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Master's Degree Thesis

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Advisor: Professor Jin Cheol Yoo, PhD

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TABLE OF CONTENTS

CHAPTER 3:RESULTS AND DISCUSSIONS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

GRAS	Generally Regarded As Safe
AMP	Anti-microbial Peptide
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
LPS	Lipopolysaccharide
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
RPM	Rotation per minute
kDa	Kilo Dalton
SDS-PAGE	Sodium Dodecyl Sulfate –Polyacrylamide Gel Electrophoresis
CFU/ML	Colony Forming Unit/Mililitre
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol tetra acetic acid
SDS	Sodium dodecyl sulfate
CHAPS	3- [(3- Cholamidopropyl)-dimethylammonio]-1-propanesulfonate
NCBI	National Centre for Biotechnology Information

ABSTRACT

A novel antimicrobial peptide from *Bacillus* strain exhibiting therapeutic potential against vancomycin resistant *Enterococcus* group of organisms.

Sandesh Panthi

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Antimicrobial peptides are widely distributed and have been found in organisms ranging from prokaryotes to plants, insects and mammals. To protect themselves from microbes, microorganisms including bacteria secrete antimicrobial peptide. They are generally considered as a part of the innate immune system and rapidly increase in concentration in the host upon challenge by pathogens. *Bacillus* is an interesting genus to be investigated for antimicrobial activity because these species produce a large number of peptides with biological activities. Kimchi is found to be perfect for antimicrobial peptide production which is a traditional Korean food fermented by various organisms such as *Bacillus and Lactobacillus*. The aim of this work was to investigate the therapeutic potential of antimicrobial substances produced by *Bacillus* species. *Bacillus* strain K1R was amended from bacterial supernatant obtained from fermentation in culture media containing carbon and nitrogen sources which is therefore called optimized media for that particular strain and then by the application of ammonium sulfate precipitation, concentration (membrane filters), dialysis, sequential column chromatography, AMP was obtained. To determine the purity and molecular weight, the purified peptide was subjected to tricine-sodium dodecyl sulfate–

polyacrylamide gel electrophoresis. Protein band around 4.6 kDa was seen in tricine-SDS-PAGE which was confirmed by in situ inhibitory activity of the gel. K1R was found to be stable over a broad range of pH (6.5-9) and thermo tolerant up to 60˚C and unaltered activity at low temperature of 0˚C and 4˚C. K1R showed better result than vancomycin and bacitracin against different MDR pathogens particularly on vancomycin resistant *enterococcus* group of organisms. Amino acid sequence of K1R was AVQGTLEDALNLSKGALNQVQKAIQNGDXLTVXGXLGTIXL AVSX. Its antagonistic effect against MDR pathogens like *Salmonella typhimurium* and *Enterococcus* sp. verified that K1R has potential application in treating infectious MDR cases. Antioxidant activity of K1R was also comparable to that of standard ascorbic acid.

국문초록

반코마이신 내성장 구균을 저해하는 바실러스 유래 신규 항세균 펩타이드

판띠산데쉬

지도교수: 유진철

조선대학교대학원약학과

항균펩타이드는원핵생물에서식물, 곤충과포유류에이르기까지다양한생명체에서발견된다. 병원균으로부터스스로를보호하기위하여박테리아를포함하는미생물은항균펩타이드를분비한다.이는선천면역시스템의일부로서병원균이침입하였을때, 숙주의체내에서그농도가급격이증가한다. Bacillus 는항균활성연구에있어서흥미 로운 속에속하는데, 이는생물학적활성을 가지는수많 은펩타이를생 산하기때문이다. 대표적인예로김치는, Bacillus 나 Lactobacillus 와같은다양한미 생물에의해발 효된한국의전통식품으로서, 항균펩타이드생산에최적인 조건을 나타내는것 으로 밝혀졌다. 본연 구의목적은, Bacillus 종에의해생산된항미생물의치 료적효능을조사하고자하였다. Bacillus 속균주인 K1R 은탄소와질소공급원을 포함하는세포 배양액의발효에의해얻어진박테리아상등액으로부 터되었고이는이균주에최적화된배지조 건이라고할수있으며황산알루미늄침전 법, 멤브레인필터 를통한농축, 여과, 일련의컬럼크로 마토그래피, AMP 에의하여획득되었다. 순도와 분자량을결 정 하기위해서정제한펩타이 드를 Tricine SDS-PAGE 겔에전기영동하였다. 단백질밴 드는 Tricine SDS-PAGE 겔에서약 4.6 kDa 에서확인되었고, 이는전기 영동된겔의항균활 성검사 를통하여확 정되었다. K1R 은넓은범위의 pH(6.5~9) 에서도안정적인것 으로밝혀졌으며, 60℃까지열내성을 가지며 0℃및

4℃의낮은온도에서도활성이변하지않았다. 특히, K1R 은반코마이 신내성장내구 균 에서 MDR 병원균에대하여반코마이신이나바시트라신보다더나은결과를보여주었다. K1R 의아미 노산서 열은 AVQGTLEDALNLSKGALNQVQKAIQNGDXLTVXGXLGTIXLAVSX 이었다.또한, K1R 은살모넬라나 장구균등의 MDR 병원균에대해서도저해효과를보였는데이는 K1R 이감 염성 MDR 의치료에 잠재적 인응용 가치가있음 을의미 한다. 이와더불어, K1R 의항산화활 성효과는아스코 르브산과비슷한수준이였다.

CHAPTER 1: INTRODUCTION

1.1 *Bacillus* sp.

Bacteria are mostly simple in form and exhibit one of three basic structures and *Bacillus* is one of them. Other two are coccus and spirillus [1]. *Bacillus* species are rod-shaped, endospore-forming obligate aerobes or facultative anaerobes, Gram-positive bacteria; in some species cultures may turn Gram-negative with age. The many species of the genus exhibit a wide range of physiologic abilities that allow them to live in every natural environment and a member of the phylum Firmicutes [2].

Bacillus species continue to be dominant bacterial workhorses in microbial fermentations.They are on the Food and Drug Administration's GRAS list. The capacity of selected *Bacillus* strains to produce and secrete large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers. The ability of different species to ferment in the acid, neutral, and alkaline pH ranges, combined with the presence of thermophiles in the genus, has lead to the development of a variety of new commercial enzyme products with the desired temperature, pH activity, and stability properties to address a variety of specific applications [3]. *B. subtilis* constitutes a highly versatile and tractable model organism for the study of generic stress responses and the expertise that has been gained can easily be transferred to the study of the cellular physiology of related Gram-positive pathogens and their patho-physiology [4].

1.2 Peptides

Peptides come from the Greek word which means “to digest". They are naturally occurring biological molecules. They are short chains of amino acid monomers linked by peptide (amide) bonds. The covalent chemical bonds are formed when the carboxyl group of one amino acid

reacts with the amino group of another. The shortest peptides are di-peptides, consisting of 2 amino acids joined by a single peptide bond, followed by tri-peptides, tetra-peptides, etc. Antimicrobial peptides are secreted by a wide range of microorganisms, including *Bacillus* sp., to protect themselves from other microbes. These peptides are composed of 12-50 residues polypeptides and affect the active element of the innate immune response. Also, AMPs have been confirmed to kill Gram-negative and Gram-positive bacteria including important clinical pathogens, mycobacteria, protozoa, viruses, fungi, and cancer cell [5]. In the response of their innate immunity, wide varieties of organisms produce biologically active antimicrobial peptides. AMPs, due to their cytotoxic nature, they provide host defense invading pathogenic microorganisms and they also serve as immune modulators in higher organisms [6]. AMPs are considered as a promising and potential drug candidate for the future due to their broad range of activity, lesser toxicity, and decreased resistance development by the target cells [7].

1.3 Classification of peptides

On the basis of synthesis, these peptides fall in two classes i.e. nonribosomally synthesized and ribosomally synthesized antimicrobial peptides [8]. Nonribosomally synthesized peptides are class of peptide secondary metabolites, synthesized by nonribosomal peptide synthetases generally produced by bacteria and fungi. These are independent of messenger RNA. Each nonribosomal peptide synthetase can synthesize only one type of peptide. These peptides have broad range of biological activities and pharmacological properties. Nonribosomal peptide antibiotics (actinomycin, bacitracin, daptomycin, vancomycin etc.), cytostatics and immunosuppressants drug are in commercial use.

Ribosomally synthesized peptides are produced by mammals, birds, amphibians, insects, plants, and microorganisms and affect crucial components of their defense systems against

microorganisms. These gene encoded peptides share a common physico-chemical property. Such as being small, cationic, amphiphilic, and often being membrane active. Even though they vary in structure, they are cationic and amphiphilic, which indicates the fact that many of them attack target cells by permeabilizing the cell membrane [9]. The action mechanisms have been investigated for some antimicrobial peptides, including defensin [10], maganin [11], nisin [12], cathclicidin and histatin [13]. Most AMPs do not target specific molecular receptors of pathogens but rather interact with each other and permeabilize microbial membrane [14].

Till now, over 2000 AMPs have been described [15]. The activity of AMPs was first demonstrated during 1950, when it was found that cationic proteins were responsible for the capability of neutrophils to kill bacteria based on oxygen-independent mechanisms [16-17].

On the basis of their structure, antimicrobial peptides are classified into four major groups: amphipathic α-helical, β-sheet, β-hairpin or loops, and extended AMPs [18-20]. Amphipathic α-helical AMPs comprise pardaxin [21], dermaseptin [22], and the extensively studied LL-37 AMP [23]. These peptides are amphipathic in nature, can form α- helical structures in the presence of model membranes or in aqueous solution. AMPs that are stabilized by the formation of disulfide bonds and are characterized by the presence of two or more β-strands are β-sheet. This class includes α-, β-, and θ-defensins [24-25]. Loops or β-hairpin AMPs are highly stable peptides that exhibit a hairpin structure interconnected by a type II β-turn. Their stability is due to the disulfide bonds formed between the β-strands. Bactenecin [26], dodecapeptide [27], and tachyplesins [28] are examples of this class of antimicrobial peptides. And, extended AMPs are peptides which are rich in histidine, arginine, or glycine and that lack a secondary structure. Examples include indolicidin, histatins [29] etc.

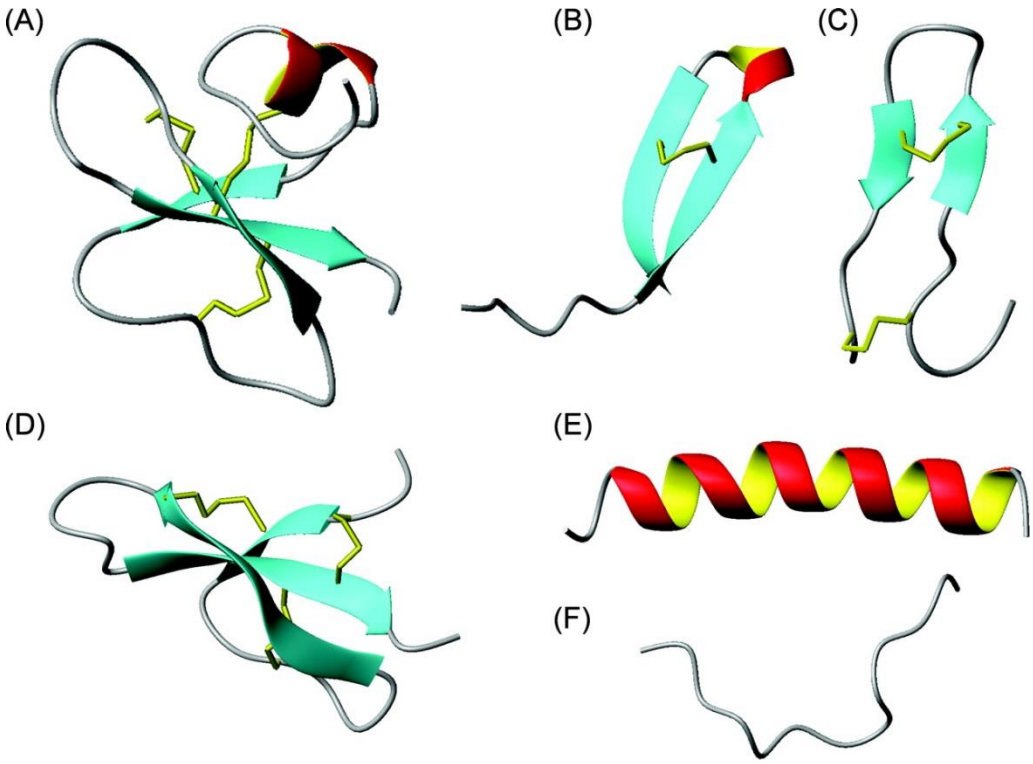


Figure 1. Structural classes of antimicrobial peptides.

(A) Mixed structure of human defensin-2 (B) looped thanatin (C) β-sheeted polyphemusin (D) rabbit kidney defensin-1 (E) α-helical magainin-2 (F) extended indolicidin. [30].

1.4 Source of peptides

AMPs have been reported from different sources such as plants, mammals, insects and marine invertebrates other than microorganisms. Cecropin A, Sarotoxin IA, ponericin G2, ceratotoxin, stomoxyn, spinigerenin, thanatin, heliomicin, sapecin, defensin A and gallerimycin are the AMPs obtained from different insects [31-32]. Amphibians like frogs and toads are rich source of peptides like japonicin-1 & 2, nigrocin-1 & 2, brevinin-20a and maximin-1[33]. Strongylocins are a novel family of cysteine-rich AMPs, recently identified in the sea urchins,

Strongylocentrotus droebachiensis and *S. Purpuratus* [34]. Thionins, plant defensins, lipid transfer proteins are the AMPs from plant sources [35]. Defensin, histatin, LL-37, indolicidin, protegrin, lactoferricin are the examples of mammalian peptides [36]. Echinocandins, aculeacins, mulundocandins, FK463, aureobasidin, leucinostatins and helioferins are the peptides derived from fungi [37]. Marine fishes also possess antimicrobial peptides as the part of their defense systems and some of them are pardaxins, misgurin, pleurocidins, parasin, oncorhyncin II and III [38]. Antimicrobial effect obtained upon combining the prokaryotic antimicrobial peptides pediocin PA-1, sakacin P, and curvacin A with the eukaryotic AMP pleurocidin was also found [39].

1.5 Mechanism of action

The most interesting character of anti-microbial peptide is their cell specificity by which they kill microbes but are nontoxic to mammalian cells. Differences in lipid composition between eukaryotic and prokaryotic cell membranes are regarded as the reason behind the relative insensitivity of eukaryotic cells to antimicrobial peptides [40-41]. AMPs bind in a identical manner to negatively charged membranes and permeate them, which results in the formation of a pathway for ions and solutes.

Hancock described as a ‘self-promoted uptake’ with respect to Gram-negative microorganisms. In this process, AMPs partly neutralize LPS via initial interaction with the surface LPS and competitively displacing the divalent polyanioniccations. This causes disruption of the outer membrane and peptides pass through the disrupted outer membrane and stretch to the negatively charged phospholipid cytoplasmic membrane [42]. Several aspects of antimicrobial peptide structure relevant to antimicrobial activity and selective toxicity are also considered. Specifically, structural parameters such as conformation, charge, hydrophobicity, hydrophobic

moment, amphipathicity, and polar angle play a vital role in the molecular basis of antimicrobial peptide mechanisms of action [43].

