be comprised of the sum of the amounts of the three aromatic amio acids mentioned above. The reason why the value losses are different from each other is that the peptide contents of the protein sources are different from each other and when they are disrupted by 3 different enzymes, they may be extracted from different peptides and amino acids without cutting from the same regions. However, the controls also suffered protein loss of 5.76%, 5.16% and 2.21% for the whey, casein and

milk, respectively. Impurities in protein sources and applied heat treatments may have affected this situation.

Lopez and his group's study showed that using 0.8 g of activated carbon to remove phenylalanine and incubation at room temperature for 30 minutes resulted in 91% success. For this reason, the same values were chosen in this study. But in our study a maximum removal rate of 79% was achieved.(93)

4.6. ACE INHIBITORY ACTIVITY

To observe the ACE inhibitor effect, fractions of 3 to 10 kDa and smaller than 3 kDa of the casein and milk peptides removed from the phenylalanine were selected. In the fractions between 3 and 10 kDa of the milk peptides, it was seen that the most activity with 82.01% was in peptides generated by hydrolysis with protease enzyme of *B. megaterium*. *B. megaterium* was followed by *V. panthothenticus* and *B. gladioli* with activities of 76.84% and 73.80%, respectively.

When it comes to casein peptides, the highest activity among the peptides between 3 and 10 kDa was attributed to the peptide degraded by the protease of *B. gladioli* with 74.81%. The results obtained from *B. megaterium* showed a very close activity to *B. gladioli* with 73.06%. Among the selected bacteria, the lowest activity has been observed in peptides that are involved in the cleavage of the *V. panthothenticus* derived protease by 68.08%.

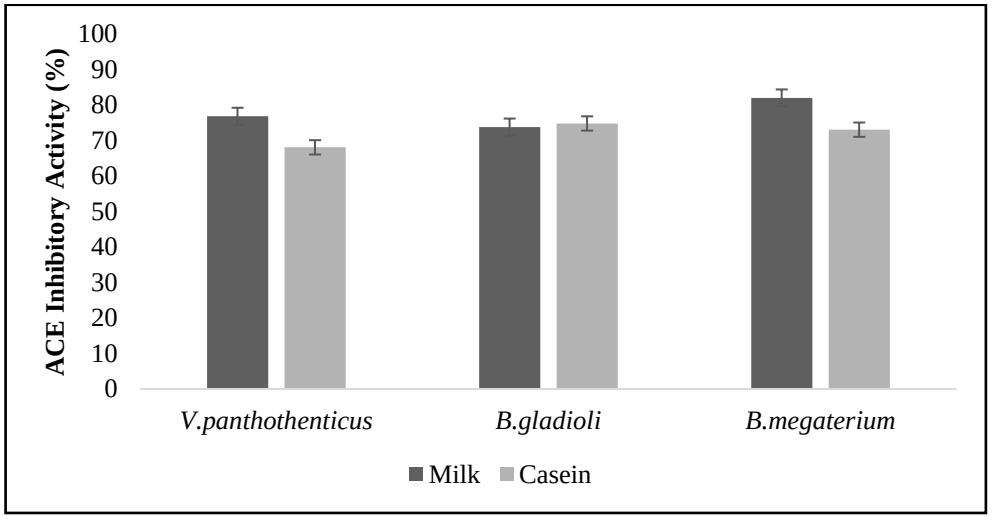

Figure 4.9. ACE inhibitory effect of peptides with a molecular weight between 3 and 10 kDa.

In the smaller than 3 kDa sizes of casein peptides, the highest ACE activity is again found in *B. gladioli* with 65.1%, followed by *B. megaterium* with 54.9% and *V. panthothenticus with* 49.08%. In same sizes of milk-derived peptides, the highest activity was observed in *B. megaterium* with 74.17%. *B. megaterium* was followed by *V. panthothenicus* with a close activity of 72.97%. *B. gladioli* showed the lowest activity with 64.11%.

