SCREENING AND CHARACTERIZATION OF PROTEIN HYDROLYSATES FROM AGRICULTURAL WASTES, AGRO-INDUSTRIAL WASTES AND FISHERY WASTES AGAINST BACTERIAL PLANT PATHOGENS

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Thesis	Screening and characterization of protein hydrolysates from agricultural wastes, agro-industrial wastes and fishery wastes against bacterial plant pathogens
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Abstract

Non-edible materials like agricultural wastes can serve as sources of antimicrobial peptides (AMPs) effective against bacterial plant pathogens. In this study, fifteen agricultural samples (six agricultural wastes, seven agro-industrial wastes and two fishery wastes) were collected and their protein hydrolysates obtained using pepsin. Peptides smaller than 3 kDa were purified by reverse-phase chromatography, cation exchange chromatography and pI-based fractionation and tested for activity against plant pathogenic bacteria at each step. Active peptides were then analyzed for putative mechanisms using nanoLC-MS/MS and the Mascot program. Ultimately, thirteen candidate peptides originating from bagasse were selected and chemically synthesized for a comparative study of growth inhibition in plant pathogenic bacteria and plant growth-promoting rhizobacteria (PGPRs). Three synthesized peptides exhibited potent activity against plant pathogenic bacteria while also supporting growth of PGPRs. Proteomics analysis revealed the peptides PQLAVF (Pro-Gln-Leu-Ala-Val-Phe) and MDRFL (Met-Asp-Arg-Phe-Leu) to act against *Xanthomonas oryzae* pv. *oryzae* via membrane-active mechanisms; whereas peptide VQLMNSL (Val-Gln-Leu-Met-Asn-Ser-Leu) acted against *Pectobacterium carotovorum* and *Agrobacterium rhizogenes* through intracellular-active mechanisms. Further study remains needed to customize peptides for more effective activity against these and other critical pathogens.

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Chapter 1

Introduction

1.1 Statement and significance of the problems

Bacterial plant pathogens have the proficiency to cause various symptoms like abnormal growths, rots, spots, wilts, cankers, and blights. Many of them can cause the dying to plant using their secreted enzymes that devastate cell wall, live in the xylem and interlope which the transport of water and nutrients in the host plant was disrupted and causing wilt disease. Phytopathogenic bacteria can cause great crop damages which may lead to severe economic losses throughout the cultivated areas. Subsequently of the negative effect on plant health, plant pathogenic bacteria also threat food security in the world. Another considerable fact is that in today life, humans and animals spread bacterial disease by cultivating, consuming, and exporting low-quality plant-based foods.

At present, the disease control relies principally on chemical pesticides. Nevertheless, their consequences on long-term environmental contamination and carcinogenic effects on living organisms significantly limit their future use (Daoubai et al., 2005). Thus, the finding for an effective and environmentally friendly approach is needed.

Antimicrobial peptides (AMPs) are advanced guards for defense against attacking pathogens and work in important function in innate immunity (Park et al., 2004). There are over 5,000 AMPs have been reported in living organisms both in eukaryotes, like plants and animals, and prokaryotes (Zhang and Gallo, 2016). Normally AMPs presented a broad activity to kill parasites, viruses, fungi and bacteria, thereupon AMPs are classified in general as antiparasitic, antiviral, antifungal and antibacterial, respectively (Zhang and Gallo, 2016). The antibacterial activity of peptides results from the amphiphilic qualification and high density of positive charged within their structure. This character allows peptide attachment and insertion into bacterial membrane to generate pore formation resulting in membrane disruption and cell lysis (Powers et al., 2004; Lee et al., 2016).

Currently, several AMPs against plant pathogens were reported such as defensins against *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv *oryzicola* (bacterial pathogen cause bacterial leaf blight and bacterial leaf streak disease on rice, respectively) (Tantong et al., 2016), defensins against phytopathogenic fungi and bacterial (Wu et al., 2011) and melittin against *X. oryzae* pv. *oryzae* (Shi et al., 2016). However, these peptides came from agricultural products,

edible parts of plants. Almost no productive AMPs from wastes have been reported to inhibit bacterial plant pathogens.

Plant growth promoting rhizobacteria (PGPRs) is a group of bacteria that being around plant roots for root exudates releasing and directly promoting plant growth by synthesis growth-promoting compound such as phytohormones, vitamins, enzymes. In relation to the indirect growth promotion, PGPRs inhibit plant pathogens by synthesize of antagonistic materials and effect to resistance against pathogens (Gray and Smith, 2005). Genus of bacteria belonging to PGPRs includes *Bacillus* and *Pseudomonas* (Bhattacharyya and Jha, 2012). Although PGPRs play an important role in agriculture, but reports in the past years explaining effect of AMPs against PGPRs were not apparently proven or appeared. Then, this research will find AMPs that affect only on plant pathogens and non-effect on PGPRs.

The major objective of the present research is to determine the antibacterial activity of AMPs from agricultural wastes, industrial wastes and fishery wastes against plant pathogenic bacteria. Its mechanism of action will be investigated at the translational level.

1.2 Objectives

1.2.1 To determine the antibacterial activity of lower 3 kDa protein hydrolysates from agricultural wastes, industrial wastes and fishery wastes against bacterial plant pathogens.

1.2.2 To purify active peptides from protein hydrolysates.

1.2.3 To study antibacterial mechanism of active peptide against plant pathogens using proteomics technique.

1.3 Research hypothesis

Protein hydrolysate from agricultural wastes, agro-industrial wastes or fishery wastes has antibacterial activity against some plant pathogens.

1.4 Scope of the study

1.4.1 Sample of study: agricultural wastes, agro-industrial wastes and fishery wastes.

1.4.2 Place of study: National Center for Genetic Engineering and Biotechnology (BIOTEC) Thailand Science Park, Phahonyothin Road Khlong Nueng, Khlong Luang, Pathum Thani 12120.

1.4.3 Duration of study: August 2019- July 2022.

1.5 Process of the study

Agricultural wastes samples were collected from different agricultural areas of Thailand and total proteins were extracted and prepared the protein hydrolysates with pepsin. Then protein hydrolysates were purified by reverse-phase chromatography, cation-exchange chromatography and off-gel fractionation, respectively, and antibacterial activity of all fragments was determined using broth dilution assay after every step of purification. Effective peptide fractions were analyzed by LC-MS/MS and peptides were synthesized and studied the mechanisms by proteomics techniques.

1.6 Definition

1.6.1 Protein hydrolysates

Protein hydrolysates are biological compound produced by chemically or biologically hydrolysis at peptide bonds of proteins. In this work, pepsin was utilized as proteolytic enzyme because of its specific cleaves location property at Phenylalanine and Leucine.

1.6.2 Agricultural wastes

Agricultural wastes are wastes or unwanted parts produced by agricultural process. Agricultural wastes used in this work are comprised of rice straw, corn cobs, corn leaves, corn cob leaves, sugarcane leaves, and bagasse.

1.6.3 Agro-industrial wastes

Agro-industrial wastes are unutilized parts produced by agricultural-based industries. For the present research, agro-industrial wastes are fermented soybean, soybean pellet, peanut seed coat, coconut residues, coffee ground, fish residues and desalted fish residues.

1.6.4 Fishery wastes

Fishery wastes are unwanted parts of fish in fishery process. For this research, two samples of fish residues: *Nile tilapia* fish fin and *Clarias* sp. fish fin.

1.6.5 Antimicrobial peptides

Antimicrobial peptides are small amino acid residues having antimicrobial property against microbes such as viruses, bacteria, fungi and parasites. In this study, antimicrobial activity against plant pathogen bacteria was significantly focused and observed.