The action of antimicrobial peptides induces membrane defects such as phase separation or membrane thinning, pore formation, promotion of nonlamellar lipid structure or bilayer disruption, depending on the molecular properties of both peptide and lipid [44]. These pathways are termed as transmembrane pores, wormholes or toroidal pores, and channel aggregates [45-48].

An interesting 'in plane diffusion' model has also been proposed, where lipid-mediated channel formation is based on the curvature stain imposed on lipid membranes in the presence of intercalated amphipathic peptides. This model is independent of peptide aggregation, which has been reported to be entropically and electrostatically disfavored even in the presence of negatively charged phospholipids [45].

Another is 'two-state model' which explains the action of both α-sheet and helical antimicrobial peptides after they bind to the plasma membranes of cells. Each peptide has two distinct physical states of binding to lipid bilayers. Peptide tends to adsorb in the lipid head group region in a functionally inactive state at low peptide-to-lipid (P/L) ratios. Above a threshold value P/L, the peptide forms a multiple-pore state that is lethal to a cell. The susceptibility of a cell to an antimicrobial peptide depends on the value of P/L that is resolved by the lipid composition of the cell membrane. This model contributes plausible explanations for the experimental findings that the susceptibility of different bacteria to a peptide is not directly dependent to its binding affinity, various peptides preferentially kill different pathogens, and peptides exhibit different levels of lytic activity against different eukaryotic

cells [49].

Two general mechanisms were originally proposed to describe the process of phospholipid membrane permeation by membrane-active peptides, the 'barrel-stave' [50] and the 'carpet' [51] mechanisms. The cartoon (figure 2) illustrates both models suggested for membrane permeation. The third mechanism, the aggregate channel formation, was also proposed for peptides without causing significant membrane depolarization.

Barrel-Stave Model:

The term "barrel-stave" describes the overall topology of a membrane channel formed in this mechanism of membrane permeabilization. In this model, a variable number of channel-forming peptides are positioned in a "barrel-like" ring around an aqueous pore. The "stave" term refers to individual transmembrane spokes within this barrel, which may be composed of individual peptides or peptide complexes [52]. A crucial step in the barrel stave mechanism requires peptides to recognize one another in the membrane bound state. Peptide assembly can occur on the surface or within the hydrophobic core of the membrane, since hydrophobic peptides can span membranes as monomers [53].

Because these peptides insert into the hydrophobic core of the membrane, they should have two important properties [54]:

(i) their interaction with the target membrane is driven predominantly by hydrophobic interactions.

(ii) if they adopt amphipathic α-helical structure, their net charge along the peptide backbone should be close to neutral.

Alternatively, they can be composed of predominantly hydrophobic amino acids [55]. As a consequence of these properties the peptides bind to phospholipid membranes irrespective of

the membrane charge, and therefore, should be toxic to both bacteria and normal mammalian cells. Indeed, functional studies revealed that pardaxin [56], alamethicin [57], and the helix α5 of δ-endotoxin [58] kill both bacteria and erythrocytes by the barrel-stave mechanism.

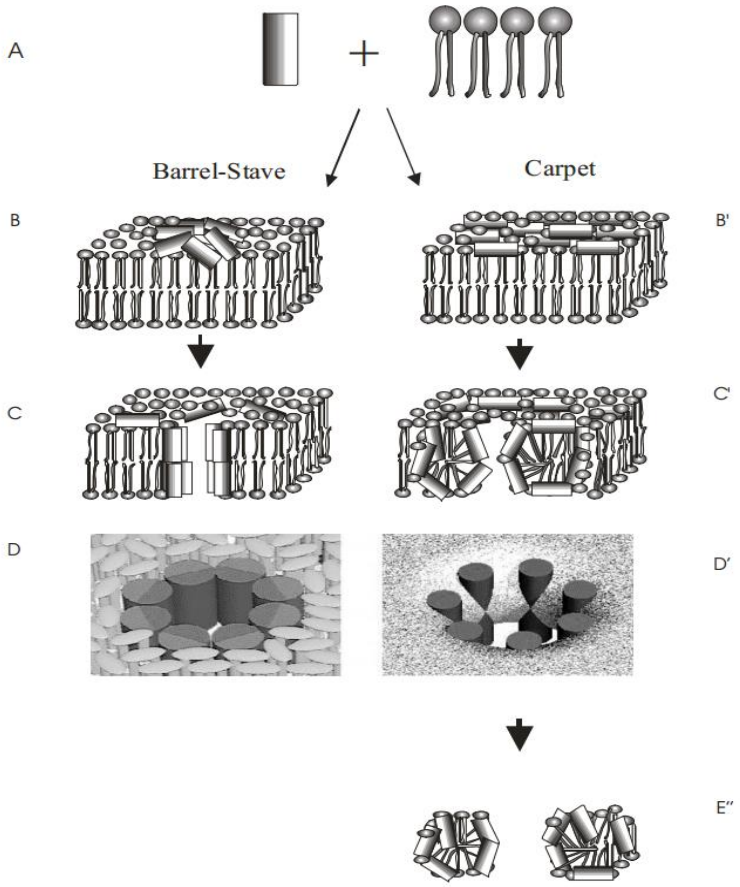


Figure 2. The barrel-stave and the carpet models presented for membrane permeation.

Carpet Model:

Some peptides may act against microorganisms through a relatively diffuse manner i.e. the carpet mechanism. But, peptides that employ this mechanism are not indiscriminate membrane detergents. According to this theory, a high density of peptides accumulates on the target membrane surface. Phospholipids bring changes in membrane fluidity and/or reductions in

membrane barrier properties subsequently lead to membrane disruption. Likewise in other theory, electrostatic interactions and carpeting the phospholipid bilayer are the process by which peptides initially bind to the membrane [54].

In the barrel-stave model peptides first assemble on the surface of the membrane (panel B), then insert into the lipid core of the membrane following recruitment of additional monomers (panel C), forming pores [52] . In the carpet model the peptides are bound to the surface of the membrane with their hydrophobic surfaces facing the membrane and their hydrophilic surfaces facing the solvent (panel B'). When a threshold concentration of peptide monomers is reached, the membrane is permeated and transient pores can be formed (panels C' and D')[52], a process that can lead also to membrane disintegration (panel E'). The spheres represent the phospholipid head groups and two legs connected with these the two acyl chains. The molecules of antimicrobial peptides are illustrated as cylinders.

The Toroid Pore/Wormhole Model:

One of the most well logically explained peptide-membrane interactions is that of the toroid pore. The basic difference between the toroid pore and barrel-stave models is that in the former, lipids are intercalated with peptide in the transmembrane channel. Therefore, this structure has been referred to as a supramolecular complex and represents a membrane-spanning pore lined with polar peptide surfaces as well as phospholipid head groups [43].

The rapid increase of bacterial resistance towards many conventional antibiotics has resulted in search for alternative antimicrobial agents [59]. Natural antimicrobial peptides have potential application in food preservation as they specifically kill microbial cells by destroying their unique membranes. Bacteriocins produced by Lactic Acid Bacteria (LAB) have been sparked by growing consumer demands for natural and minimally-processed foods. LAB bacteriocins

have well-documented lethal activity against foodborne pathogens and spoilage microorganisms [60], and can play a vital role in the design and application of food preservation technology [61-62]. Nisin is approved as a food preservative in more than 40 countries worldwide [63]. Intrabiotics, peptide D2A21, P113L and P113D (derived from histatins) has shown their effectiveness and passed clinical trials [64-66]. The first peptide to undergo clinical trials was pexiganan (derivative of magainin) for the topical treatment of diabetic foot ulcers [67]. Due to the ability of AMPs to bind lipopolysaccharide, sequences are being studied for septic shock treatment [68-69]. Some AMPs are created for treatment of pneumonia and for oral indications [70-71]. Enfuvirtide, has been approved for treating HIV [72]. Still some peptides are under study due to their synergistic effect with traditional antibiotics [7, 73-74]. The anticancer treatment has shown different side effects but these effects are found to be overcome by peptides by using them to generate therapeutics for enhancing cellular uptake, drug targeting and vaccination [75].

However, they are not found to be stable for oral administration which leads this therapeutics to be administrated via IV route. But, transdermal delivery and inhalation routes are considered to solve the problem of oral administration [76-77].

AMPs produced by microbes such as *Bacillus* spp. play a major role in therapeutic applications. The *Bacillus* genus is a genus of Gram positive, rod shaped, endospore forming bacteria spread into the environment and other members of the *B. subtilis* group are considered as safe and have “generally recognized as safe’’ status. *Bacillus* spp., particularly *B. subtilis*, are usually found in foods such as dry cured sausages, cheeses, traditional fermented milks, sourdough, etc.

Also, *Bacillus* has been widely used in the fermentation industry for the production of antibiotics as well as several extracellular enzymes. A large number of peptides with biological activities have been increasingly reported from this group and have become a centre of

attention for antimicrobial study [78-79]. Most ofthe peptides produced by *Bacillus* are antibacterial and a few are antifungal, antitumor, fibrinolysis-promoting, immunosuppressive, amylases, lipases and proteases.

CHAPTER 2:MATERIALS AND METHODS

2.1 Materials

Sephadex G-50 and Sephadex G-25 were purchased from Pharmacia (Uppsala, Sweden). All the other reagents were of analytical grades.

2.2 Isolation and screening

The bacterial strain K1R was isolated from traditional Korean food kimchi. For isolation, 1 gm of kimchi was mixed with 9 ml of 0.85% NaCl and kept in incubator at 37°C for 24 hour. After diluting up to 10^7 times with distilled water, the diluted sample was inoculated in Mueller-Hinton agar plates. After growing, the plate was subjected for screening and identification. This was done according to the Bergey's manual of systematic bacteriology. Many research has demonstrated kimchi as a source of many bacterial strains capable of producing AMPs.[80-82]

2.3 Sequence similarities

The BLAST program (www.ncbi.nlm.nih.gov/blast) was employed in order to assess the degree of DNA similarity.

2.4 Optimization of culture media

Optimization of culture media has great significance for the better production of antimicrobial peptide. Different researchers suggest different types of media for the enhanced production of bacteriocins. Bacteriocins are usually produced in complex media [83-84]. Physicochemical factors have a dramatic effect on the production of bacteriocins. [85] Standard culture media are not always the good producer of peptide as well as enriched media. [86-87]. Media optimization was performed with different carbon sources (Lactose, Sorbitol, Sucrose,

Glucose, Mannitol, Starch, Fructose, Maltose), nitrogen sources (Tryptone, Yeast extract, Peptone, Oat Meal, Beef extract, Malt, Soymeal) and metal ions (KH_2PO_4, $FeSO_4$, NaCl, Na_2HPO_4, $CaCl_2$, NaH_2PO_4, $MgSO_4$, $ZnSO_4$, $MgCl_2$). MRS was taken as a control media and the amount of three sources was varied. Finally, 2% Beef extract and 2% Maltose were established as the optimized media and fermentation was carried out on 250ml erlenmeyer flasks containing 20% of media with constant shaking at 160 rpm at 37ºC. No effect of metal ions was seen.

2.5 Peptide production and purification

Seed culture was incubated for 16 hours at 37ºC at 160rpm. This was transferred into mass culture containing optimized medium and incubated for 30 hours. After 30 hours, harvesting was executed in cold centrifugation at 10000 × g for 35 min.

Ammonium sulfate precipitation method was used to retrieve peptide from harvested broth [88], followed by centrifugation at 10000 × g at 4 °C for 50 minutes. The obtained precipitate was dialyzed against 10 mMTris/HCl (pH 7.4). Ultra-filtration membrane of 30 kDa and 10 kDa (Millipore Corp.) was then used for desalting procedure. Following dialysis & filtration, it was purified with a Sephadex G-50 column (1.5 cm× 65cm) using the same buffer. The active fractions were pooled, concentrated and further purified with a Sephadex G-25 column (1.4 cm × 25 cm) using the same buffer system.

2.6 Protein estimation, molecular weight determination

Protein concentration was estimated by Bradford method [89], using bovine serum albumin as a standard. Molecular weight determination was done by Tricine SDS-PAGE [90].

2.7 In-situ analysis

In-situ analysis was done against indicator organism *Mycobacterium smegmatis* ATCC 9341 ($1.5X10^8$cfu/ml) by overlaying the processed gel from Tricine SDS-PAGE after been washed with 50 mM Tris–HCl buffer (pH 7.5) containing 2.5% Triton X-100 for several times to 0.6% agar on Mueller-Hinton media and incubated at 37°C.

2.8 Effects of temperature and pH on antimicrobial activity:

Thermal stability of K1R was determined by exposing to 0, 10, 20, 37, 50, 60, 70, 80°C for 30 minutes and at standard autoclave condition before analyzing residual activity. Similarly, pH stability was analyzed at various pH values (4.0-12.5) using 100mM pH buffers: citric acid/phosphate (4-5.5), tris/hydrochloric acid (6.5-9.5) and potassium chloride/sodium hydroxide (10-13.5).

2.9 Effect of chemicals on antimicrobial activity:

Effect of different chemicals like oxidizing agents (hydrogen peroxide and sodium perborate), reducing agent (β- mercaptoethanol), chelating agents (EDTA and EGTA), detergents (SDS, CHAPS, Triton X-100, Tween 20, Tween 80 and deoxycholic acid), metal ions (Ca^{++}, Mg^{++}, Co^{++}, Cu^{++}, Ni^{++}, Zn^{++}, Mn^{++} and Ba^{++}) and solvents (acetone, chloroform, dimethyl sulfoxide, methanol, ethanol, 2-propanol, 1-butanol, toluene, diethyl ether, TCA, sodium chloride and potassium chloride) on antimicrobial activity of AMP was seen.

2.10 Antimicrobial activity of K1R

2.10.1 Minimum inhibitory concentration test:

Agar dilution method was used for the determination of minimum inhibitory concentration [91]. Bacitracin and Vancomycin were used as reference standard antibiotics.

Indicator organisms selected for the test are as follows:

1. Gram's negative bacteria: *Escherichia coli* KCTC 1923, *Salmonella typhimurium* KCTC 1925, *Pseudomonas aeruginosa* KCTC 1637, *Alcaligenes faecalis* ATCC 1004.
2. Gram's positive bacteria: *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Mycobacterium smegmatis* ATCC 9341, Vancomycin Resistant *Enterococci* 4 (VRE 4), Methilicine resistant *Staphylococcus aureus* 5-3 (MRSA 5-3), Methilicine resistant *Staphylococcus aureus* 4-5 (MRSA 4-5), Vancomycin resistant *Satphylococcus aureus* (VRSA), Vancomycin Resistant *Enterococci* 98 (VRE 98), Vancomycin Resistant *Enterococci* 89 (VRE89), Vancomycin Resistant *Enterococci* 82 (VRE 82), *Staphylococcus aureus* KCTC 1928, *Micrococcus luteus* ATCC 9341.

Inoculation of test organisms ($1.5X10^8$cfu/ml) in different concentration antibiotics plates along with no antibiotic plate as control was incubated at 37^0C. Results were observed after 12 hours in comparison with control plate.