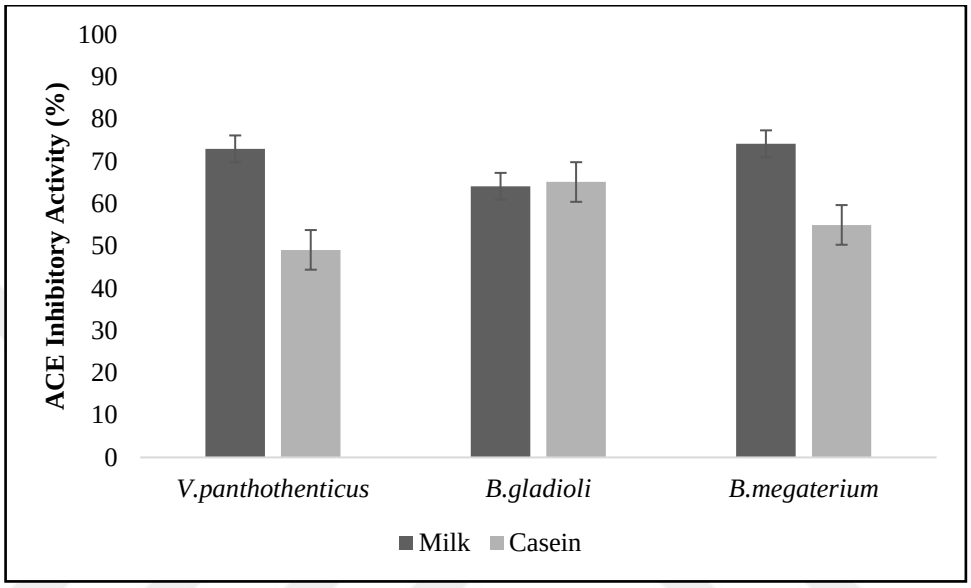

Figure 4.10. ACE inhibitory effect of peptides with a molecular weight below 3 kDa.

The ACE inhibition of bioactive milk hydrolysates between 10 and 3 kDa eluted by fractionation varied between 82% and 74%. This result is close to a study in which the <1 kDa fraction of an Alaskan pollock protein hydrolyzate has the highest ACE inhibitory activity (87.6%). For casein hydrolysates, the activity varies between 74% and 68% with lower values than the milk hydrolysates [92].

In one study, the ACE inhibition activity was elevated when the kDa of hydrolyzates were decreased. On the contrary, in our study, ACE inhibition activities of hydrolysates smaller than 3 kDa are lower than hydrolysates of higher kDa [95].

Table 4.4. ACEI peptides isolated from bovine β-casein [96]

Matrix	**Peptidic sequence**
Casein hydrolysate obtained by proteolysis with extra cellular protease from *Lactobacillus helveticus* CP790	Lys-Tyr-Pro-Val-Gln-Pro-Phe-Thr-Glu-Ser-Gln-Ser-Leu-Thr-Leu
	Ser-Lys-Val-Leu-Pro-Val-Pro-Glu
	Pro-Pro-Gln-Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Glu
	Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe
	Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val
	Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val-Ser-Lys
	Leu-Leu-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val
	Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser
Casein hydrolysate incubation with protease	Lys-Val-Leu-Pro-Val-Pro-Gln
	Leu-Gln-Ser-Trp
	Arg-Glu-Leu-Glu-Glu-Leu
	Leu-Leu-Tyr-Gln-Gln-Pro-Val
Hydrolysis mediated by PR4 protease extracted from Lactobacillus helveticus	Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Glu-Asn-Ile-Pro-Pro
Cow milk	Val-Pro-Pro
	Asn-Ile-Pro-Pro-Leu-Thr-Glu-Thr-Pro-Val
	Val-Pro-Pro-Phe-Leu-Glu-Pro

There are numerous ACEI peptides belonging to cow-based, isolated and described, such as goat's milk. These peptides are released from protein precursors during transit through the gastrointestinal tract.

While the functional-structural relationship of ACEI peptides is still unclear, these peptides appear to exhibit a number of common properties. The enzyme conjugation is strongly

controlled by the tripeptide C-terminal sequence of the peptides which caused the ACE has more affinity for competitive inhibitors with hydrophobic amino acids. This principle applies to very active short chain peptides.

4.7. ANTIRADICAL ACTIVITY

In the investigation of antiradical activity, for the peptides between 3 kDa to 10 kDa showed higher activity for the peptides derived from milk than the casein for all three bacteria. Highest activity was belong to *V.panthothenticus* 77.3% followed by B.megaterium with 70.4% and *B.gladioli* 53.5% for milk peptides; while, for the peptides obtained from the casein *B. megaterium* showed the highest activity with 68.3%. *V. panthothenticus* was in second place with 61.6% activity and *B. gladioli* had lowest activity with 49.8%.