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Chapter 2

Literature reviews

2.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are small peptides from 5 – 50 amino acids long having antimicrobial activity. AMPs have broad targeted organisms such as viruses, bacteria, fungi and parasites.

At present, more than 5,000 AMPs have been discovered or synthesized (Zhao et al., 2013). AMPs can be found in both prokaryotes (bacteria) and eukaryotes (fungi, plants, and animals). In animals, AMPs are the first barrier of innate immune against viruses, bacteria, and fungi, essentially found in the tissues that are exposed to airborne pathogens. As it has been reported, AMPs play an important role in blocking most infections before they cause any symptoms in hosts (Radek and Gallo, 2007).

Some AMPs target the lipopolysaccharide layer of microbe cell membrane. Some AMPs can kill microorganisms after contact with cell membrane in seconds (Loeffler et al., 2001). Some AMPs kill microbes in the same way as antibiotics that target specific cellular activities such as synthesis of DNA or proteins; whereas some AMPs can improve the activities of antibiotics through synergistic effects compared to using antibiotics alone (Naghmouchi et al., 2012).

2.1.1 Classification of AMPs

There are many classes of AMPs based on their target including bacteria, fungi, viruses, and parasites.

At the present, antibacterial peptides are the most studied AMPs. Most of them are cationic and amphipathic AMPs that target bacterial cell membranes and cause pore formation of the lipid bilayer structure. Amphipathic structure (both hydrophilic and hydrophobic domains) provides AMPs the capability to bind lipid compounds (hydrophobic region) and phospholipid groups (hydrophilic region) (Shai, 2002). Some AMPs at low concentrations can destroy bacteria without changing the membrane structure. Instead of attack with membrane, these AMPs inhibit some important pathways inside the cell such as DNA replication and protein synthesis (Brogden, 2005).

Antifungal AMPs can destroy fungi by targeting either the chitin in cell wall or intracellular components (Pushpanathan et al., 2012). Either way, antiviral AMPs neutralize viruses by combining with either the viral envelope or the host cell membrane. Previous studies have shown that both enveloped RNA and DNA viruses can be targeted by antiviral AMPs (Horne et al., 2005).

The last group of AMPs is antiparasitic peptides. These are a smaller class compared to other classes. Although some parasites are multicellular, the mode of action of antiparasitic peptides is the same as other AMPs. They destroy cells by directly interacting with cell membrane (Park et al., 2004).

2.1.2 Mechanisms of AMPs

As described above, AMPs affect cells by interrupting membrane structure (interaction with negatively charged cell membrane). By this, they result in the blocking process of proteins, DNA and RNA synthesis as well as interact with intracellular targets.

Normally each AMP is only effective against one class of microorganisms but there are exceptions, some AMPs have different modes of action to different types of microorganisms. AMPs' rapid killing effect does not only generate from membrane disruption, but they can also result from inhibition of these functional proteins. Thus, two modes of action, membrane active and intracellular active, are interacted.

Most membrane-active AMPs are amphipathic, which have both cationic and hydrophobic parts. This characteristic certifies the initial electrostatic interaction with the negatively charged cell membrane and the insertion into cell membrane. As shown in figure 2.1, there are three mechanism models of membrane-active AMPs. Barrel-stave model, AMP molecules insert themselves into the membrane vertically, while carpet model show small parts of the membrane are covered with hydrophobic sides of AMPs. Toroidal pore model, AMPs make a peptide-microbe complex in membrane (Bahar and Ren, 2013).

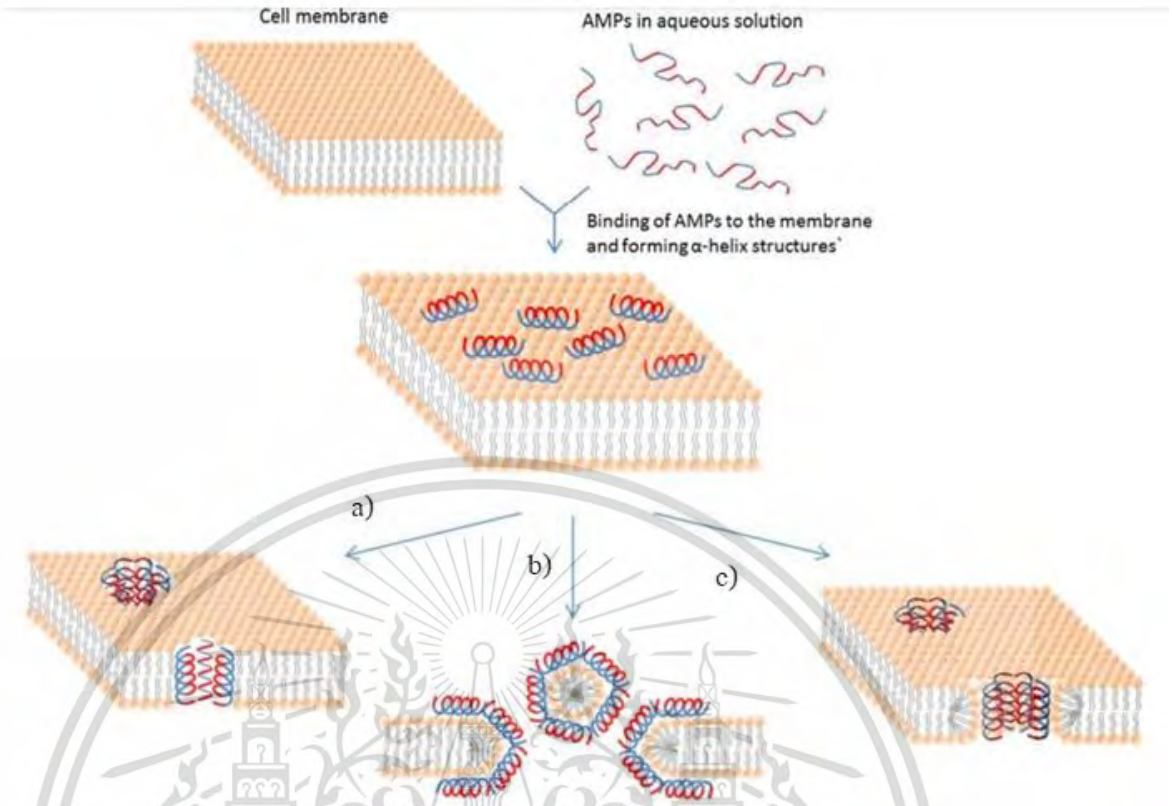

Figure 2.1 Mechanism models of membrane-active AMPs a) barrel-stave pore b) carpet mechanism and c) toroidal pore (Bahar and Ren, 2013).

Intracellularly active AMPs can destroy microbial cells without causing membrane permeabilization. These AMPs target inside the cells. Some AMPs can block DNA and protein synthesis. Some AMPs can also inhibit proteases of microorganisms (Otvos, 2005).

AMP from *Apis mellifera*, melittin, was investigated by examining its consequences on cell membranes, energy metabolism, and nucleic acid synthesis. The melittin disrupts membrane by making holes resulting in the leakage of cytoplasm. In addition, melittin may inhibit biosynthesis in both DNA and proteins (Shi et al., 2016).

2.1.3 Physiochemical properties of AMPs

Some properties of AMPs structure are required for improve antimicrobial activity (Tossi et al., 2000; Lee et al., 2002). The size of an AMP is one of important characteristic because at least 7 – 8 amino acid residues are necessary to form amphipathic structures. The length for an

AMP to interact lipid bilayer of bacteria in the barrel-stave model (a prototype of peptide-induced transmembrane pores) should be at least 22 amino acids for α-helical AMPs, while eight amino acids are needed for β-sheet AMPs. Moreover, the size of an AMP may also affect its cytotoxicity (Figure 2.2).