2.10.2 Agar disk diffusion assay:

The antimicrobial activity of peptide K1R was detected by agar disk diffusion assay [92] and was tested against all indicators explained as in minimum inhibitory concentration test. An aliquot of 50 µl K1R was applied on disks (8 mm) on agarplates previously inoculated with each individual indicator strain.

2.11 Amino acid sequence analysis

The amino acid sequence of K1R was determined by Edman degradation using a Procise® Model 491 HT Protein Sequencer (Applied Biosystems, USA). The molecular weight of peptide was also verified by utilizing sequence [93].

2.12 Anti-oxidant activity of K1R

DPPH method was employed to check the anti-oxidant activity using the process described by Zhan *et al.* with some modification. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. Various concentrations of peptide solutions 100µl were added to 100µl of DPPH (0.3mM) in methanol solution. The mixture was kept in dark place at room temperature for 30 min and absorbance at 517nm measured on spectrophotometer. Methanol was used for the blank whereas distilled water was used for negative control. Ascorbic acid was used as positive control.

DPPH radical scavenging ability was calculated by following equation:

$\% = (1- Abs_{sample}/ Abs_{blank})$

where Abs_{sample} and Abs_{blank} are the absorbance of sample and blank respectively.

CHAPTER 3:RESULTS AND DISCUSSION

Morphology of colonies, biochemical characteristics and the utilization of nutrients suggested the selected strain as a *Bacillus* species. Purified products from fermented food alter dietary items pre-consumption, and in turn, the ways in which fermentation-enriched chemicals act upon our own intestinal microbiota profile [94]. The functional activities of those products include antimutagenic/anticancer [95], antiobesity [96], antiatherosclerotic, and immunomodulatory effects [97].

3.1 Identification of *Bacillus* strain:

For molecular phylogeny, the 16S rRNA sequence of the local isolate was compared to sequence of 10 *Bacillus* species. In order to determine the relation of the local isolate to these *Bacillus* strains, multiple sequence alignment was done between the sequences of the 16S rRNA gene of various *Bacillus* species and local isolate. Computer assisted RNA searches against bacterial database similarly revealed that the 16S rRNA sequence was identical over 99% with those 10 *Bacillus* strains (Table 1). K1R's 16S rRNA sequence was deposited in GenBank under accession no. AYTO01000043.

AACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCT
CCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGAC
TGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGG
TTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTA
GCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAG
GGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGA
TGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAA

TAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGG
GCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGG
GTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGT
AGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG
GTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTT
AGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTG
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGA
TAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGC
CAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAAT
GGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTT
CTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAAT
CGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCACGGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCA
GCCGCCGAAGGTGGGACAGATGATTGGG

Figure 3. 16S rRNA sequence of *Bacillus* sp. K1R

Rank	Name	Strain	Accession	Pairwise Similarity (%)	Diff/Total nt
1	*Bacillus subtilis subsp. inaquosorum*	KCTC 13429(T)	AMXN01000021	99.93	1/1465
2	*Bacillus tequilensis*	KCTC 13622(T)	AYTO01000043	99.93	1/1465
3	*Bacillus subtilis subsp. subtilis*	NCIB 3610(T)	ABQL01000001	99.86	2/1465

4	*Bacillus subtilis subsp. spizizenii*	NRRL B-23049(T)	CP002905	99.8	3/1465
5	*Brevibacterium halotolerans*	DSM 8802(T)	AM747812	99.66	5/1465
6	*Bacillus vallismortis*	DV1-F-3(T)	JH600273	99.59	6/1465
7	*Bacillus mojavensis*	RO-H-1(T)	JH600280	99.59	6/1465
8	*Bacillus siamensis*	KCTC 13613(T)	AJVF01000043	99.45	8/1465
9	*Bacillus amyloliquefaciens subsp. plantarum*	FZB42(T)	CP000560	99.39	9/1465
10	*Bacillus methylotrophicus*	CBMB205(T)	EU194897	99.38	9/1441

Table 1. Similarity of 16S rRNA gene sequence of K1R.

3.2 Optimization of the culture media:

Many bacteria can be grown the laboratory in culture media which are designed to provide all the essential nutrients in solution for bacterial growth but for the maximum production of antimicrobial compound nutrients and growth factors should be optimized [98].

a. Optimization of carbon, nitrogen and metal ion source:

In case of carbon source, *Bacillus* strain K1R was inoculated in culture media containing different carbon sources and incubated up to 108 hours and sample was withdrawn in every 12 hours. Disk diffusion assay was performed to check the activity of antimicrobial peptide produced by *Bacillus* strain K1R against indicator organism and graph was plotted. Incubation time vs zone of inhibition was plotted and it was seen that maltose was the best carbon source for this strain. Similarly, this process was repeated to choose best nitrogen source and metal ion source. In case of nitrogen source, beef extract turn out to be the best one but in case of metal ion, none of them had any good effect in the antimicrobial activity. So, maltose and beef extract were choosen as the best carbon and nitrogen source.

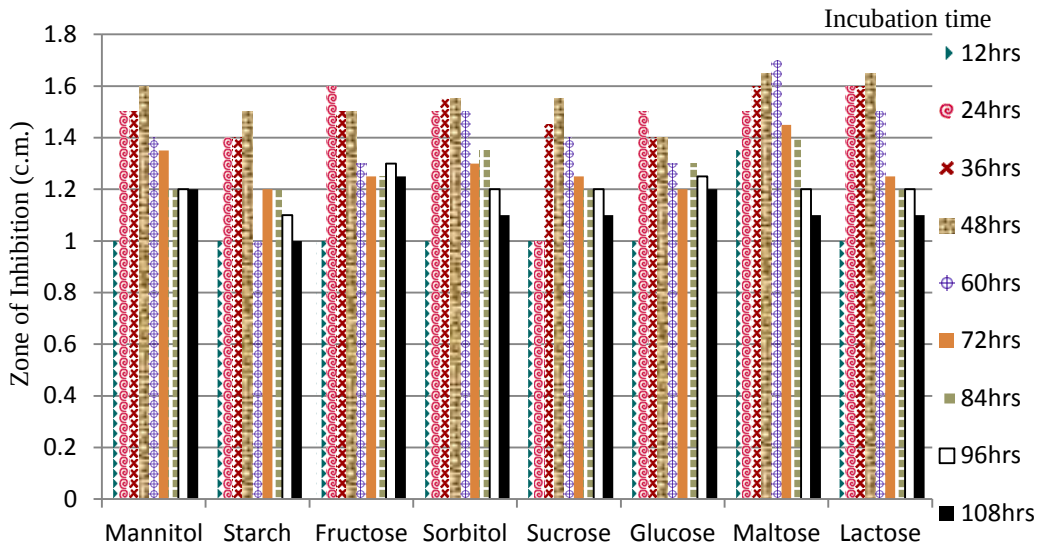


Figure 4. Effect of various carbon sources on the activity of antimicrobial peptide produced by *Bacillus* strain K1R.

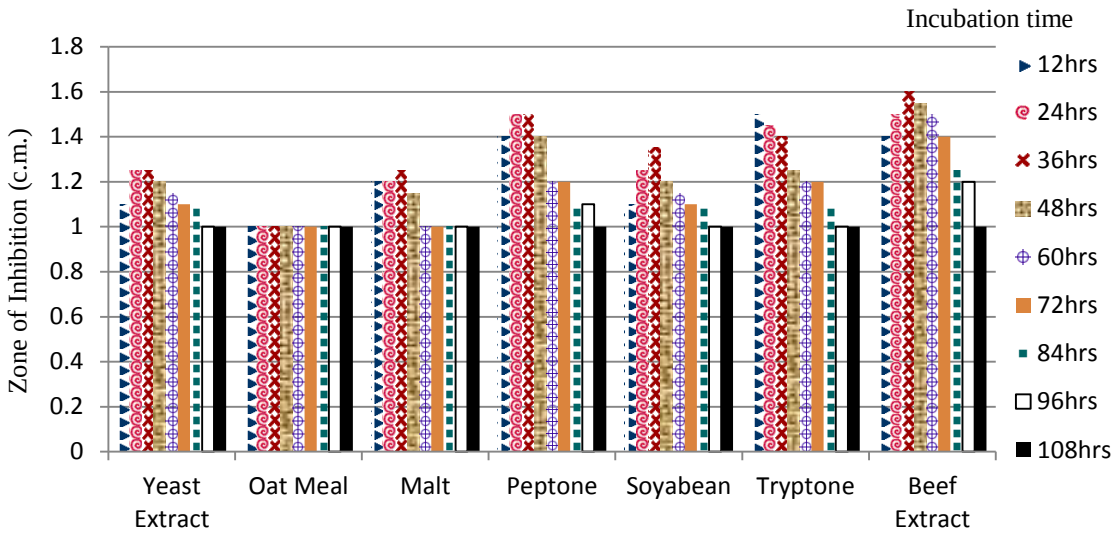


Figure 5. Effect of various nitrogen sources on the activity of antimicrobial peptide produced by *Bacillus* strain K1R.

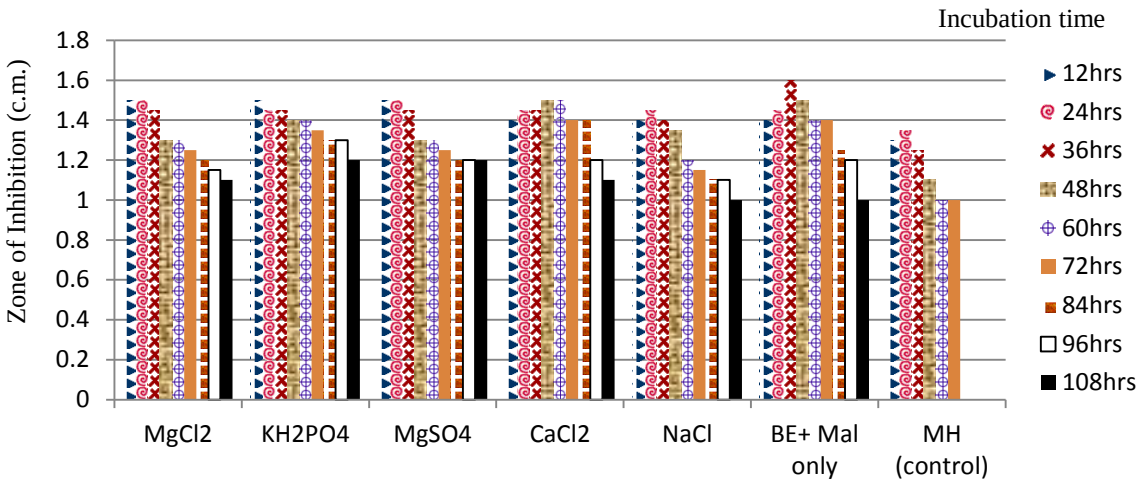


Figure 6. Effect of various metal ions on the activity of antimicrobial peptide produced by *Bacillus* strain K1R.

Final Optimization:

Final optimization is done by varying the amount of beef extract and maltose. Form figure 7, it is clear that peptide produced by Bacillus strain K1R shows its maximum activity at 30th hour of inoculation, when 2% maltose and 2% beef extract is present in media. In this research, *Mycobacterium smegmatis* ATCC 9341 was chosen as the indicator organism.

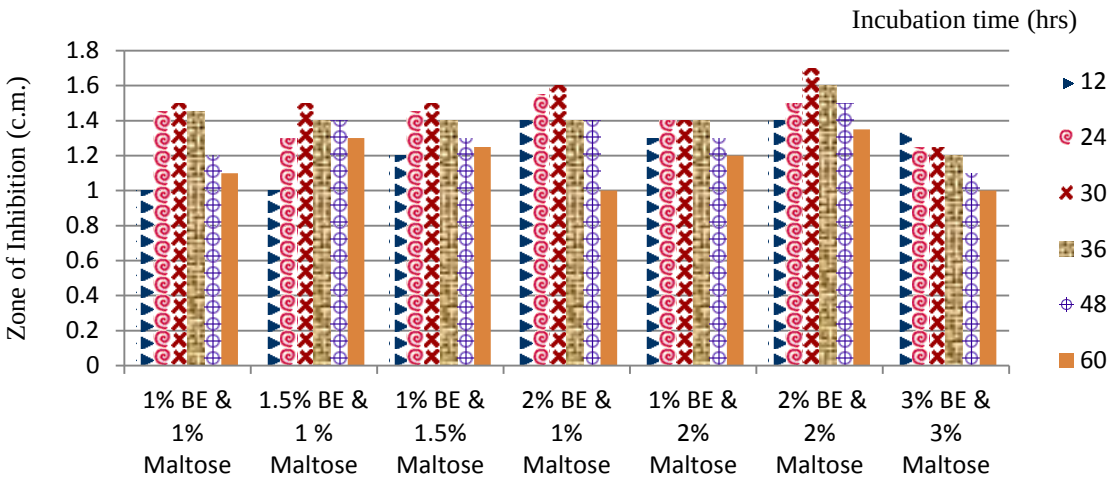


Figure 7. Effect of different amount of beef extract and maltose on the activity of antimicrobial peptide produced by *Bacillus* strain K1R.

3.3 Production of antimicrobial peptide:

Seed culture was carried out in MRS media and after16 hours of growth; it was transferred to main culture containing optimized media and conditions and allowed to grow for 30 hour. Antimicrobial peptide secreted by the strain K1R displayed antimicrobial activity against *Mycobacterium smegmatis* ATCC 9341, *Enterococcus faecalis* ATCC 29212 and *Micrococcus luteus* ATCC 9341.

3.4 Purification of antimicrobial peptide:

1. Ammonium sulfate precipitation pethod:

After 30 hour of growth, broth of strain K1R was harvested. Cell free culture supernatant was precipitated using ammonium sulfate (80% w/v) and stored at 4°C overnight with continuous stirring. The pellet was collected, suspended and dialysed in Tris-HCl buffer (pH 7.4).

2. Ultrafiltration technique:

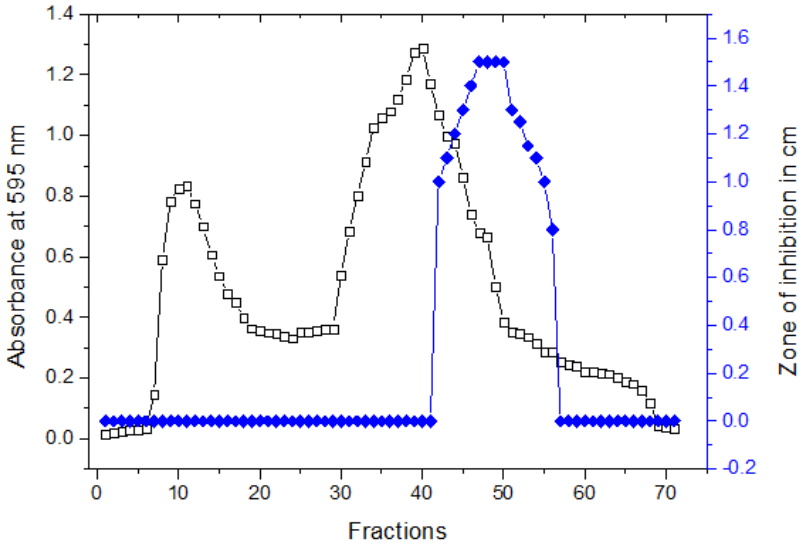
Amicon ultrafiltration technique was used after ammonium sulfate precipitation method to purify the suspened pellets in buffer. 30 kDa & 10 kDa filter papers were used in this process respectively.