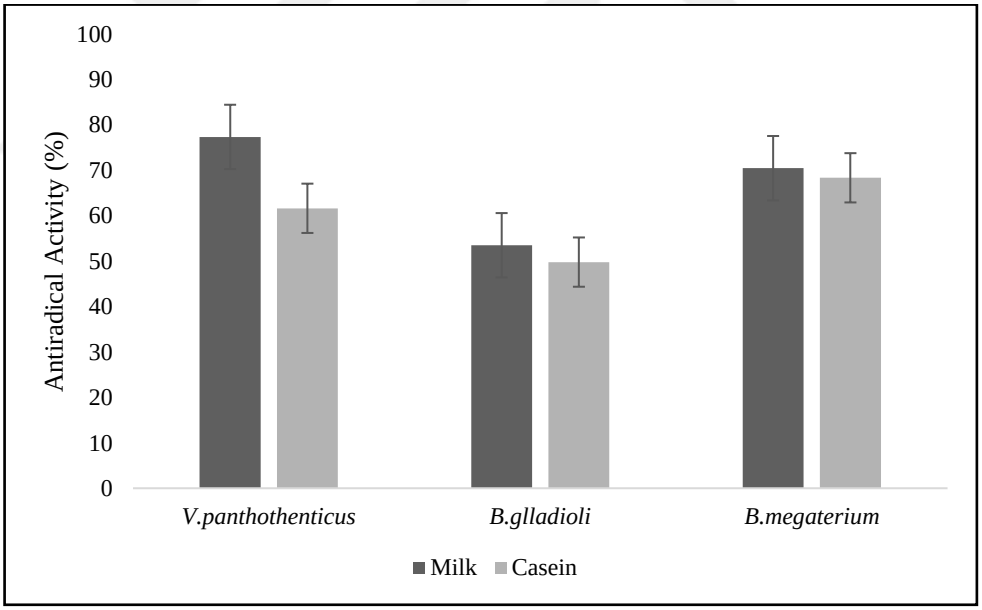

Figure 4.11. Antiradical activity of peptides with a molecular weight between 3 and 10 kDa.

In the smaller than 3 kDa sizes the highest ACE activity was shown in *V. panthothenticus* with 75.99% for the milk derived peptides while *B. gladioli* showed 64% activity and *B. megaterium* showed 62.58% activity. In same sizes of casein derived peptides, the highest activity was observed in *B. gladioli* with 67.30%. *B. gladioli* was followed by *B.*

megaterium with a close activity of 67.44%. *V. panthothenticus* showed the lowest activity with 57.91%.

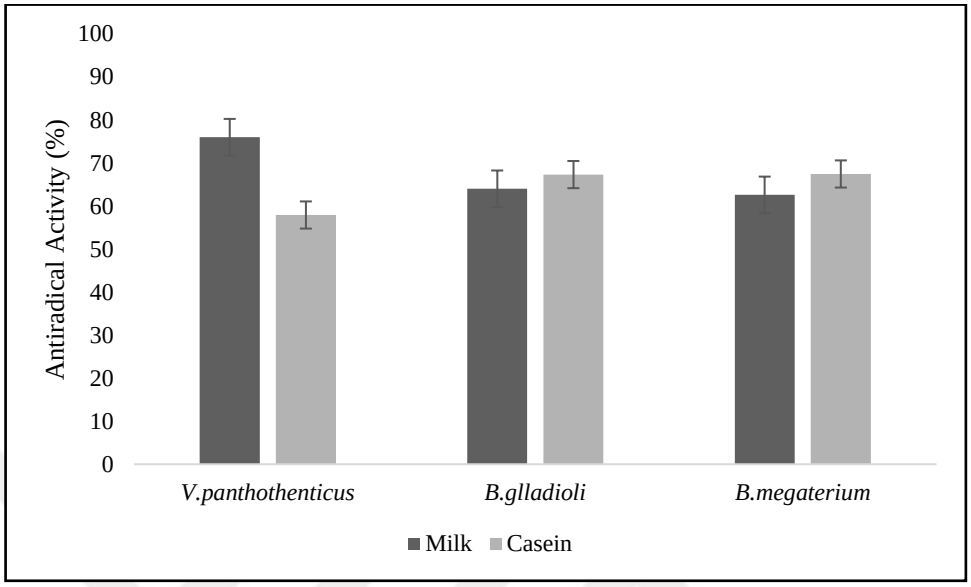

Figure 4.12. Antiradical activity of peptides with a molecular weight below 3 kDa.