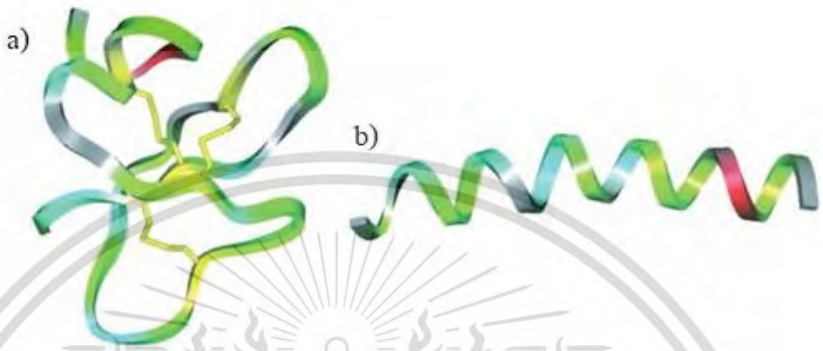

Figure 2.2 Structure of AMPs. a) β-sheet AMPs and b) α-helical AMPs (Dösler, 2017).

The net charge of AMPs is the main portion for primary interaction with negatively charged cell membranes. Modification of the AMP net charge, antimicrobial and hemolytic activities can be differed. Hydrophobic property of AMPs also plays a key role to control the activity and selectivity of AMP molecules. Almost 50% of normal AMPs are hydrophobic residues. Many studies found that increasing hydrophobicity on the positively charge can increase its antimicrobial activity, while decreasing hydrophobicity can reduce antimicrobial activity. Moreover, AMPs also need to be soluble in aqueous environments to interact on or enter through lipid membranes (Lee et al., 2002).

2.1.4 Application of AMPs in agricultural works

Plant diseases are significant matters in agriculture, and AMPs are known as possible alternatives to chemical control because of their effective broad-spectrum antimicrobial activity with low toxicity. Most AMPs can target pathogens in an unspecific way, and pathogens hardly develop resistance to AMPs, which makes AMPs quite appropriate for developing plant disease resistance. The ability of AMPs to battle plant pathogens has been revealed by the expression of AMPs in transgenic plants (Goyal and Mattoo, 2014). For examples, defensin Mj-AMP1 in tomato enhanced tomato resistance to *Alternaria solani* (Schaefer et al., 2005), defensin Rs-AFP2 in

tobacco and tomato enhanced the resistance to *Amanita longipes* (Terras et al., 1995). In 2021, amphipathic helical peptides were designed with different spatial distributions of positive charges and found that AMPs that had a special pattern of B (basic residue) and H (hydrophobic residue). The sequence "BBHBBHHBBH" showed excellent bactericidal and fungicidal activities against important plant pathogens like *Pectobacterium carotovorum* subsp. *carotovorum* and *Botrytis cinerea* (Chen et al., 2021).

Moreover, studies on deletion or overexpression of the defensin DEF2 in tomatoes showed that it could considerably affect tomato pollen viability, seed production, and plant morphology (Stotz et al., 2009). the α-hairpinin TK-AMP in *Triticum kiharae* can be induced by abiotic and biological stresses (Utkina et al., 2013). These studies showed that AMPs do not only behave as innate immunity against bacterial plant pathogens, but they are also involved in many aspects of plant lives.

There are several patents investigated protein hydrolysates preparation and their applications in antibacterial roles. In 2012, Murugesan et al. studied agro-industrial wastes as high-protein sources for protein hydrolysates preparation. They hydrolyzed agro-industrial wastes by microbial deproteinization using *Bacillus megaterium* PB4 as an enzyme producer. Therefore, they found that the deproteinization efficiency was 83.50%. After the effective experiment, the protein hydrolysates with pH 4.72 was obtained. Seven essential amino acids and four non-essential amino acids also found. Then they studied the effect of protein hydrolysates on growth and development of numbers of crops and animals, and found that protein hydrolysates enhanced plant height, root length, leaf area and chlorophyll content of plants. Moreover, waste-protein hydrolysates also performed better quality as an animal food.

In 2000, proteins from Korean native goat milk were hydrolyzed by pepsin to obtained protein hydrolysates and peptides. Then, purification using methods of chromatography was applied to get the strongest antibacterial activity protein hydrolysates compared with lactoferrin, protein-carbohydrate compound extracted from cow milk. These protein hydrolysates were used in antibiotics, feed additives, human food, cosmetics and antiseptics (Myeong et al., 2000). In 2017, chilli seed was interesting because of their low cost, abundant and high protein composition. Protein from chilli seed was prepared for hydrolyzed to be protein hydrolysates. Initially, crude protein from chilli seed was extracted by greased to be powder and hydrolyzed by enzyme. Then antibacterial activity was experimented. The chilli seed protein hydrolysates were purified by gel

chromatography and deionized water elution. This method was selected to study because of low cost, high efficiency and fast preparation (Haiwei, 2017).

2.2 Bacterial plant diseases

Plant pathogenic bacteria cause many serious diseases of plants throughout the world. Infected plants develop visible symptoms after certain latent periods. Four bacterial plant pathogens will be studied in this research include *X. oryzae* pv. *oryzae*, *X. citri*, *Pectobacterium carotovorum*, and *Agrobacterium rhizogenes.*

2.2.1 *X. oryzae* pv. *oryzae* causing bacterial leaf blight disease

X. oryzae pv. *oryzae* cause bacterial leaf blight disease (BLB) on rice, one of the most serious diseases of rice, which results in immense losses in rice yield in much Asia and parts of Africa. *X. oryzae* pv. *oryzae* cause the disease by invading vascular tissue. Over 30 races of *X. oryzae* pv. *oryzae* have been reported and it exhibits genetic variation among isolates (Rajeshwari et al., 2005).

2.2.1.1 BLB symptoms and dissemination

X. oryzae pv. *oryzae* infects rice through wounds, stomata or hydathodes at the leaf tip and leaf margin or straight through openings caused by emerging roots at leaf sheath base, moves to xylem vessels and multiply in the intercellular spaces. Inside xylem, *X. oryzae* pv. *oryzae* likely interacts with xylem parenchyma cells (Hilaire et al., 2001). Within a few days, xylem vessel is filled with bacterial cells and extracellular polysaccharide (EPS). Ooze, droplets composed mostly of bacteria, is leaked out from hydathodes and forming droplets of exudate on leaf margin, this is the specific sign of BLB and the beginning of secondary inoculum (Goto, 1992). BLB has three definite symptoms: leaf blight, kresek (seedling blight that occurs quickly after transplant from nurseries to the ground) and pale-yellow leaves. Leaf symptoms of BLB normally become obviously at the tillering stage, green drench spots at the tips and margins of completely developed leaves. The spots extend along the vessels and then necrotic, become greyish-white colored lesions that regularly expand from leaf tip vertically along the leaves (Mew, 1987). While pale-yellow leaf is noticed in aged plants, older leaves appear normal green, whereas younger leaves are equally pale or whitish yellow. Moreover, tillers do not grow completely (Goto, 1992).