3. Column Chromatography:

The crude extracts were applied to Sephadex G-50 column, eluted with 10mM Tris-HCl buffer (pH 7.5) as a mobile phase. Fractions were collected and monitored for antimicrobial activity against indicator bacteria *Mycobacterium smegmatis* ATCC 9341. Active fractions were concentrated by lyophilizing, loaded onto a Sephadex G-25 column and eluted with same

buffer used before. The fractions positive for antimicrobial activity were pooled and stored at 4ºC. Protein content was estimated using Bradford Method [89]. Figure 8 gives the comprehensible concept of both chromatographic steps involved during purification and the selection of the active fractions by observing the antimicrobial activity versus protein content plot i.e. zone of inhibition vs absorbance at 595 nm.

a)



b)

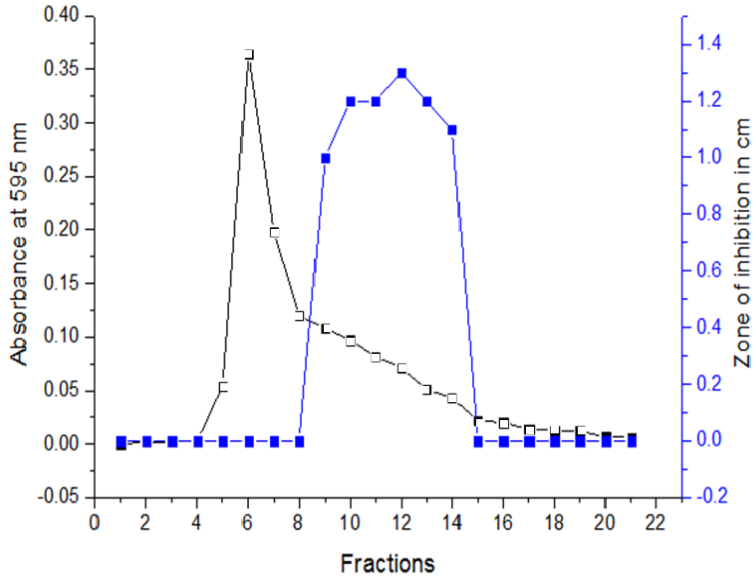


Figure 8. Elution profile of K1R antimicrobial peptide.

a) Gel filtration chromatography with Sephadex G-50 column (1.5 cm× 65cm) with flow rate of 0.20 ml/min.

b) Gel filtration chromatography with Sephadex G-25 column (1.4 cm× 25cm) with flow rate of 0.20 ml/min.

3.5 Determination of molecular weight

Antimicrobial peptide was separated by Tricine SDS-PAGE. Ultra low molecular weight marker proteins (1.7 to 42 kDa) were used to determine the approximate molecular size of active protein band. After electrophoresis, one gel was stained with coomassie brilliant blue and another was kept for identification of AMPs band on an unstained gel for in-situ examination. The antimicrobial compound was confirmed by the presence of single band in first gel having molecular weight around 4.6 kDa and in situ examination result showed a distinct zone of inhibition spotagainst indicator organism.

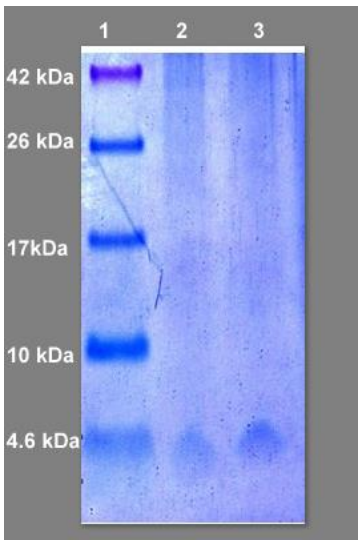


Figure 9. Tricine SDS-PAGE of K1R.

Lane 1. Protein size marker with the corresponding value in kDa on the left;

Lane 2 and 3, purified K1R peptide

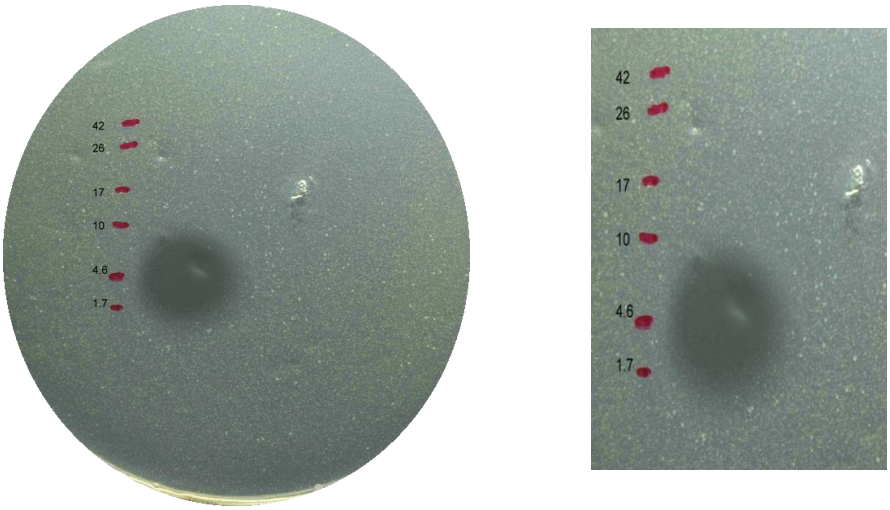


Figure 10. Activity staining of K1R peptide showing the distinct zone of inhibition spot against indicator organism.

3.6 Effects of temperature and pH on antimicrobial activity:

The influence of temperature and pH in the antimicrobial activity of peptide is represented in figure 11 and 12. K1R was found to be much stable around 30-60 ºC and loose its activity about 25% at 70 ºC. Autoclaved sample lost its activity completely.

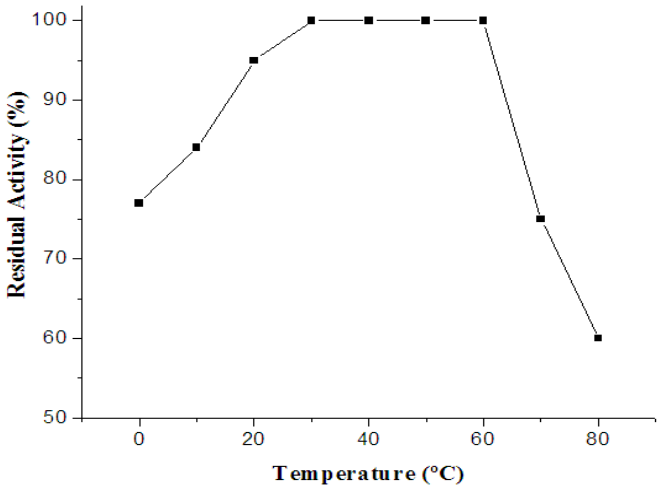


Figure 11. Effect of temperature in the antimicrobial activity of K1R.

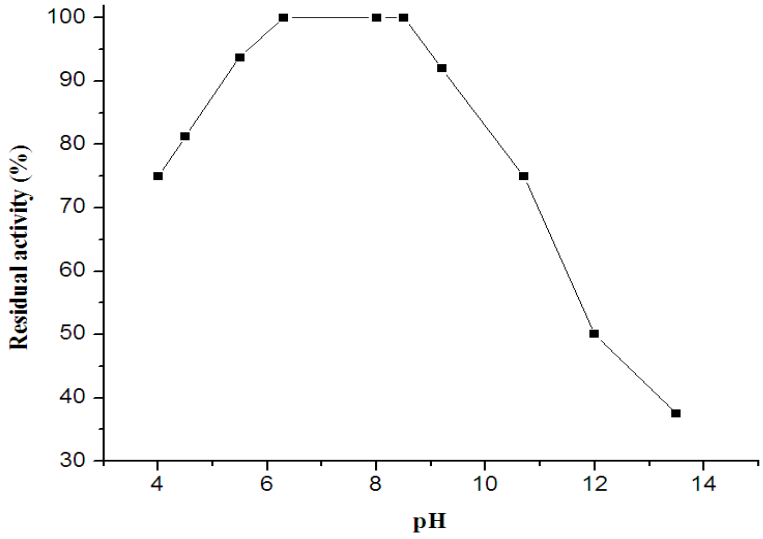


Figure 12. Effect of pH in the antimicrobial activity of K1R

Analysing the residual activity, it was seen that AMP from K1R was found to be highly stable around pH 6.5-9. It lost 50% of activity when pH is increased up to 12. Finally it showed 35% of residual activity at pH 13.5.

3.7 Effect of chemicals on antimicrobial activity:

Effect of various chemicals in terms of residual activity is represented in Table 2.

Metal Ion	Residual Activity (%)
Calcium	53
Magnesium	53
Cobalt	89
Copper	71
Nickel	59
Zinc	83
Manganese	71
Barium	89
Solvents/Chemicals	
Acetone	83
Chloroform	71
Dimethyl Sulfoxide	83
Methanol	71
Ethanol	83
2-propanol	71
1-Butanol	71
Toluene	95
DiEthyl Ether	83
Trichloroacetic Acid	83
Sodium Chloride	59
Potassium Chloride	71

Oxidizing Agents	Residual Activity (%)
Hydrogen Peroxide	100
Sodium Perborate	100
Reducing Agents	
β- mercaptoethanol	83
Chelating Agents	
EDTA	83
EGTA	77
Detergents	
Triton X-100	95
Tween-20	71
Deoxycholic Acid	100
SDS	100
CHAPS	77
Tween- 80	69

Table 2.Effect of different chemicals in antimicrobial activity of K1R.

From the table, it can be seen that the intact activity of antimicrobial peptide produced by *Bacillus* strain K1R remained stable in presence of oxidizing agents like hydrogen peroxide and sodium perborate & detergents like deoxycholic acid and SDS. Triton X-100 and toluene had similar type of effect. Metal ions like calcium and magnesium inhibited activity.

3.8 Antimicrobial activity of K1R

3.8.1 Minimum inhibitory concentration test:

Antimicrobial activity of K1R in terms of minimum inhibitory concentration (MIC) is shown in table 3. Emergence of multi-drug resistant has created a challenge to pharmaceutical

researches. In our study, we carried antimicrobial activity study to know the efficacy of our newly extracted antimicrobial peptide in comparison to the already known drugs like

Microorganism	K1R (µg/ml)	Bacitracin (µg/ml)	Vancomycin (µg/ml)
Gram negative bacteria *Alcaligenes faecalis* ATCC 1004	>128	>128	>128
Salmonella typhimurium KCTC 1925	16	>128	>128
Escherichia coli KCTC 1923	>128	>128	>128
Pseudomonas aeruginosa KCTC 1637	>128	>128	>128
Gram positive bacteria *Enterococcus faecalis* ATCC 29212	4	8	4
Bacillus subtilis ATCC 6633	>128	64	1
Staphylococcus aureus KCTC 1928	64	>128	8
*Micrococcus lu*teus ATCC 9341	8	64	4
Mycobacterium smegmatis ATCC 9341	64	>128	2
MRSA 4-5	>128	2	2
MRSA 5-3	>128	2	2
VRE 4	32	64	>128
VRE 82	16	>128	>128
VRE 89	16	64	>128
VRE 98	32	>128	>128
VRSA	>128	>128	>128

Table 3. Antimicrobial spectrum of antimicrobial peptide with the standard drugs.

vancomycin and bacitracin. K1R displayed antagonistic effect against different multi-drug resistant pathogens. K1R shows strong effect on pathogens like Vancomycin Resistant *Enterococci* 4, Vancomycin Resistant *Enterococci* 82, Vancomycin Resistant *Enterococci* 89, and Vancomycin Resistant *Enterococci* 98 than shown by vancomycin, bacitracin.

Its effect was similar with vancomycin against *Enterococcus faecalis* ATCC 29212 and better than bacitracin against the same pathogen. The MIC value of K1R against *Micrococcus luteus* ATCC 9341 was found to be effective than that of bacitracin. K1R resultant effect in

comparison to the MDR pathogens shows good impact with gram positive organism having resistant pattern with vancomycin. Strains of *staphylococcus* and *enterococcus* resistant with vancomycin show promising result.

3.8.1 Agar disk diffusion assay:

Inhibitory spectrum of K1R on the basis of agar disk diffusion assay is shown in Table 4. The pattern of antimicrobial activity determined via agar disk diffusion assay supports the results of MIC test.

Microorganism	Zone of inhibition (mm)
Gram negative bacteria *Alcaligenes faecalis* ATCC 1004	14
Salmonella typhimurium KCTC 1925	18
Escherichia coli KCTC 1923	14
Pseudomonas aeruginosa KCTC 1637	14
Gram positive bacteria *Enterococcus faecalis* ATCC 29212	22
Bacillus subtilis ATCC 6633	14
Staphylococcus aureus KCTC 1928	16
Micrococcus luteus ATCC 9341	20
Mycobacterium smegmatis ATCC 9341	16
MRSA 4-5	14
MRSA 5-3	14
VRE 4	17
VRE 82	18
VRE 89	18
VRE 98	17
VRSA	14

Table 4. Inhibitory spectrum of K1R against different organisms.

3.9 Amino acid sequence analysis:

The amino acid sequence of K1R was found to be AVQGTLEDALNLSKGALNQVQK AIQNGDXLTVXGXLGTIXLAVSX. This sequence did not show significant homology with the reported peptides of similar type but showed similarity with the sequence of hypothetical proteins from *Bacillus* sp. in the National Centre for Biotechnology Information (NCBI) protein database using BLAST (basic local alignment search tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the amino acid sequence and by the use of protein identification and analysis tools on the ExPASy Server, the molecular weight of peptide was found to be ~4578.23 dalton which also verifies the data from Tricine SDS-PAGE and in-situ analysis.

Hypothetical protein from organism	Sequence	Similarity Percentage
Bacillus subtilis	SVQGCLEDALNLSKGALNQVQKAIQNGDWLTVVGFLGTIGLAVS	87%
Bacillus tequilensis	SVQGCLEDALNLSNGALNQVQKAIEKGDWLTVVGFLGTIGLAVS	82%
Bacillus pumilus	VQRCLEDALGITKGALNQVQKAIEKGEWLSVLGFLTLTGIAVS	61%
Bacillus amyloliquefaciens	VRRCLEDAIGISKGALDKVQKAIEKGDWWTVLEYLGKLGVAIS	54%
K1R	**AVQGTLEDALNLSKGALNQVQKAIQNGDXLTVXGXLGTIXLAVSX**	**(Current study)**

Table 5. Similarity of amino acid sequence of K1R with other hypothetical proteins.