In a similar study, by DPPH assay, the scavening activity of the goat milk hydrolysates derved by *Bacillus spp.* was measured, and the most active peptide was a fraction smaller than 3kDa at a ratio of about 20%. The 30 kDa, 10-30 kDa and 3-10 kDa peptide fractions for both 30 and 60 min hydrolyzate had similar scavening activity at about 10%. In this study it was seen that the scavenging activities were higher for each kDa interval than the activity obtained from aforementioned study.

5. CONCLUSION AND FUTURE PERSPECTIVES

Milk has been shown to be an important source of natural bioactive components for human nutrition and health. The bioactive peptides are released by proteases during hydrolysis. Researches have proved that these bioactive peptides have different physiological functions.

In this thesis, three different bacteria, *V. panthothenticus*, *B. megaterium*, and *B.gladioli*, among several different bacteria were selected for protease production to obtain peptides in different fragments than milk and milk proteins. Protease activities were visualized by skimmed milk agar method.

In our study, protease activity was the highest at 40°C, but at 30°C, results were close to those obtained at 40°C. At high temperatures, protease of *V. panthothenticus* was found to be more tolerant to high temperatures than proteases produced by other bacteria. In our study, when the effect of different pH values on the enzyme activity was observed, it was observed that the activity of the protease increased as pH value was increased.

When the activity of the proteases produced is examined in the detergent presence activities, it is determined that the activities do not decrease too much and these enzymes are compatible with the usage of the detergent industry. Although the presence of hydrogen peroxide does not cause low hydrolysis activity in enzymes, the negative interaction of enzymes with SDS, which is frequently used in detergents, restricts the use of enzymes in the detergent industry.

Proteins were digested with unpurified enzymes and then fractionated by centrifugal filtration. Although, the percentage of hydrolysis is unknown, the fraction of protein obtained after fractionation corresponds to the amount of protein at the beginning. For this reason, it can be said that the degradation process has been performed successfully. In subsequent studies, degree of hydrolysis determination methods can be used to calculate the hydrolysis activity of these enzymes, and degradation can be developed.

The produced bioactive peptides have been found to have antiradical activities. Promising results have also been obtained when the ACE inhibitor effect is observed and these peptides can be used in the treatment of cardiovascular diseases in the future.

When it was desired to remove the phenylalanine, the success rate was high. If this result is developed, it can be regarded as a beginning to ensure that patients with phenylketonuria can use milk and dairy products.

In this context, scientifically demonstrating the antibacterial, antiviral, anticancerogenic, antioxidant and hypocholesterolemic properties of bioactive peptides with biological activity and serum proteins with physiological activity indicate that they will be indispensable components of both new and currently consumed foods in the future.

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APPENDIX A: LIST OF MICROORGANISMS

Table 6.1. List of microorganisms in which protease activity is determined

Number	Collection Code	Species	Protease Activity
1	1,2,3	*Exiguobacterium indicum*	++++
2		*Bacillus cereus*	++++
3	As 12	*Bacillus subtilis*	++++
4	72.3	*Virgibacillus pantothenticus*	++++
5	As 9	*Virgibacillus pantothenticus*	++++
6	83.7	*Paenibacillus polymyxia*	++++
7	As 10	*Paenibacillus lentimorbus*	++++
8	5 YS 37	*Brevibacillus parabrevis*	++++
9	5 YS 47	*Pseudomonas gladioli*	++++
10	5 YS 39	*Bacillus flexus*	++++
11	49.4		++++
12	26.3		++++
13	36.4		++++
14	As 13		++++
15	27.1		++++
16	72.2		++++
17	49.1		++++
18	74.2		++++
19	77.3		++++
20	35.3		++++
21	1.1.5		++++
22	Fc 4		++++
23	33.3		++++
24	3.2.2		++++
25	1.1.1		++++
26	42.2		++++
27	15.1		++++
28	As 5		++++

29	26.2		++++
30	5M4		++++
31	54.3		++++
32	24.2.1		++++
33	1 YS 32		++++
34	1 YS 33		++++
35	1 YS 39		++++
36	1 YS 49		++++
37	1 YS 50		++++
38	1 YS 52		++++
39	1 YS 55		++++
40	2 YS 54		++++
41	2 YS 55		++++
42	2 YS 63		++++
43	2 YS 72		++++
44	2 YS 75		++++
45	2 YS 79		++++
46	5 YS 48		++++
47	6 YS 1		++++
48	6 YS 17		++++
49	7 YS 63		++++
50	8 YS 20		++++
51	8 YS 21		++++
52	8 YS 22		++++
53	1,2,4	*Bacillus cereus*	+++
54	Fc 13	*Bacillus subtilis*	+++
55	Fc1	*Bacillus cereus*	+++
56	75.7	*Bacillus cereus*	+++
57	Fc 3	*Bacillus subtilis*	+++
58	77.4	*Bacillus cereus*	+++
59	As 16	*Bacillus megetarium*	+++
60	1 YS 57(77.4)	*Bacillus cereus*	+++