Hosts of *X. oryzae* pv. *oryzae* are several wild rice species (*Oryza sativa, O. rufipogon, O. australiensis*) and various gramineous grasses (*Leersia oryzoides*, *Zizania latifolia*, *Leptochloa* spp., *Cyperus* spp.). In the tropical zone, *X. oryzae* pv. *oryzae* survives throughout the year by the exist of high temperature, humidity and plentiful of host plants. While in temperate zone, the pathogen remains in soil for a few months depending on acidity and moisture. Eruption of BLB is probably occur pending rainy or monsoon season of south-east Asia than other times of the year because wind and rain blow bacteria from infected rice plants to other hosts. Moreover, the primary inoculum comes from contaminated rice stumps from previous season. Severe widespread of BLB often occurs during fierce winds or typhoons. Human and animals also be a dispersal helper as well as irrigation water (Sakthivel et al., 2001).

2.2.1.2 The control of BLB disease

Disease management for BLB consist of cultural practices, biological and chemical controls. Depending on disease incidence records and the farm location, benefits of cultural practices differ. The proper culture practices begin with management in nursery stage include seed disinfection, suitable nursery drainage, removal of diseased weeds and debris. Before transplanting, disinfected field by burning rice straw from previous season is necessary. Weeds are removed from water-supply to reduce natural pathogen habitats and prevent disease dispersal through irrigation water. The careful fertilization and proper plant spacing are also suggested culture manners of control. Appropriate fertilization by avoiding from access of nitrogen because it stimulates speedy plant growth, that support disease development. Usual practices of fertilizer application are getting rich in phosphorus and potassium at the height tiller stage after a flood or a typhoon (Goto, 1992).

For biological control, specific bacterial strains have been used for BLB biological control. For example, powder formulations and bacterial suspension of plant growth-promoting rhizobacteria such as *Bacillus* sp. and *Pseudomonas* sp. These ways of control are suitable for disease control because they are environmentally friendly. However, biological controls are limited from pathogen diversity and the lack of proper biological agent (Kim et al., 2016).

Farmers have used chemicals to cope with BLB since 1950s with application of copper compounds, mercuric compounds and several antibiotics. The results from laboratory testing indicated that mercuric compounds and streptomycin derivatives were most productive, however, they were found ineffective when sprayed at the heading stage (the panicles are observed)

in the field (Mizukami and Wakimoto, 1969; McManus et al., 2002). Disinfection of plant seeds using mercuric compounds, antibiotics and hot water is workable in many countries in southeast Asia. While temperate regions apply chemical control with probenazole to agricultural water before and after transplant stage for restrain bacterial multiplication. In addition, phenazine oxide, tecloftalam and nickel dimethyldithiocarbamate were used to spray directly on rice plants (Goto, 1992; Mizukami and Wakimoto, 1969). Application of bleaching powder containing 30% chlorine also reduced leaf blight lesion in rice (Chand et al., 1979). Several antibiotics such as ampicillin, kanamycin, streptomycin, chloramphenicol, oxytetracycline, gentamycin, sinobionic and benzylpenicillin were also examined *in vitro* and found that they could inhibit growth of *X. oryzae* pv. *oryzae* (Khan et al., 2012).

2.2.2 *X. citri* causing citrus canker disease

Citrus canker is a bacterial disease caused by *X. citri* influencing all economic citrus varieties. This has been concentratedly studied in the past several years. Citrus canker is normally classed into three types: A, B, and C. By this, type A is believed to have initiated in Asia, being the most frequently and causing the most significant financial damage. Type B (or false canker) was first identified in Argentina in 1923, and present only in Argentina, Paraguay, and Uruguay, while type C is confined to the state of São Paulo, Brazil. Types B and C are determined attenuated forms of type A (Graham et al., 2004).

2.2.2.1 Citrus canker symptoms and dissemination

Canker regularly appears on trees of susceptible hosts both young and adult in most citrus growing regions from late summer through to autumn. Canker lesions are occurred on the fruit peels, leaves, stems and shoots of citrus looks like scabs or crater-like lesions. The lesion may disperse one by one or many lesions may happen together over the fruit peel. Gummy exudation may be observed on unripe infected fruits. In moist conditions, the lesion enlarges more rapidly than in dry condition. Conversely, the wound remains unruptured and is greasy at the edge. On leaves, light yellow blemishes are found on the underside, changed into brownish lesions on both sides of the leaves, then rough and cracked. The severe symptom relies on the susceptibility of the host plant cultivars. The spotted area may differ in shape and in size from 0.5 to 1 cm. Other bacteria like *X. alfalfae* subsp. *citrumelonis* and *X. fuscans* subsp. *aurantifolii* can cause citrus

canker-like symptoms, however, both have a limited host range and cause less violence (Schaad et al., 2006).

About dissemination capability, *X. citri* subsp. *citri* can survive in diseased plant tissues as epiphyte on host and as saprophyte in soil. When windy rain and spattering of water come, the bacteria are circulated by rain pouring over the surface of lesions and then splashing onto healthy hosts. Moreover, wounds can made by thorns and insects, even from attacks of *Phyllocnistis citrella.* The movement of contaminated plant material, such as young rootstocks and budded trees, has been involved in long-term dispersal. There has not been reported as seed-borne pathogen (Hall et al., 2010).

2.2.2.2 The control of citrus canker disease

Periodic spraying of insecticides is performed to control leaf miner. Copper compounds or streptomycin compounds may be applied by spraying every week during the active growth of new shoots. These control measures are focused on preventing primary infection on spring shoots. Control of the beginning stage is mainly performed by using abamectin and neonicotinoid insecticides (Powell et al., 2007; Stein et al., 2007).

2.2.3 *P. carotovorum* causing bacterial soft rot (BSR) disease

P. carotovorum (was formerly classified as *Erwinia carotovorum*) is an opportunistic pathogen that require proper environmental conditions and a susceptible host to cause BSR disease. The disease occurs in several crop plants especially in carrots, potatoes, corns and ornamental plants. It can be generally found in the root zones of various crops. Ornamentals host plants include ornamental pepper, begonia, geranium, dahlia hybrids, and cactus (Gardan et al. 2003, Glasner et al. 2008, Jittikornkul et al., 2017).

2.2.3.1 BSR symptoms and dissemination

P. carotovorum is a member of soft rot group bacteria. When cells infect and colonize in the intercellular spaces of plant cells, they send an influential effector molecules AVR to plant cells through the type III secretion system, causing specific symptoms. The major symptoms are wilting and water-soaked lesions on the outside of fruits and vegetables, finally these lesions will sink and form pits which may lead to stem collapse. The pathogen may penetrate susceptible hosts through wounds in the petiole, which will move to the stem. This is another way

leads to the collapse of the plant. BSR symptoms may include pith browning or darkening, especially in infected ripe fruits. Changes rapidly occur after stems collapse, skin folding and cracking then developing of creamy white ooze. These symptoms occur within 48 hours after infection (Nazerian et al., 2011).

Expanded periods of soil soaking in agricultural area can simplify *P. carotovorum* infection. High humidity and heavy rainfall are required for the dissemination of this pathogen. Contaminated irrigation water is an essentially source of inoculum. Other sources may be contaminated potting media or soil, infected plant debris or insect vectors. Bruising also allows for entry of the pathogen. Unsanitary storage facilities also induce the pathogen into the plant. Moreover, hosts injured from fungal diseases are also become infected with this pathogen. (Nazerian et al., 2011).

2.2.3.2 The control of BSR disease

The disease may be controlled by cultivating resistance cultivars if they are available. Farmers should plan crop rotation and apply field sanitary. Farming practices are carefully performed not to injure crops. Control measures are taken against insects. Field is well drained. The chemical control, oxolinic acid and copper compounds can be regularly sprayed as a solution in a period of 7 to 10 days when disease inflicts (Waleron et al., 2014).