3.10 Anti-oxidant activity of K1R

To obtain the information about the mechanisms of the antioxidative effects of AMP, the radical scavenging effect was examined by measuring changes in absorbance of DPPH radical at 517 nm. Ascorbic acid and K1R both showed concentration dependent scavenging of DPPH radicals. The effect of K1R was similar and comparable to ascorbic acid for all concentrations tested (1-1000 µg/ml). Figure 13, shows the results of the free radical scavenging activity in % inhibition. K1R showed a percentage inhibition of 70.97 at 1000 µg/ml whereas standard ascorbic acid exhibited 75.36% of inhibition at same concentration. In both cases, graded increase in percentage of inhibition was observed for the increase in the concentration of ascorbic acid. All determinations were done in triplicate and the mean values were determined.

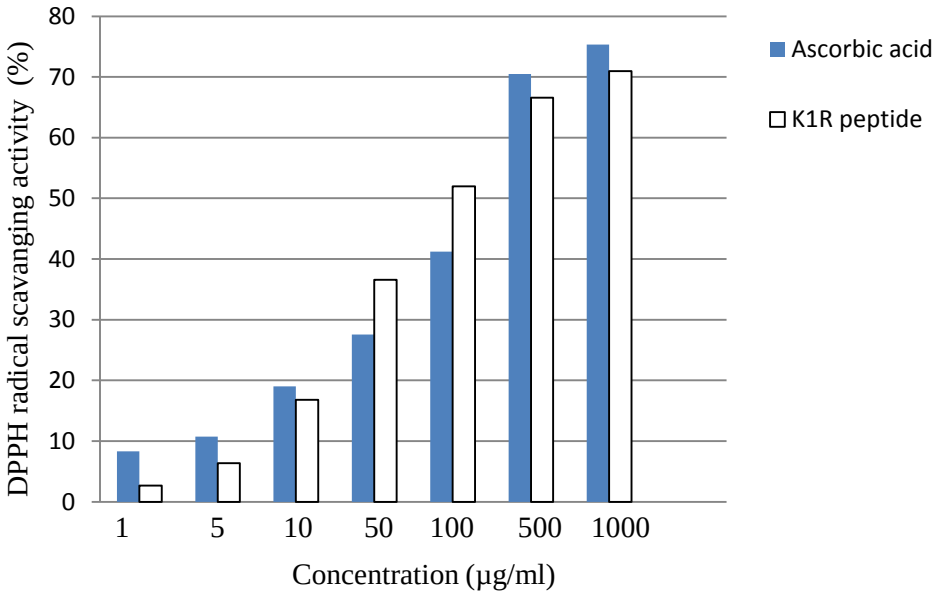


Figure 13. DPPH radical scavenging activity of K1R in different concentration

CHAPTER 4: CONCLUSIONS

In this study, a potent antimicrobial peptide was produced from microbial strain and identified as *Bacillus* from 16S rRNA gene sequences analysis. This study was aimed to determine the antimicrobial activity of K1R with characterization. Beef extract and maltose were found the suitable nutrient sources for the maximum peptide production. AMP was purified using Sephadex G-50 and Spehadex G-25 gel filtration column chromatography respectively. It was found to be stable over wide range of temperature (30-60ºC) and pH (6.5-9) conditions. The molecular weight of K1R was found to be ~4600 Dalton which was also verified by the result from the amino acid sequence using ExPASy server. It remained stable in presence of oxidizing agents like hydrogen peroxide and sodium perborate & detergents like deoxycholic acid and SDS. Antimicrobial effects, in terms of MIC values of the purified peptide were obtained and two well known reference antibiotics named bacitracin and vancomycin were used. It is remarkable that K1R displayed antagonistic effect against different multi-drug resistant pathogens like Vancomycin Resistant *Enterococci* 4, Vancomycin Resistant *Enterococci* 82, Vancomycin Resistant *Enterococci* 89, Vancomycin Resistant *Enterococci* 98 including *Enterococcus faecalis* and *Salmonella Typhimurium* where MIC was in range of 4-32 µg/ml. Disk diffusion assay also verified the MIC result. The antioxidant activities of the K1R was tested using free radical DPPH method. Antioxidant activity of K1R was comparable to the standard ascorbic acid, which siginifies its activity in terms of hydrogen atom donating capacity/electron transfer capability.

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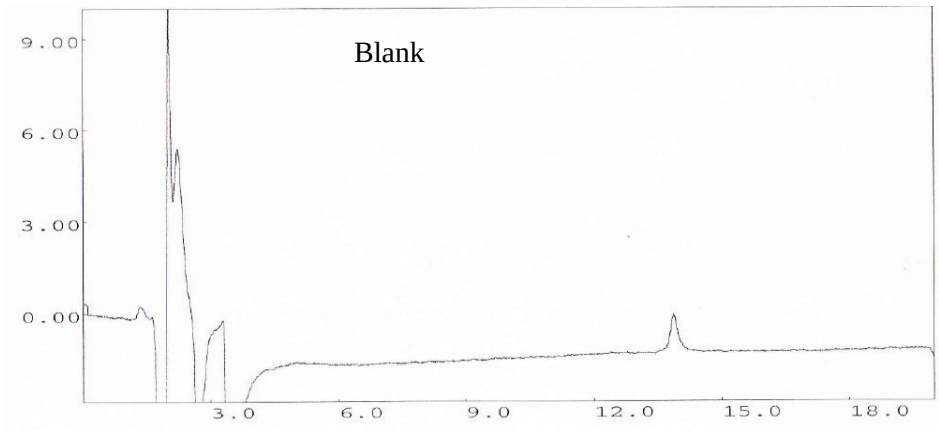
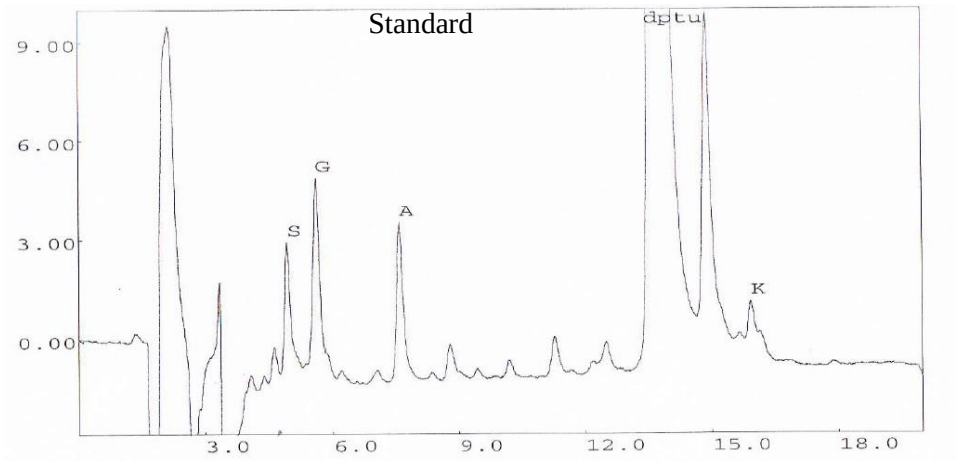
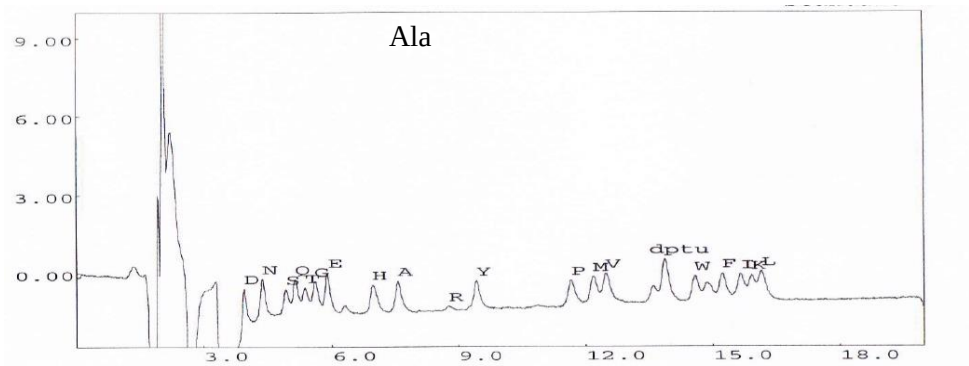
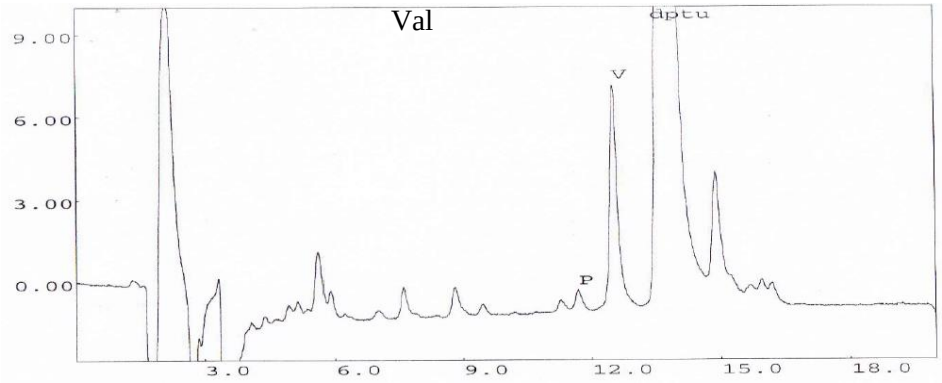
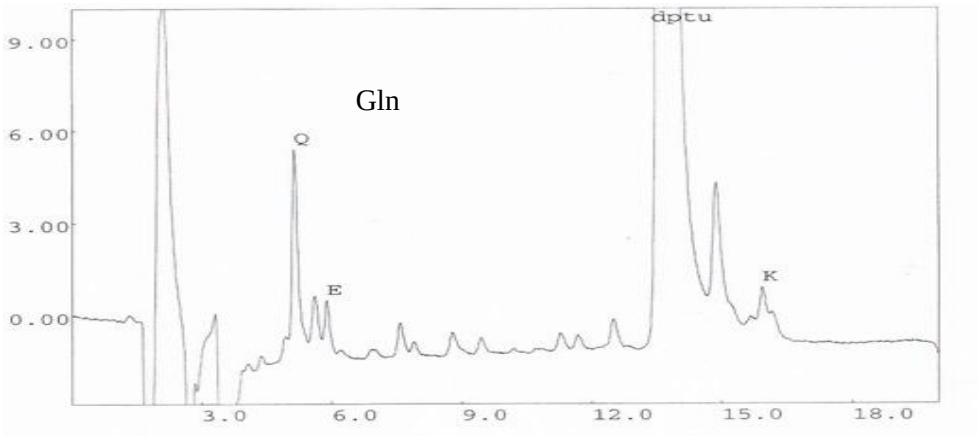
Appendix: N-terminal sequences of K1R