61	83.9		+++
62	2.2.2 sarı		+++
63	27.3		+++
64	58.5		+++
65	Fc 10		+++
66	16.6		+++
67	62.1.a		+++
68	39.2.5		+++
69	83.3		+++
71	10.5		+++
72	As 8		+++
73	As 2		+++
74	1 YS 25		+++
75	1 YS 53		+++
76	1 YS 80		+++
77	2 YS 42		+++
78	2 YS 58		+++
79	2 YS 60		+++
80	2 YS 77		+++
81	3 YS 4		+++
82	3 YS 53		+++
83	4 YS 33		+++
84	4 YS 57		+++
85	4 YS 78		+++
86	5 YS 54		+++
87	6 YS 10		+++
88	6 YS 21		+++
89	6 YS 25		+++
90	6 YS 27		+++
91	6 YS 78		+++
92	7 YS 61		+++
93	1,2,1	*Paenibacillus polymyxia*	++

94		*Bacillus subtilis*	++
95		*Bacillus pumilis*	++
96	83.6	*Bacillus megetarium*	++
97	X	*Bacillus subtilis*	++
98	6 YS 37	*Pseudomonas putida*	++
99	93.3		++
100	77.6		++
101	28.5		++
102	72.09		++
103	47.1		++
104	61.5		++
105	59.2		++
106	57.1		++
107	8.2		++
108	Fc 17		++
109	28.5		++
110	CER KH 4	*Ewingella americana*	+
111	FK 24	*Bacillus pumilis*	+
112	1 YS 28		--
113	1 YS 29		--
114	1 YS 30		--
115	1 YS 31		--
116	1 YS 34		--
117	1 YS 37		--
118	1 YS 38		--
119	1 YS 40		--
120	1 YS 44		--
121	1 YS 51		--
122	1 YS 63(75.1)		--
123	1 YS 65(AA 36)		--
124	1 YS 66		--
125	1 YS 67		--

126	1 YS 68		--
127	1 YS 69		--
128	1 YS 72		--
129	2 YS 1		--
130	2 YS 3		--
131	2 YS 4		--
132	2 YS 5		--
133	2 YS 6		--
134	2 YS 7		--
135	2 YS 8		--
136	2 YS 9		--
137	2 YS 10		--
138	2 YS 11		--
139	2 YS 15		--
140	2 YS 48		-
141	2 YS 49		-
142	2 YS 51		-
143	2 YS 52		-
144	2 YS 53		-
145	2 YS 56		-
146	2 YS 59		-
147	2 YS 61		-
148	2 YS 62		-
149	2 YS 64		-
150	2 YS 65		-
151	2 YS 71		-
152	2 YS 73		-
153	2 YS 74		-
154	2 YS 78		-
155	3 YS 1		-
156	3 YS 2		-
157	3 YS 6		-

158	3 YS 7		-
159	3 YS 8		-
160	3 YS 9		-
161	3 YS 10		-
162	3 YS 12		-
163	3 YS 13		-
164	3 YS 14		-
165	3 YS 15		-
166	3 YS 16		-
167	3 YS 17		-
168	3 YS 18		-
169	3 YS 19		-
170	3 YS 20		-
171	3 YS 21		-
172	3 YS 22		-
173	3 YS 23		-
174	3 YS 25		-
175	3 YS 26		-
176	3 YS 27		-
177	3 YS 28		-
178	3 YS 29		-
179	3 YS 30		-
180	3 YS 31		-
181	3 YS 32		-
182	3 YS 33		-
183	3 YS 34		-
184	3 YS 35		-
185	3 YS 36		-
186	3 YS 37		-
187	3 YS 38		-
188	3 YS 39		-
189	3 YS 40		-

190	3 YS 41		-
191	3 YS 42		-
192	3 YS 46		-
193	3 YS 48		-
194	3 YS 49		-
195	3 YS 50		-
196	3 YS 51		-
197	3 YS 54		-
198	3 YS 55		-
199	3 YS 56		-
200	3YS 57		-
201	3 YS 59		-
202	3 YS 61		-
203	3 YS 63		-
204	3 YS 64		-
205	3 YS 65		-
206	3 YS 66		-