2.2.4 *A. rhizogenes* causing hairy root disease

A. rhizogenes is a soil-borne pathogen that cause of hairy root disease. The pathogenicity of this pathogen is caused by transferring of DNA from the bacteria to wounded plant cells. This transfer-DNA (T-DNA) contains oncogenes whose expression transforms the plant recipient cell into a rapidly dividing cell as well-known in *A. tumefaciens*.

2.2.4.1 Symptoms and dissemination

The hairy root disease is characterized by a heavy growth of adventitious roots at the site of infection. The most important *A. rhizogenes* oncogenes (*rol* genes) are also encoding proteins involved in the regulation of plant hormone metabolism. Importantly, the transformed roots will exhibit excessive root (crazy roots) structure and growth is the risk of disease. The *rol* genes are potential activators of the secondary metabolism regulation in transformed cells from the Solanaceae, Araliaceae, Rubiaceae, Vitaceae and Rosaceae families. In greenhouse vegetables, the

roots overgrow in the substrate, and in severe epidemics the drippers get clogged. In tomato, excessive vegetative growth occurs (Bulgakov et al., 2008).

The beginning of infection by *Rhizobium rhizogenes* has not yet been vividly determined. The assumption is the bacteria can survive in soil, but it is possible that first infection is from the propagation material. Furthermore, infection is also taken place via wounds. These are dispersed by rain and wind or may be humans and tools (Bulgakov et al., 2008).

2.2.4.2 The control of hairy roots disease

The most popular strategy to control the hairy roots disease is good agricultural practices. Using clean propagation material and a strict hygiene protocol, including disinfection of tools are recommended. Adjustment of the work order in greenhouses to fit with the hygiene, disinfection of the nutrient solution in order to prevent biofilm growth inside the irrigation system and prevention of contact between the plant roots and the gutters in greenhouses are also the good practices (Chen and Otten, 2017).

2.3 Wastes

The Pollution Control Department (PCD) reported in 2015 that the water quality of major rivers flowing into the upper Gulf of Thailand had seriously polluted in the last several years. The department found the lower Chao Phraya River, which flows through Bangkok, contains bacteria and nutrient pollution from biological compounds of phosphorus, and nitrogen. Nutrient pollution causes algae to grow faster and damaging water quality. The consequence also decreases the oxygen that fish need for their survived. PCD categorised water quality at the mouth of Chao Phraya river at Bangkok's Bang Khun Thian District as "very poor" and worse than in the last five years. PCD findings indicated large amounts of wastewater were discharged into the river from households, industry, and agriculture (Wangkiat, 2016). In this research, three groups of wastes (agricultural wastes, agro-industrial wastes and fishery wastes) will be gathered for study.

In agricultural areas, after harvesting there will be lots of agricultural waste left. Expanding agricultural production has absolutely resulted in enlargements of agricultural wastes and agro-industrial by-products. A common agricultural remain is rice straw from rice farms, other plant residues include leaves and bagasse from sugar cane, leaves, cobs and cob leaves from corn. According to the residue potential of main agriculture products assessment in 2001, total

agricultural wastes was about 66 million tons. By this, 22 million tons were used as fuel; whereas about 44 million tons were approximately left unused (Srisovanna, 2004).

During agro-industrial process, large amounts of by-products and wastes generated. Most of these organic wastes contain macro- and micronutrients such as proteins, carbohydrates, fats and minerals. In this study, seven agro-industrial based wastes will be used such as fermented soybean from soy sauce production, soybean pellet from soybean milk production, peanut seed coat from peanut-based snack production, coconut residue from coconut milk production, coffee ground from coffee drink maker, and fish residue from fish sauce production. Moreover, fish fins from fresh-food market will be categorized for the study as fishery wastes.

2.4 Proteomics technique and peptide studies

Proteomics have been introduced for 25 years after they were originally proposed in 1994 at the Conference on genome and Protein Maps at Siena, Italy (Wilkins et al., 1995). This term was known as the "PROTEin complement expressed by a genOME". Therefore, this technique is expected to represent an inclusive consider of all proteins expressed at a given time, in a given condition, and in a given organism. The important advantage of proteomics is that it concentrates on the functional translated part of genome (Komatsu and Ahsan, 2009). Peptides are smaller versions of proteins. Many health products contain different peptides for many uses. Recent research indicates that some types of peptides could have a beneficial role in destroying microbes (Shi et al., 2016; Sornwattana et al. 2013; Wu et al., 2011). In this research, reverse-phase chromatography, ion-exchange chromatography and pI-based fractionation were used for peptide purification. Then Liquid chromatography with tandem mass spectrometry was used to analyze peptide sequences and study their mechanisms.

2.4.1 Peptide purification by reverse-phase chromatography

In reversed-phase method, peptides are separated by interacting with hydrophobic surface of silica particles packed in columns. Silica is normally used for being hydrophobic substance because it is physically strong, stable under most solvent conditions and can be made into rounded particles of different sizes with pores of different diameters. Silica modified with hydrocarbon molecules to create a hydrophobic surface. The most common modification is attachment of a linear, aliphatic eighteen carbon chain resulting in a C18 type column. This creates a fairly-thick layer of hydrocarbon on silica surface that proteins and peptides adsorb. The C18

column is especially useful for the separation of peptides or protein hydrolysates smaller than 3,000 daltons. While long column gives higher resolution than short column. The column size between 15 – 25 centimeter long is recommended for peptide separation. There are various column diameters; 4.6 mm (analytical column), 2.0 (narrow bore column), 1.0 (microbore column), and smaller than 0.1 mm (capillary column). The standard diameter of analytical column is 4.6 mm, this column is best run at a flow rate of 1 ml/min (Geng and Regnier, 1984; Lu et al., 2001).

Proteins or peptides are desorbed from the hydrophobic surface when the organic modifier concentration reaches a specific value. Acetonitrile is an organic solvent that commonly used in peptide reverse phase chromatography as a mobile phase because it is volatile and is easily removed from samples, it has low viscosity, quite transparent to low wavelength UV light and has a long history of successful separations. Peptides are almost always eluted using a solvent gradient where the relative concentration of organic solvent is slowly increased during the separation. Moreover, the reverse-phase chromatography requires an "ion-pair" reagent added to mobile phase to achieve good peak shape. It is believed that the absence of ion-pair reagent produces poor peak of peptides while silica has low impurity. Generally, 0.2-0.01% varying of trifluoroacetic acid (TFA) concentration may improve the selectivity of peptide separations on high purity silica. However, some columns may not be steady at TFA concentration higher than 0.1%. The detection of reverse-phase chromatography is normally by UV absorption at 214-215 nm. The peptide bond absorbs well in this range of wavelength and provides the most sensitive detection for polypeptides of all types. Acetonitrile does not absorb UV light at 215 nm but TFA absorbs slightly in this wavelength, results in shifting in the absorbance baseline. A common practice to avoid the upward drift of baseline is to reduce the TFA concentration in organic solvent. This results in a flatter baseline during peptide map elution (Lu et al., 2001; Dillon et al., 2004).

2.4.2 Ion exchange chromatography

Ion exchange chromatography is one of the most common approaches used in chromatographic method for protein purification due to its high resolution of protein separation, ease of use, reproducibility and low cost of materials. The ion exchange column allows protein sample to bind even when a large volume of buffer is applied, then this method specifically useful for protein purification (Bollag et al., 1996; Yoo et al., 2014).

Purification of proteins using ion exchange chromatography is based on differences in the ionic properties of surface amino acids. Histidine, arginine and lysine residues are typically

positively charged, while glutamic and aspartic residues are negatively charged at neutral pH. Separation of proteins in ion exchange column requires differential binding of the proteins to the ion exchange matrix. After proteins are applied to column, no affinity proteins are removed during washing step, then the adsorbed or bound proteins are removed in an elution step (Bollag et al., 1996).