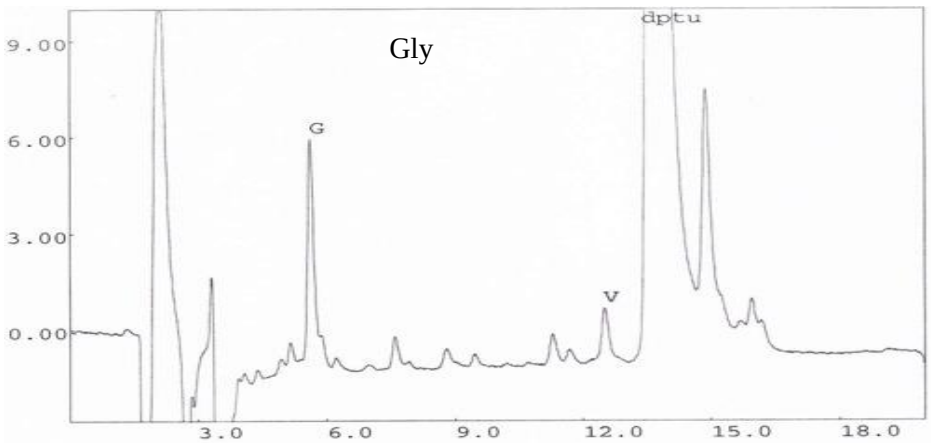


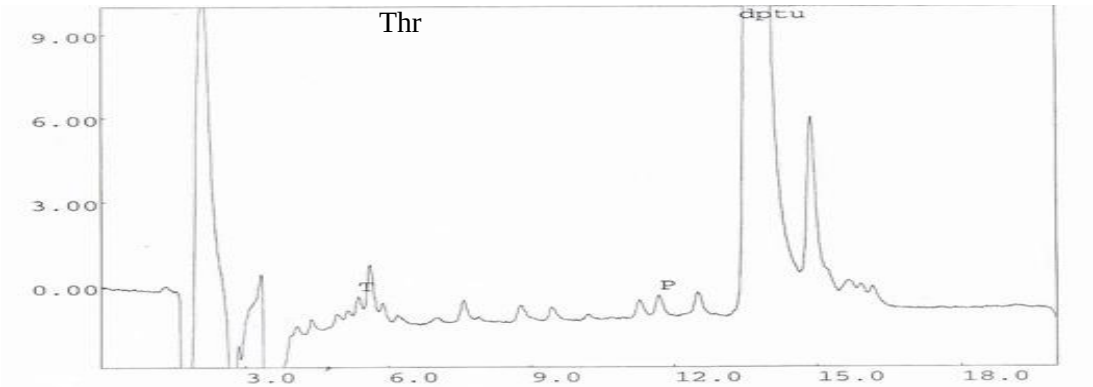


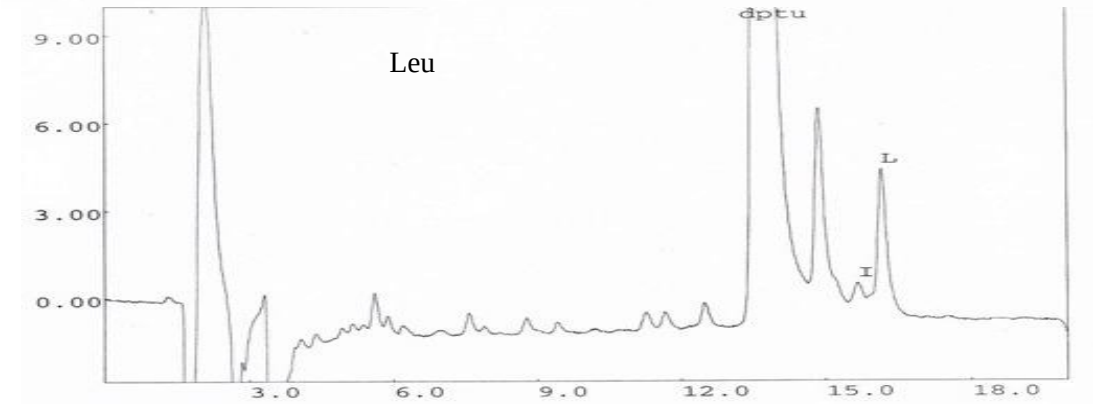


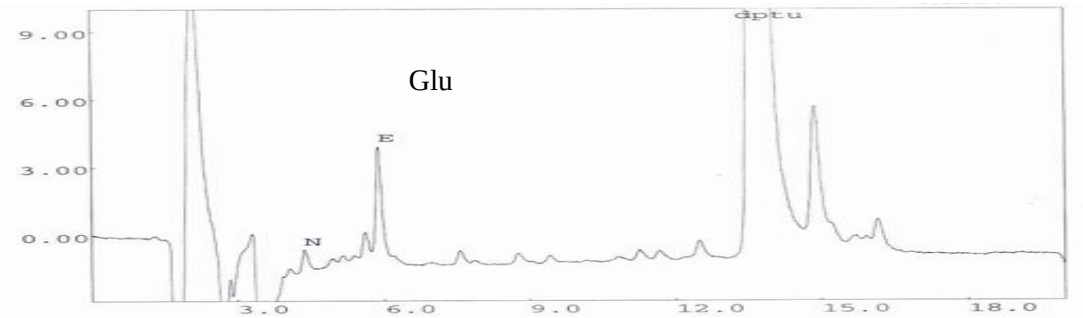


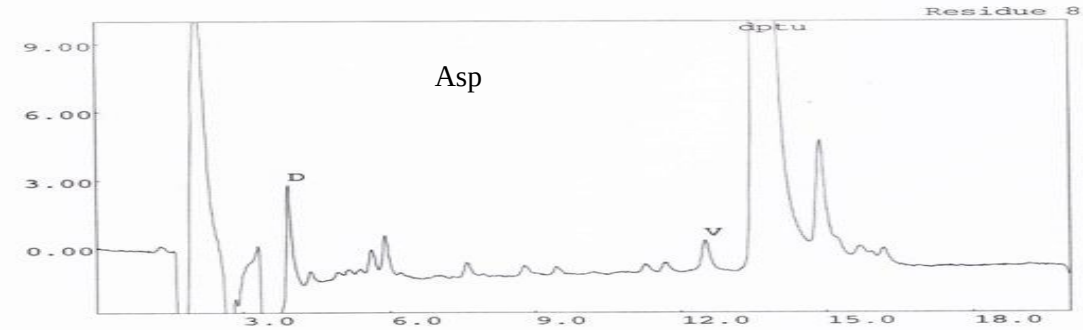


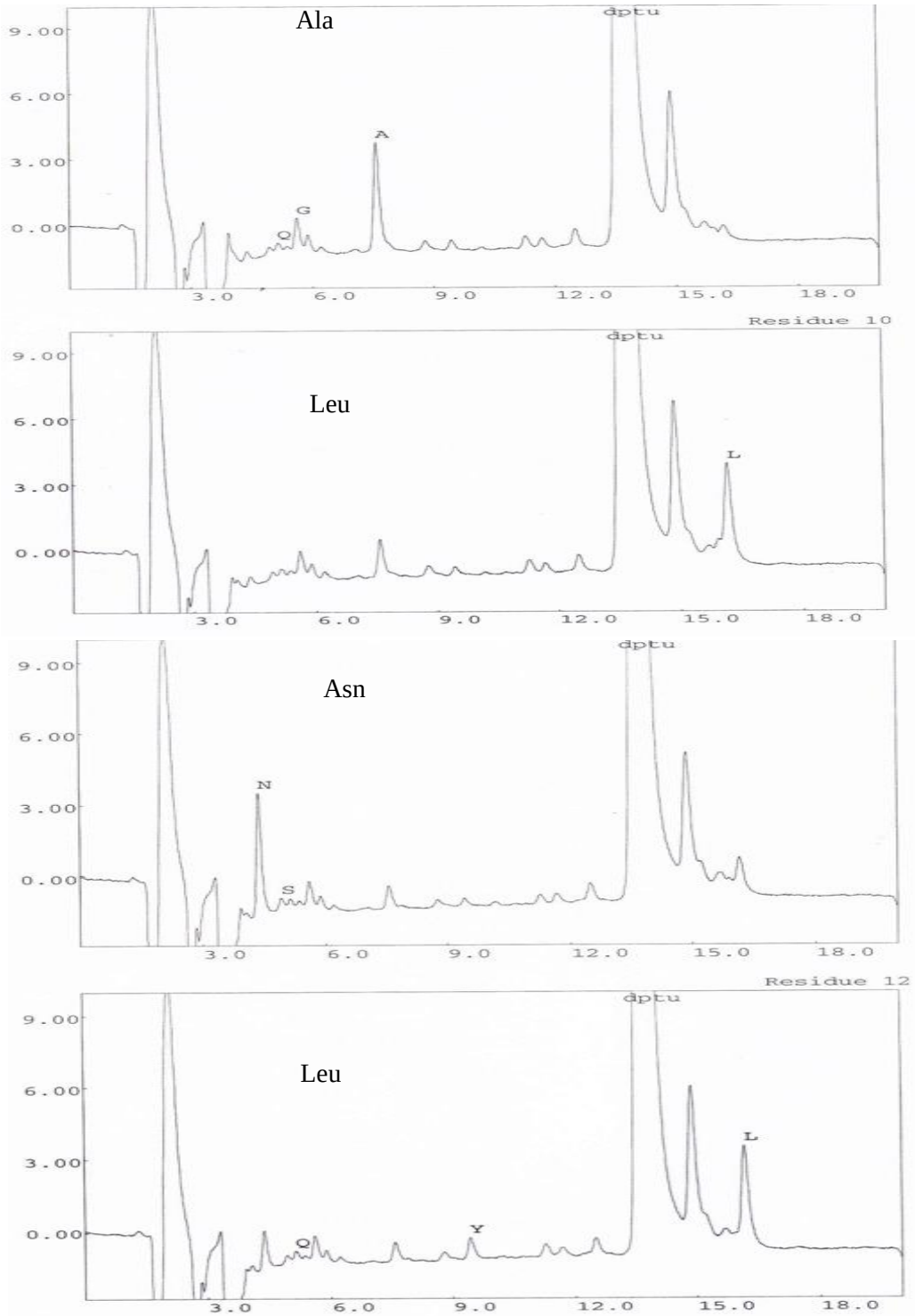


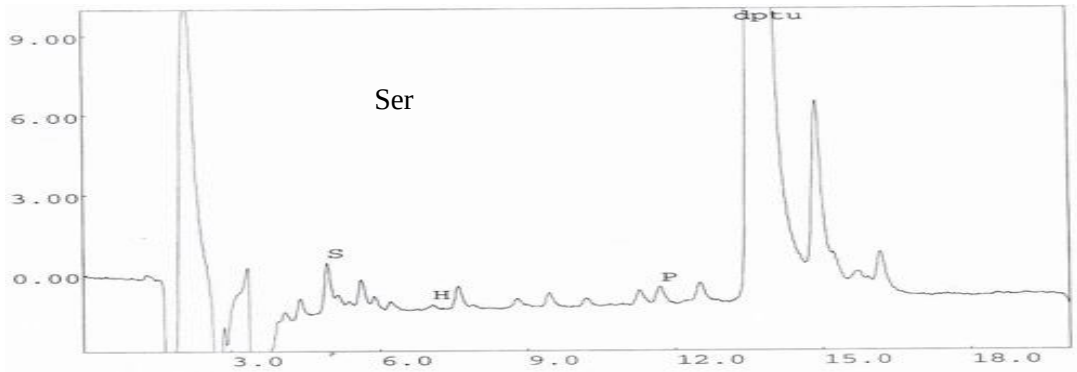


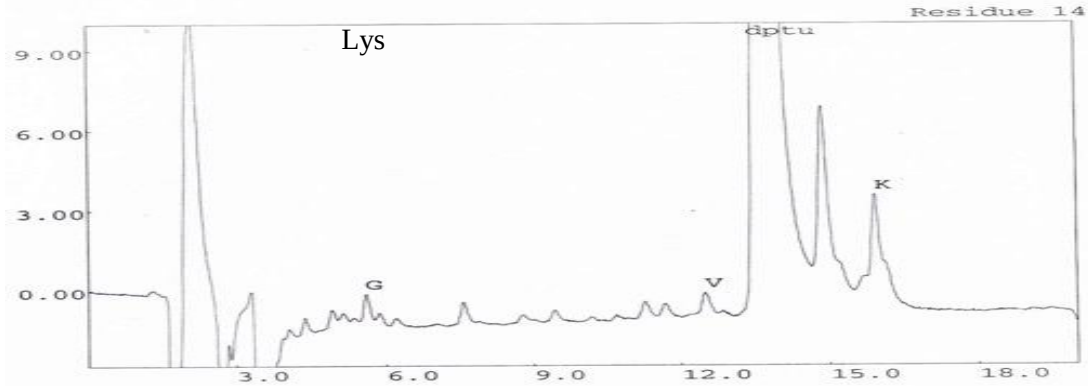


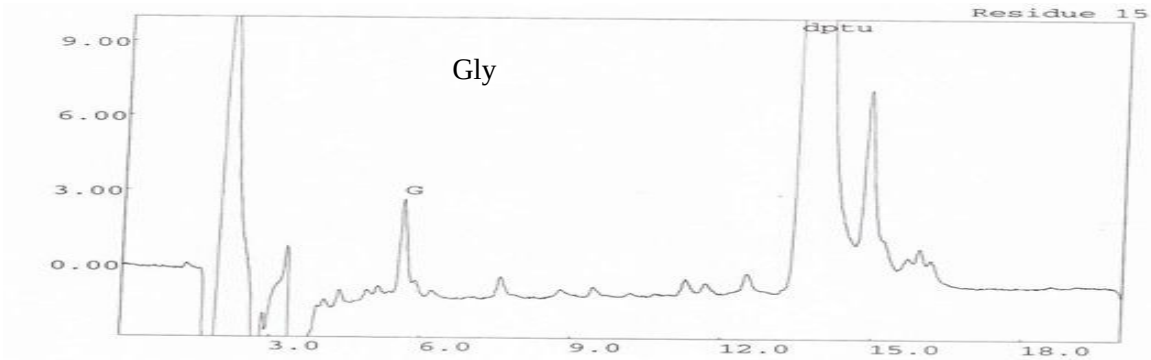


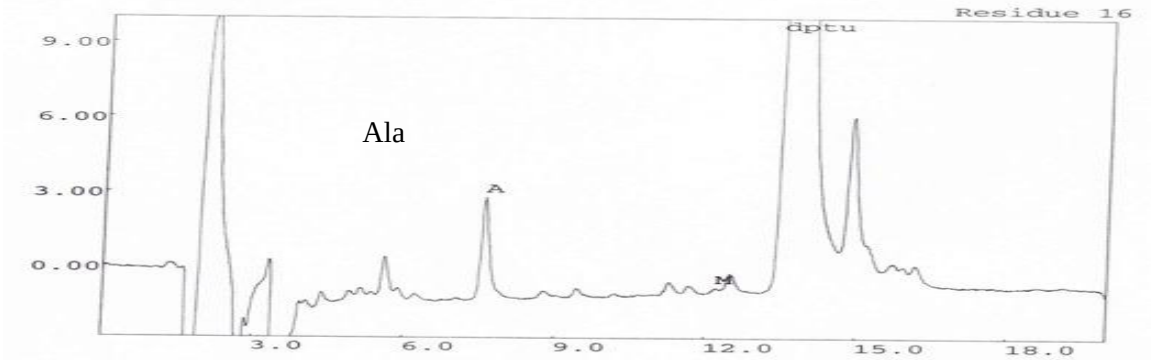