2.4.3 The pI-based fractionation

Sample pre-fractionation procedures are broad used to reduce the complexity of proteomic samples before passing to liquid chromatography and tandem mass spectrometry (LC-MS/ MS) analysis.

The pI-based fractionation is a mass spectrometry compatible isoelectric trapping instrument that provides isoelectric point (pI) based separations of peptide mixtures. Peptide separation is executed by moving the peptide ions through membranes having fixed pH values until the peptide pI is bracketed by the pH values of adjacent membranes (Righetti et al., 2003). One of the most highly applied pI-based separation instruments for proteomic studies is the OFFGEL™ Fractionator (Agilent Technologies). The OFFGEL™ is a pleasing method for peptide fractionation in the view of its multiplex abilities-running several strips simultaneously, small sample volume and low sample quantity requirements. Efficient reduction of sample complexity increases sensitivity of downstream analysis (Heller et al., 2005).

2.4.4 Liquid Chromatography with tandem mass spectrometry (LC-MS/MS)

Mass spectrometry-based procedures for the protein identification have become a standard method in proteomics. The most approved MS-based strategies depend on proteolytic digestion of proteins into peptides before launch into the mass spectrometer. Digestion of proteins into similar size is better for solubility and is readily ionized in the mass spectrometer. Peptide ions are first measured as entire fragment ions then selected based on their ion mass to charge ratio (m/z) and subject to collisionally induced dissociation (CID), in a process known as tandem mass spectrometry (MS/MS) (Harrison and Cotter, 1990; McLuckey, 1992).

Liquid Chromatography with tandem mass spectrometry or LC-MS/MS is a proficient analytical technique that integrates the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (MS). Liquid chromatography (LC) in

association with tandem mass spectrometry (MS/MS) presents a robust, reliable, economical methodology for quantitative analysis and profiling of biological samples (Yang et al., 2009).

Chapter 3

Research methodology

3.1 Preparation of wastes samples

3.1.1 Waste Samples

Total of waste samples were collected from different parts of Thailand and classified into 3 groups as follows; 6 samples of agricultural wastes, 7 samples of agro-industrial wastes and 2 samples of fishery wastes as shown in table 3.1 and figure 3.1.

Table 3.1 Classification, given code, sources and details of sample used in this research.

Sample	Classification	Given Code	Source	Detail
Rice straw	Agricultural wastes	AW1	Mueang Chachoengsao District Agricultural Extension Office	RD47
Corn cob	Agricultural wastes	AW2	Khlong Had, Sakaeo	Pacific 328
Corn leaves	Agricultural wastes	AW3	Khlong Had, Sakaeo	Pacific 328
Corn cob leaves	Agricultural wastes	AW4	Khlong Had, Sakaeo	Pacific 328
Sugarcane leaves	Agricultural wastes	AW5	Bangkok	Suphan Buri 90
Bagasse	Agricultural wastes	AW6	Chachoengsao	Suphan Buri 90
Fermented soybean	Agro-industrial wastes	IW1	Hi-q Food Products Co., Ltd, Chachoengsao	Residues from light soy sauce productions
Soybean pellet	Agro-industrial wastes	IW2	Market in Chachoengsao	Residues from soybean milk productions
Peanut seed coat	Agro-industrial wastes	IW3	Mae-Ruay Snack Food Factory Co Ltd, Bangkok	Residues from peanut based snack productions
Coconut residue	Agro-industrial wastes	IW4	Market in Chachoengsao	Residues from coconut milk productions
Coffee ground	Agro-industrial wastes	IW5	Rosetta Coffee Shop, Chachoengsao	Arabica ground, main coffee industry residue
Fish residue	Agro-industrial wastes	IW6	King Mongkut's University of Technology Thonburi	Residues from fish sauce productions

Sample	Classification	Given Code	Source	Detail
Fish residue (desalted)	Agro-industrial wastes	IW7	King Mongkut's University of Technology Thonburi	Residues from fish sauce productions, rinsed by water
Nile tilapia fish fin	Fishery wastes	FW1	Market in Chachoengsao	-
Clarias sp. fish fin	Fishery wastes	FW2	Market in Chachoengsao	-

Figure 3.1 Waste samples A) agricultural wastes B) agro-industrial wastes and C) fishery wastes. While AW codes for agricultural wastes, IW codes for agro-industrial wastes and FW codes for fishery wastes.

3.1.2 Plant pathogens

Four plant pathogens were selected for study including *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas citri*, *Pectobacterium carotovorum*, and *Agrobacterium rhizogenes*. Moreover, 3 isolates of plant growth promoting rhizobacterium (PGPR) also gathered for testing in antibacterial activity as shown in table 3.2.

Table 3.2 Groups of selected bacteria.

Bacteria	Plant disease	PGPRs
Xanthomonas oryzae pv. *oryzae*	✓	
Xanthomonas citri DOA-BC902	✓	
Pectobacterium carotovorum DOA-BC681	✓	
Agrobacterium rhizogenes TISTR 511	✓	
Pseudomonas aeruginosa ATCC27853		✓
Pseudomonas fluorescens TISTR2630		✓
Bacillus subtilis ATCC6633		✓

3.2 Preparation of protein hydrolysates and peptides

Total protein from 50 g of samples were extracted using 0.05 M sodium acetate, pH 4.0, with shake at room temperature (25±2 °C) 200 rpm for 1 h and heat at 121°C 15 min for heat tolerance protein selection. The total protein concentration of the supernatant was measured by the method of Lowry assay (Lowry et al., 1951) using bovine serum albumin as standard. After that, the proteins were hydrolyzed with pepsin in ratio 1: 20 (pepsin: sample) at 37 ºC 200 rpm for 16 h, and then reaction was stopped by boiling for 30 min. The crude hydrolysates were centrifuged at 8,000 rpm for 5 min and supernatant was kept. The hydrolysates were cutoff using Vivaspin 20 at 3 kDa MWCO (GE Healthcare, UK) and lower than 3 kDa peptides were frozen at -20 ºC until used.

3.3 Antibacterial activity determination

Microbe samples were prepared for 24 h in Tryptic soy agar (TSA), 28 ºC. Then, single colony was selected to culture in tryptic soy broth (TSB) medium for 12 – 16 h in flask to grow an

inoculum of approximately 0.05 at OD_{600}. The antibacterial activity of filtrated lower 3 kDa peptides was determined against pathogens using broth dilution method in triplicate in 96-well plates. Peptides from each sample were used as treatment, while bacteria in TSB, Phosphate-buffered saline (PBS), and antibiotic (ampicillin and kanamycin) were used as controls. The final concentration of peptides and antibiotic was 100 µg/ml. The OD_{600} after incubation for 0, 2, 4, 6 and 8 h was observed using a microplate reader (Synergy H1 Hybrid Multi-Mode Reader, BioTek, United State).

3.4 Purification of peptides by reverse-phase chromatography method

The active peptides in protein hydrolysates containing antibacterial activity were purified by reverse-phase chromatography method using a Delta-Pak C18 column, 100 Å, 3.9 mm x 150 mm (Interlink Scientific Services Ltd., United Kingdom) equilibrated beforehand with 0.1% trifluoroacetic acid (TFA) in acetonitrile (ACN). The column was washed and eluted respectively as shown in table 3.3. Both hydrophobic and hydrophilic fractions were determined for antimicrobial activity as described in 3.3, and the active fractions were then pooled.

Table 3.3 Steps of purification by reverse-phase chromatography method using a Delta-Pak C18 column.

Step	Solution	Column volume (CV)
Starting condition	ACN with 0.1% TFA	5
Washing column	Sterile water with 0.1% TFA	5
Binding sample	Sample with 0.1% TFA	Total volume of sample
Elution of hydrophilic molecules	Sterile water with 0.1% TFA	5
Elution of hydrophobic molecules	ACN with 0.1% TFA	5

3.5 Purification of peptides by cation exchange chromatography

The active fractions from Delta-Pak C18 column were purified by cation exchange chromatography using AKTA™ start (GE Healthcare, Sweden). Essentially, all samples were conductivity checked before inject sample into ion exchange column. If conductivity was detected over 3 mS, all impurities should be removed by desalting column before chromatographic step using P-6 desalting column 50 ml (Bio-Rad Laboratories, Inc., United State). Then, salt-removed

samples were pH 4 adjusted before molecule separated based on their net surface charge through HiTrap column, SP Sepharose FF 1 ml (Cytiva, United State) cation chromatography exchange column follow method shown in table 3.4. Cation and anion were separated in this step. Afterward, each fraction was exchanged buffer from 50mM NaOAc pH 4 with gradient of 1M NaCl to sterile water before antibacterial activity testing.

Table 3.4 Steps of purification by cation chromatography exchange column.

Step	Detail
Setting	HiTrap column, SP Sepharose FF 1 ml, flow rate 1 ml/min
Prime and equilibrate	Equilibrate 5 CV, flow rate 1 ml/min
Sample	flow rate 1 ml/min, fraction volume 1 ml
Wash out unbound fraction	Wash out 3 CV, flow rate 0.5 ml/min, fraction volume 1 ml
Elution of bound fraction	1M NaCl gradient 0-100, linear 3 CV, fixed fraction volume 0.5 ml

3.6 Purification of peptides by off-gel fractionation

Active fraction of peptides from cation exchange chromatography was separated according to their isoelectric points (pI). For pI-based peptide separation, the 3100 OFFGEL Fractionator with an 18-well (18 cm) setup was used according to the protocol of the supplier as shown in table 3.5. The peptides in each fraction were further analyzed by nanoLC-MS/MS.

Table 3.5 Guideline for running 18 cm OFFGEL unit.

pH interval	Step voltage mode	Voltage (V)	Time (h: min)	kVh
3-10	1 Step and hold	500	1:00 (8:00)	0.5
	2 Gradient	1000	1:00	0.8
	3a Gradient	8000	3:00	13.5
	4a Step and hold	8000	0:46-1:30	6.2-12.2
	3b Gradient	10000	3:00	16.5
	4b Step and hold	10000	0:20-0:55	3.2-9.2
	Total			**21.0-27.0**

3.7 Peptide synthesis and antimicrobial activity test

The peptides were chemically synthesized by sending to peptide synthesis services, National science and technology development agency. Then the synthesized peptide samples were partially purified by Sep-Pak C18 Cartridges before antimicrobial activity assay as described in 3.3.

3.8 Study of peptide-microbe interaction mechanism

The peptide showing best antibacterial activity was selected for study of peptide-microbe interaction mechanism. The protein profile of pathogen that treated and non-treated by effective peptide were compared by LC-MS/MS. After tryptic digestion, peptides were injected into the Ultimate 3000 LC system (Dionex) coupled to ESI-Ion Trap MS (HCT ultra PTM Discovery System, Bruker Daltonik) with electrospray. The MS/MS spectra results were analyzed with DeCyder MS 2.0 differential analysis software (GE Healthcare). The analyzed MS/MS data from DeCyder MS was submitted to a database search against the NCBI database using Mascot software (Matrix Science, London, UK, (Perkins et al., 1999). All proteins were functionality identified by GoCat (http://eagl.unige.ch/GoCat).

Mongkut's Institute of Technology Ladkrabang

Chapter 4

Results

4.1 Protein concentration

The total protein was extracted from 50 g of waste samples and the protein concentration was determined according to the procedure of Lowry (1951). At high level of confidence (R^2=0.99), protein concentration (percent protein by weight) of all samples was 3.59 in average as demonstrated in table 4.1. It was noticed that protein concentration from rice straw and corn leaves were in the highest level among all samples (10.10 and 9.87%, respectively) while corn cob leaves showed lowest protein concentration at 1.14%.

Table 4.1 Concentration of protein samples and their source.

Code	Sample	percent protein (weight by weight)*
AW1	Rice straw	10.10
AW2	Corn cob	2.15
AW3	Corn leaves	9.87
AW4	Corn cob leaves	1.14
AW5	Sugarcane leaves	4.56
AW6	Bagasse	1.36
IW1	Fermented soybean	4.22
IW2	Soybean pellet	1.82
IW3	Peanut seed coat	2.14
IW4	Coconut residue	2.39
IW5	Coffee ground	2.88
IW6	Fish residue	3.26
IW7	Fish residue (desalted)	2.03
FW1	Nile tilapia fin	3.17
FW2	Snake-head fish fin	2.72
	Means	3.59±2.76

***** Wet weight.

4.2 Screening of antibacterial property

Broth dilution experiment was carried out to determine whether protein hydrolysates at the final concentration of 100 µg/ml showed antibacterial activity against bacterial plant pathogens. After well-mixing between protein hydrolysates and suspension of bacterial pathogens, the 96-well plates were incubated under suitable conditions to grow microorganism. The result was observed after incubation for 0, 2, 4, 6 and 8 h as presented in figure 4.1. In this experiment, kanamycin and ampicillin were used as positive control for antibacterial activity and found that kanamycin showed the highest activity. It was noted that bacterial growth in TSB without kanamycin (control 1) was continuously grown from 0 to 8 hours, while the growth of bacterial pathogens with hydrolysates from some samples were almost unchanged throughout experimental time compared with kanamycin. For obviously, inhibitory percentage of all antibacterial activity tests were measured at 6 hour and shown in figure 4.2.