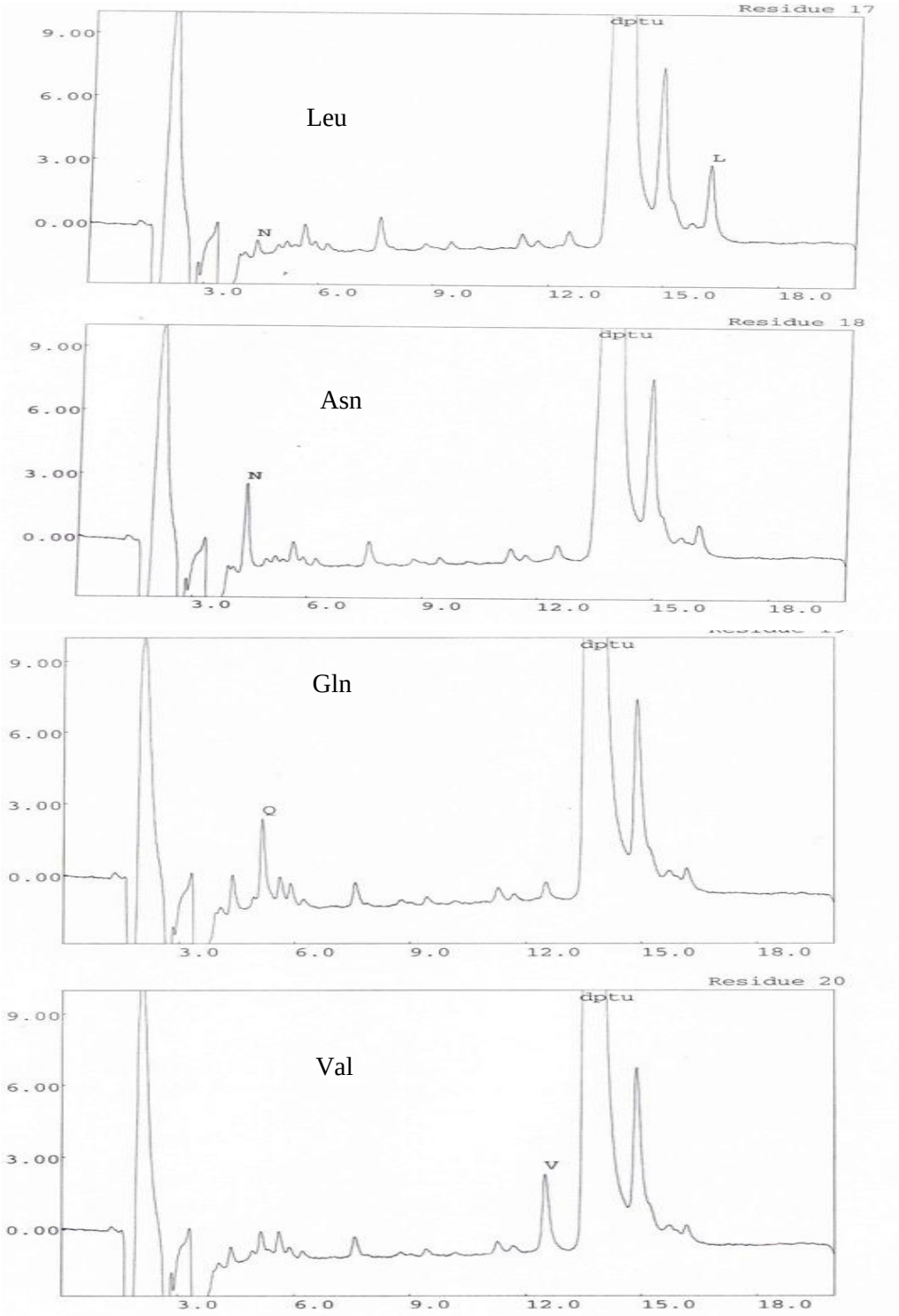


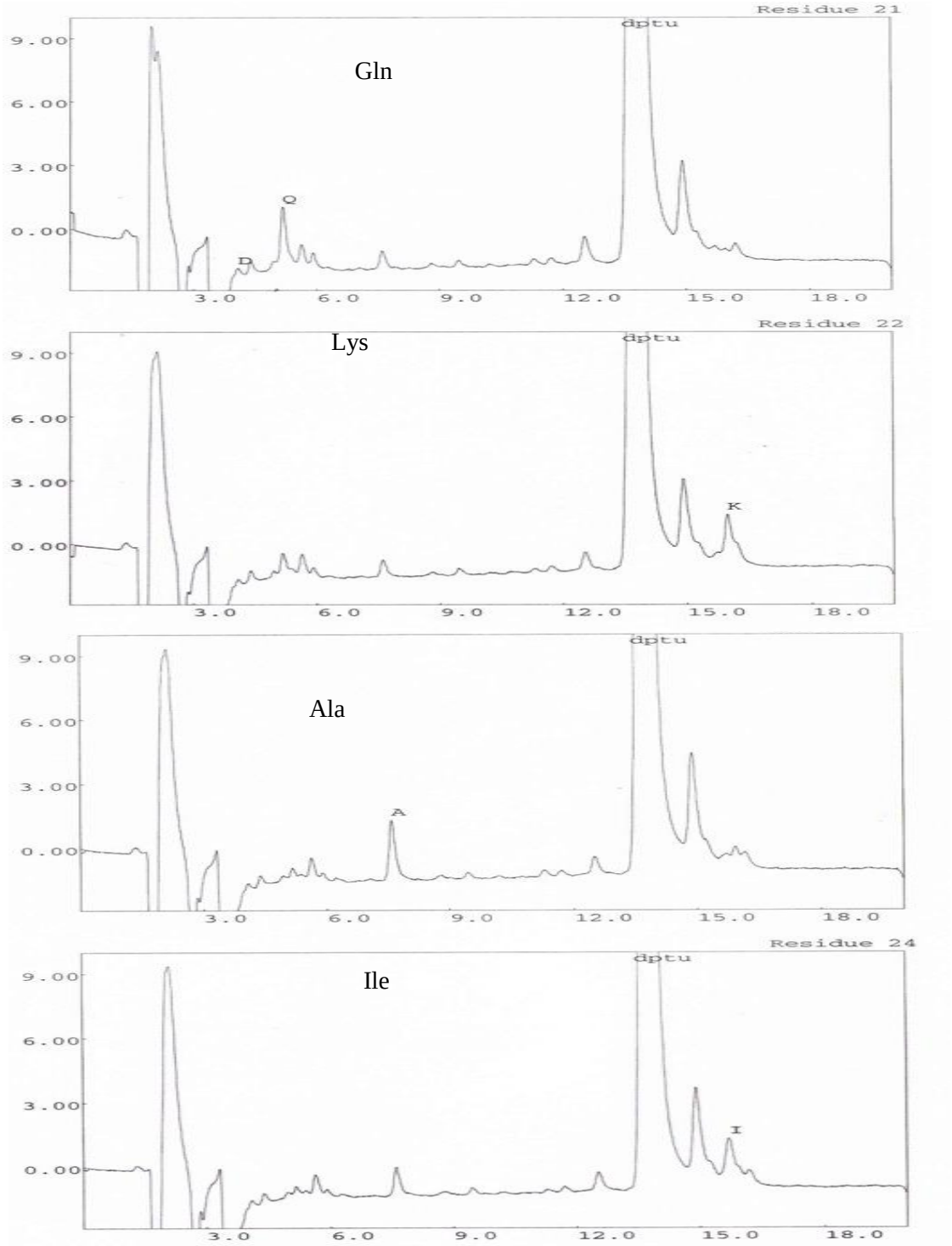


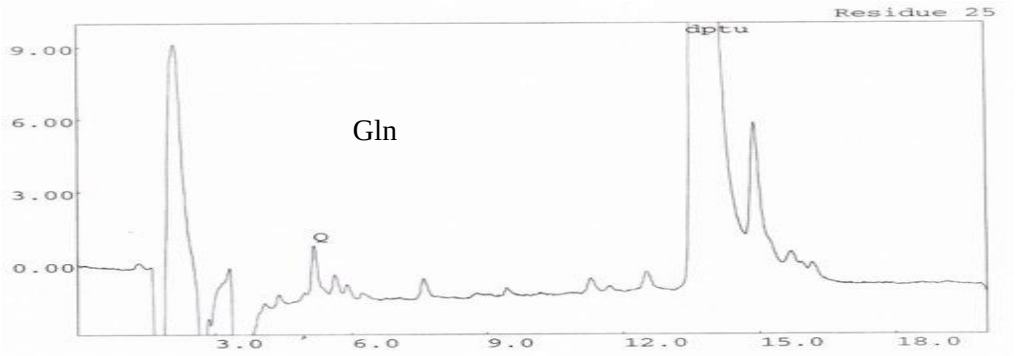


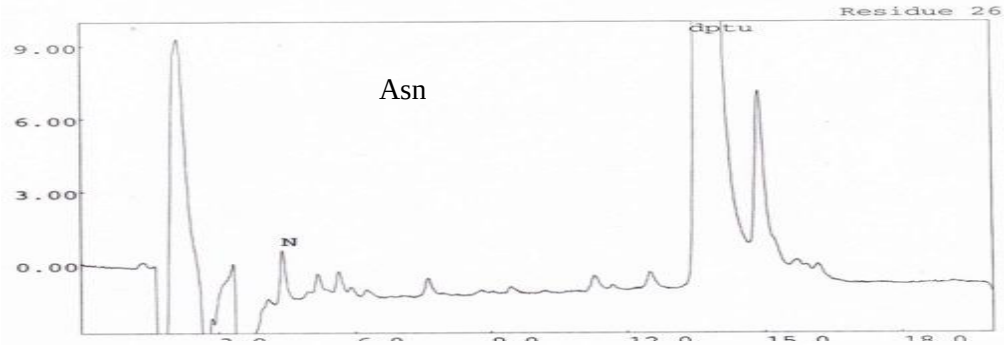


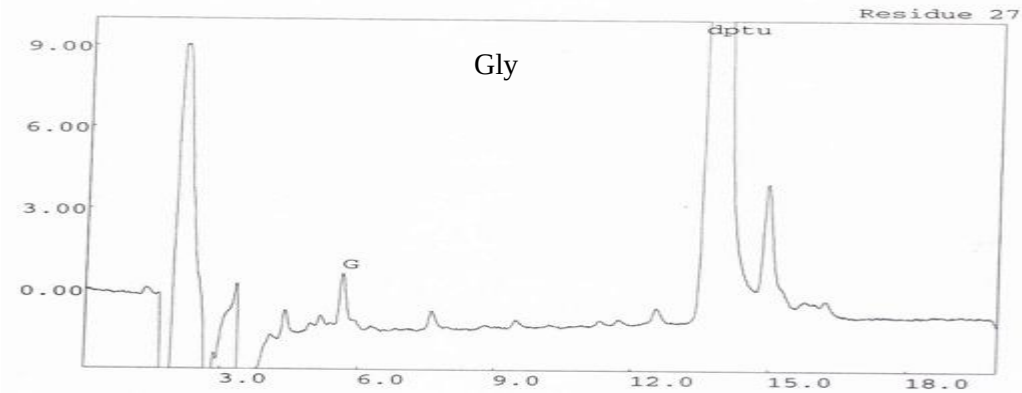


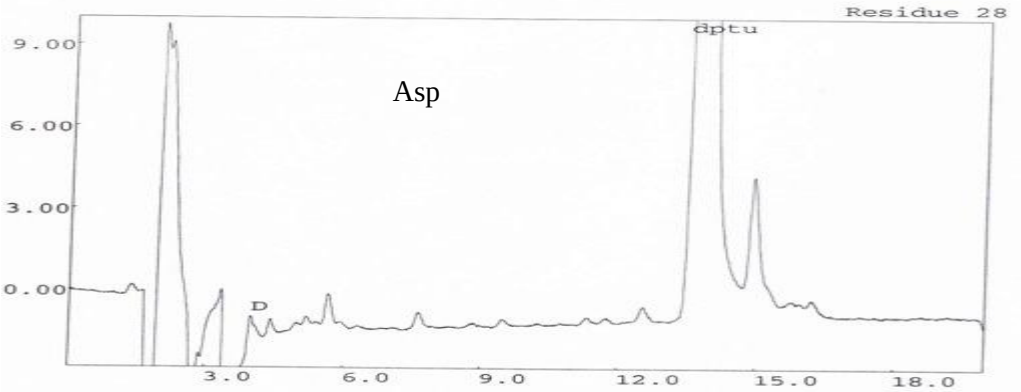


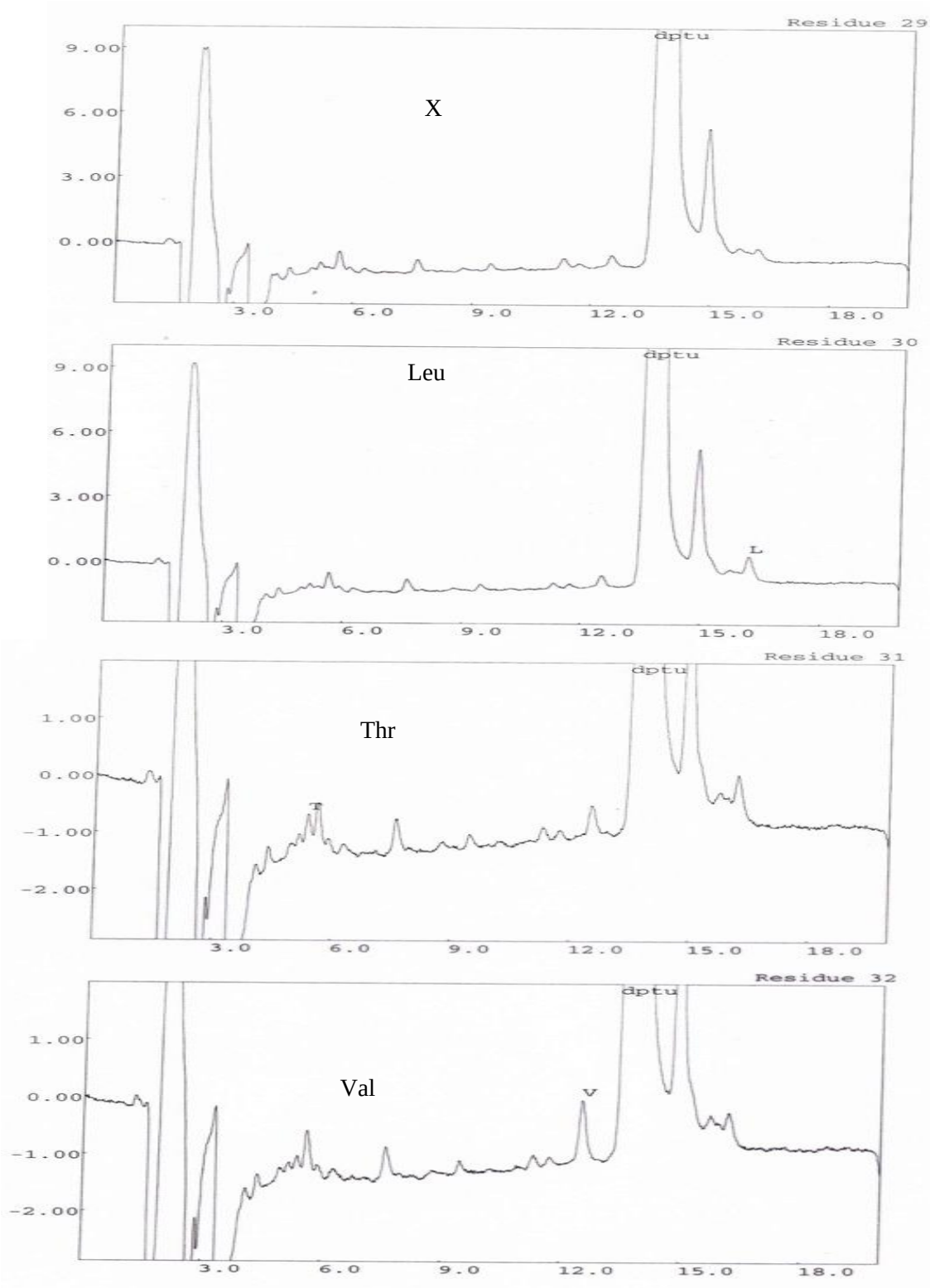


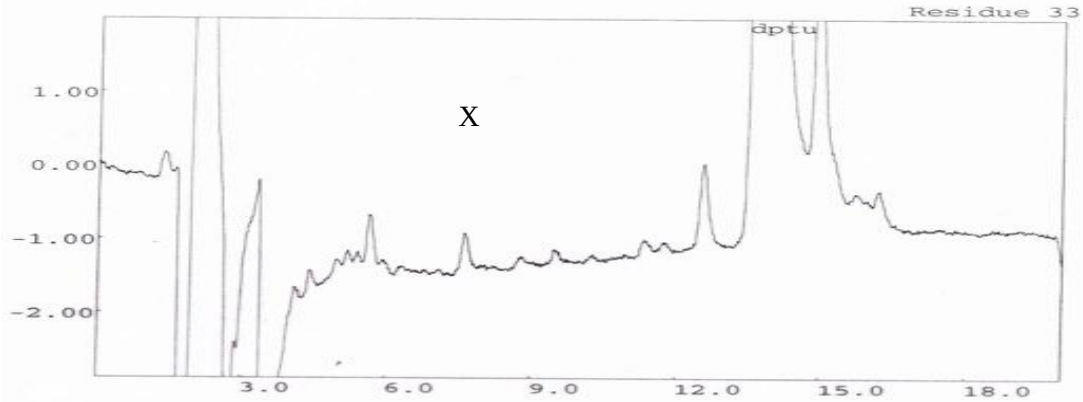


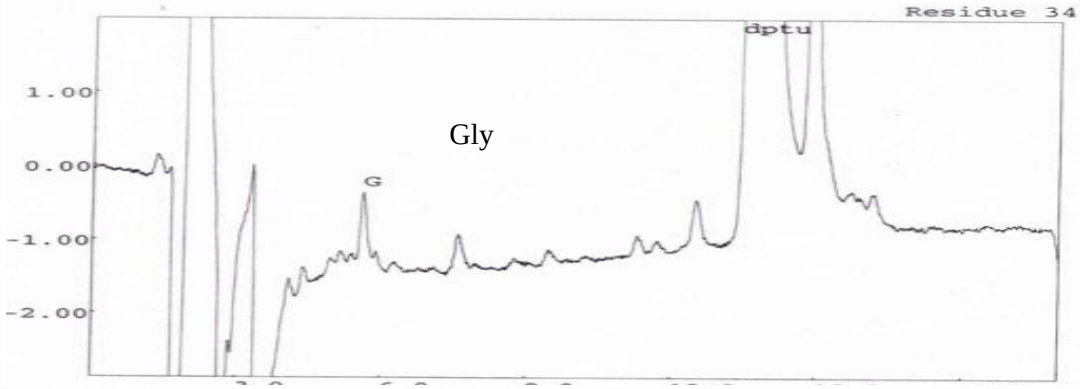


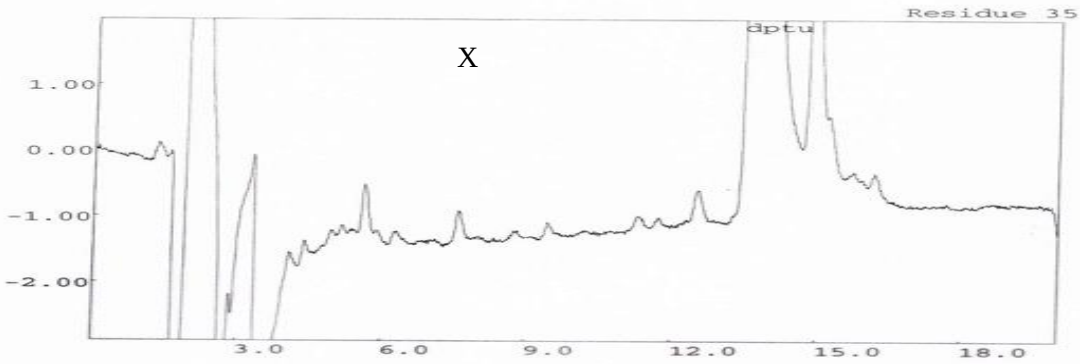


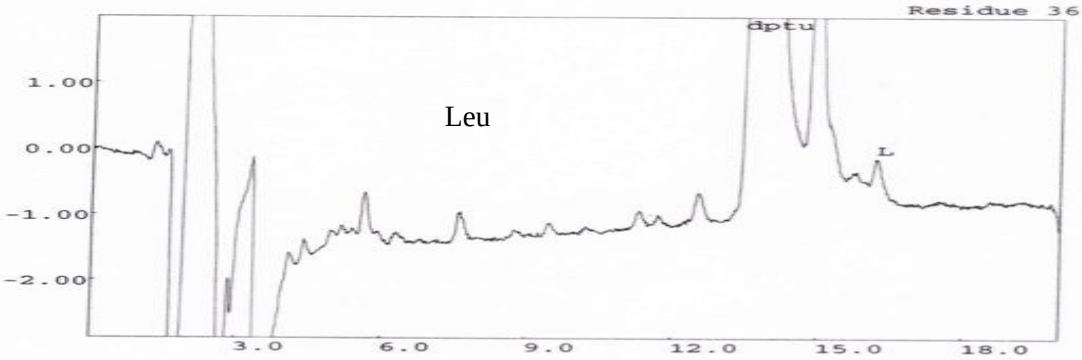


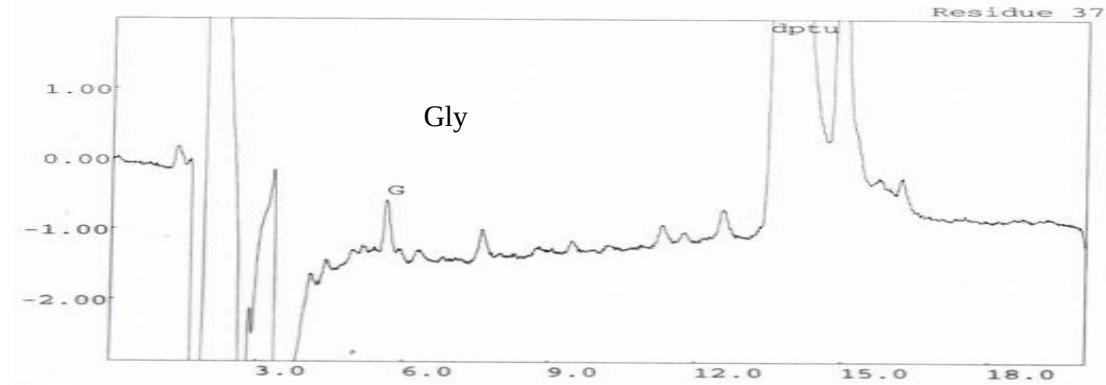


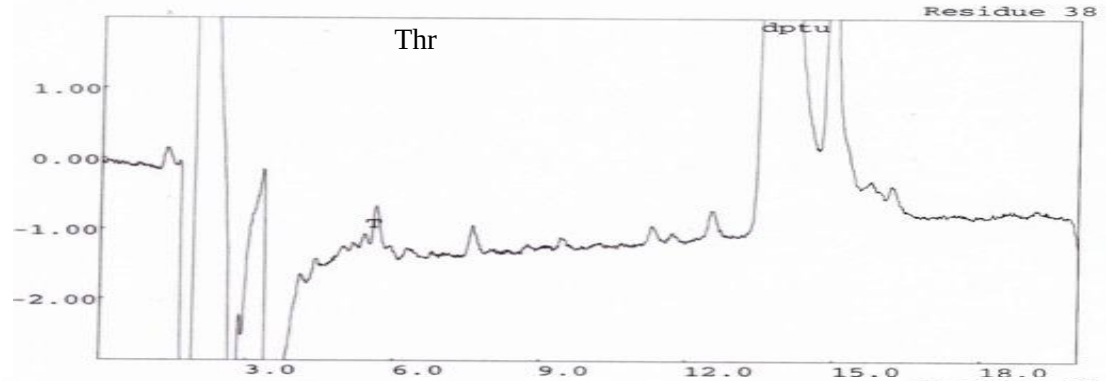


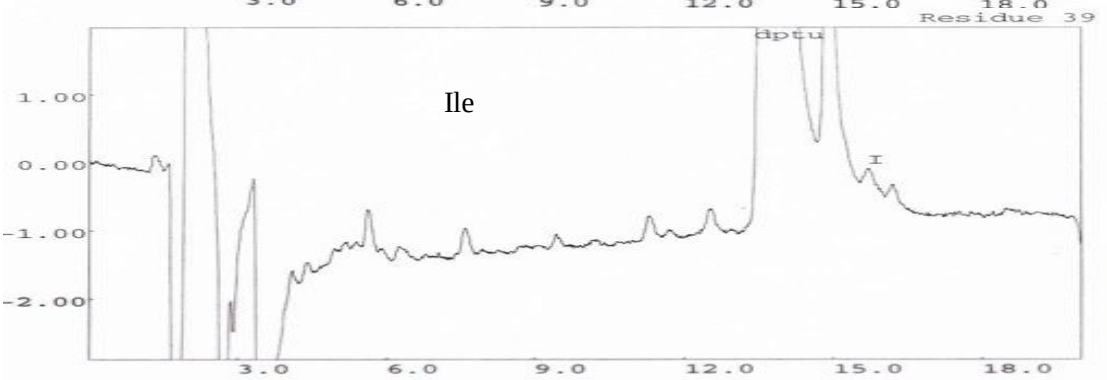


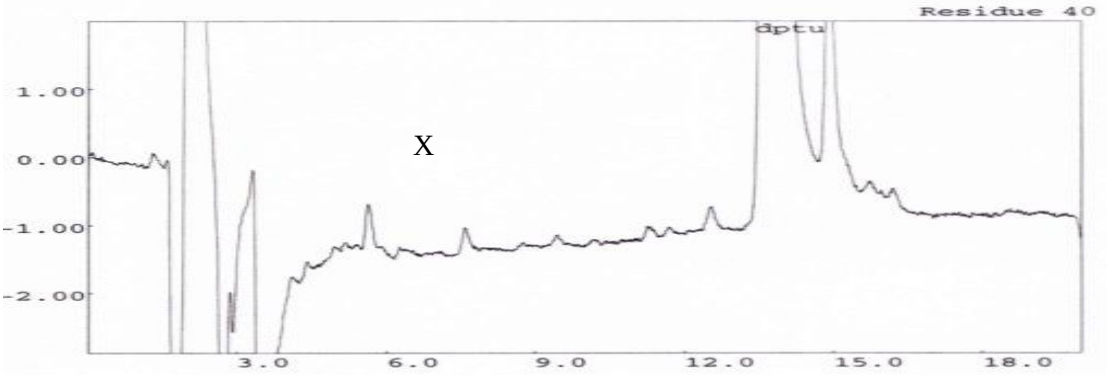


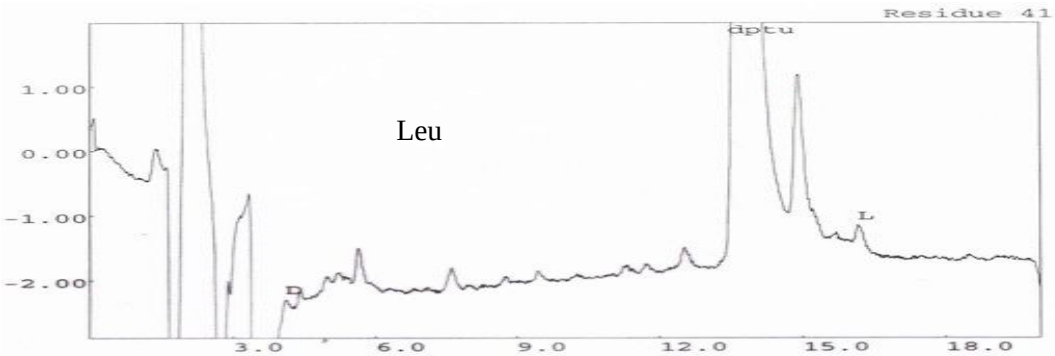


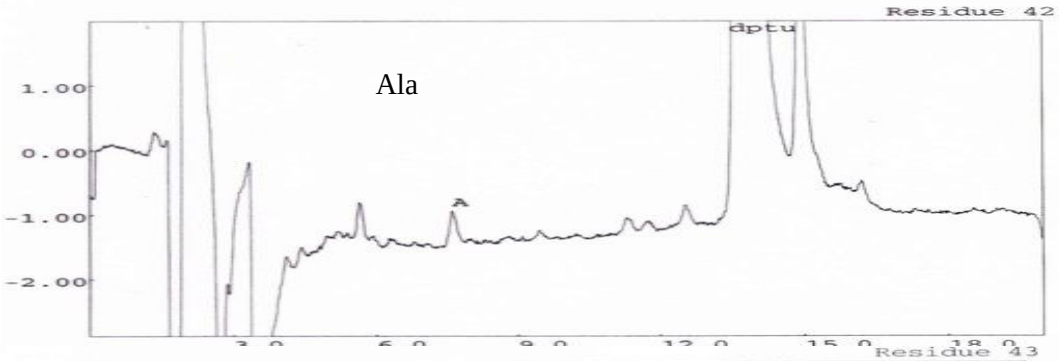


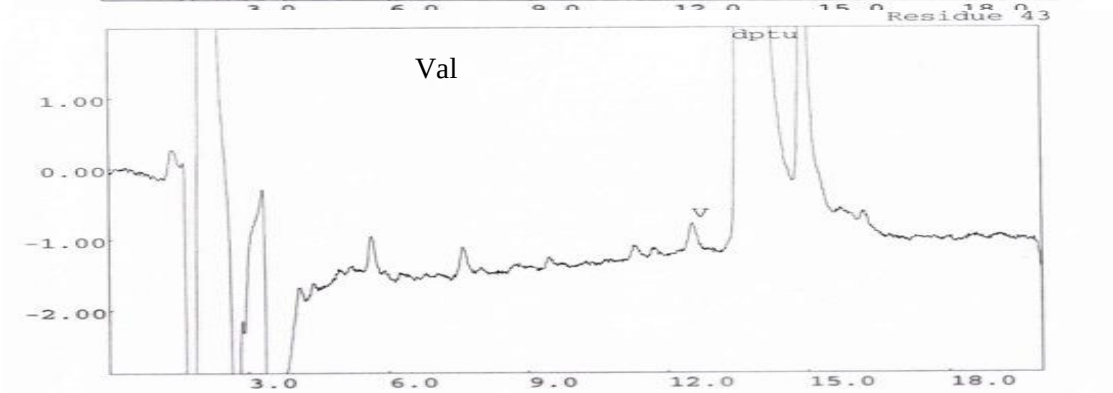


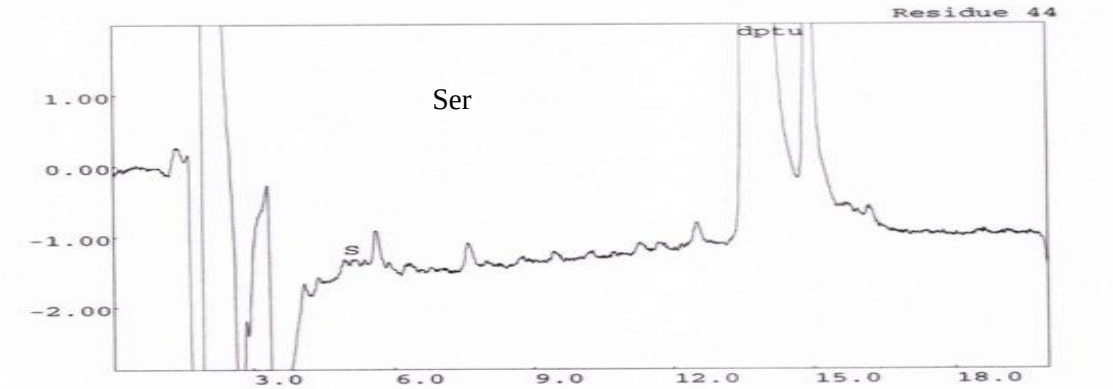


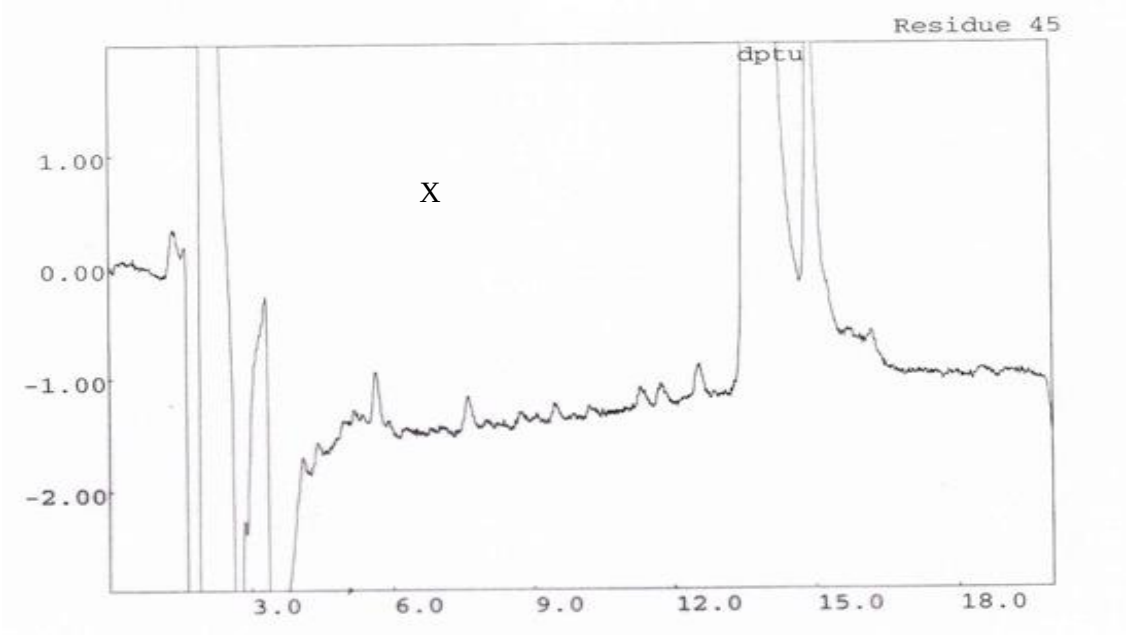












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