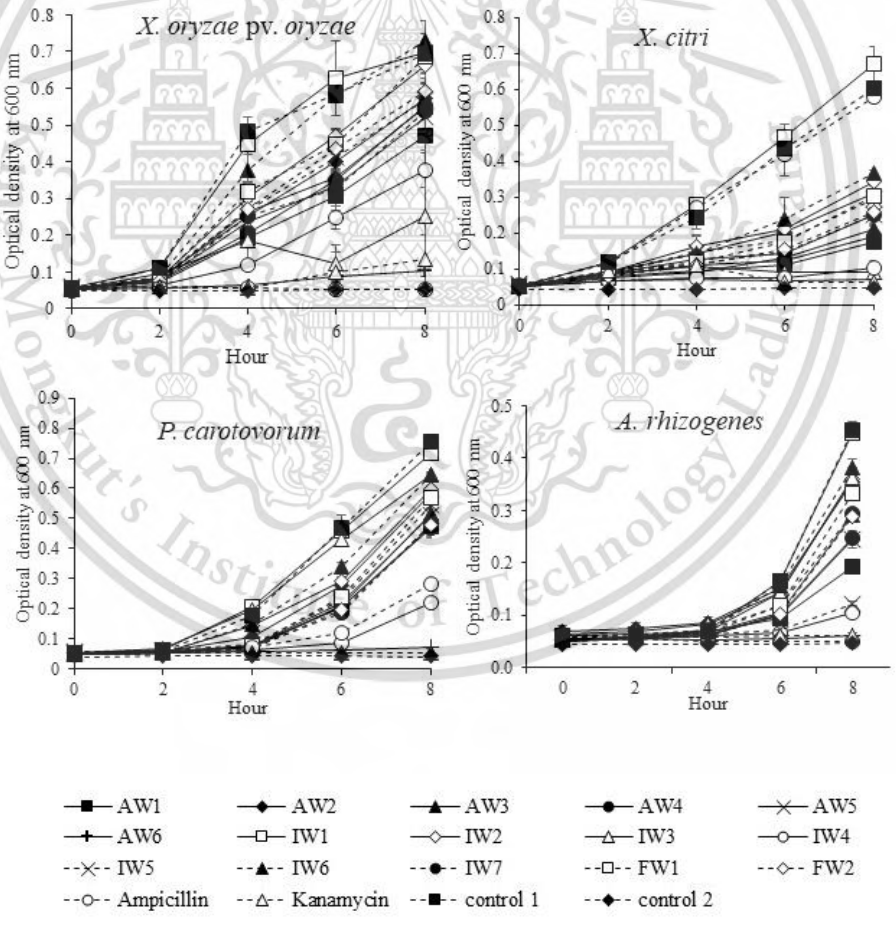

Figure 4.1 Antibacterial activity of 100 µg/ml protein hydrolysates from wastes against the bacterial plant pathogens; *X. oryzae* pv. *oryzae*, *X. citri*, *P. carotovorum* and *A. rhizogenes* while control 1 was bacterial growth without antibiotic and control 2 was PBS.

Antibacterial activity by broth dilution assay showed some samples as potential sources of antibacterial protein hydrolysates with inhibition level of 50% or higher on at least one targeted pathogen after exposure for 6 h. Four from fifteen hydrolysate samples including AW1, AW6, IW3 and IW4 showed obviously higher potential than others as shown in table 4.2. It was pointed out that AW6 ranked first in every targeted pathogens, while IW4 ranked second in all targets except *X. oryzae* pv. *oryzae*. IW3 ranked second in *X. oryzae* pv. *oryzae* and ranked third in *X. citri*, while AW1 ranked fourth in *X. citri*.

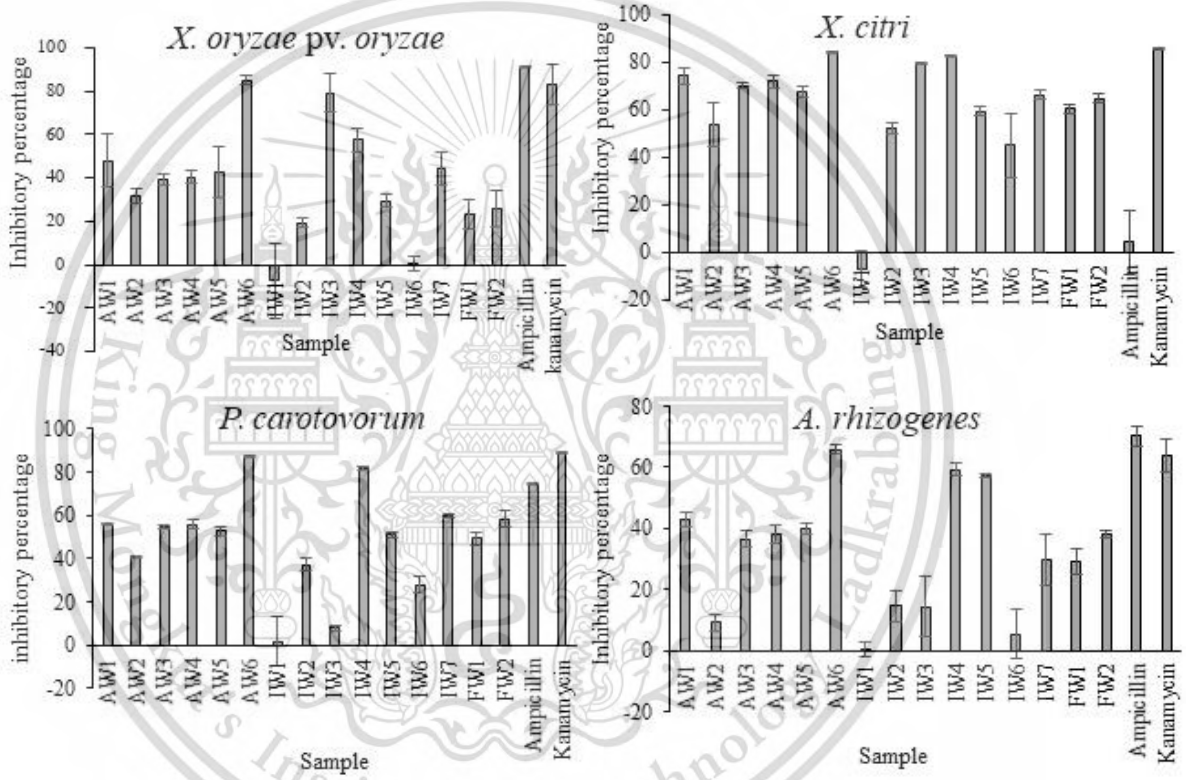

Figure 4.2 Inhibitory percentage at 6 h of protein hydrolysates from wastes against the bacterial plant pathogens; *X. oryzae* pv. *oryzae*, *X. citri*, *P. carotovorum* and *A. rhizogenes.*

Table 4.2 Ranking of inhibitory percentage of protein hydrolysate samples against plant pathogenic bacterial growth.

Antibacterial activity ranking	Inhibitory percentage on target organism							
	Xoo	Sample	*Xc*	sample	*Pc*	sample	*Ar*	Sample
1	84.90 ± 1.98	AW6	84.21 ± 0.40	AW6	87.06 ± 0.33	AW6	65.87 ± 1.58	AW6
2	79.20 ± 8.73	IW3	82.30 ± 0.58	IW4	81.95 ± 0.75	IW4	59.28 ± 2.16	IW4
3	57.61 ± 5.48	IW4	79.41 ± 0.46	IW3	60.06 ± 0.86	IW7	57.09 ± 0.69	IW5
4	47.81 ± 12.29	AW1	74.07 ± 3.35	AW1	58.42 ± 3.99	FW2	43.11 ± 2.40	AW1
5	44.50 ± 7.76	IW7	71.62 ± 2.86	AW4	55.93 ± 0.25	AW1	39.92 ± 1.92	AW5
6	42.74 ± 11.70	AW5	69.95 ± 0.95	AW3	55.93 ± 2.45	AW4	38.32 ± 1.20	FW2
7	40.40 ± 2.68	AW4	67.12 ± 2.43	AW5	54.66 ± 1.01	AW3	37.92 ± 3.01	AW4
8	39.20 ± 2.68	AW3	66.06 ± 1.72	IW7	52.74 ± 2.18	AW5	36.53 ± 2.74	AW3
9	31.68 ± 3.53	AW2	64.53 ± 2.06	FW2	51.10 ± 1.09	IW5	29.54 ± 3.99	IW7
10	29.52 ± 2.94	IW5	60.11 ± 1.69	FW1	49.25 ± 3.10	FW1	29.14 ± 8.50	FW1
11	25.47 ± 8.38	FW2	59.19 ± 1.66	IW5	40.94 ± 0.56	AW2	14.57 ± 5.02	IW2
12	23.42 ± 6.94	FW1	53.17 ± 9.22	AW2	37.38 ± 2.89	IW2	14.37 ± 9.97	IW3
13	19.26 ± 2.11	IW2	52.10 ± 2.20	IW2	28.00 ± 3.63	IW6	9.18 ± 2.83	AW2
14	0.74 ± 3.17	IW6	44.85 ± 13.37	IW6	7.89 ± 1.48	IW3	5.39 ± 7.83	IW6
15	-7.35 ± 17.30	IW1	-7.09 ± 7.73	IW1	1.85 ± 11.24	IW1	0.20 ± 2.42	IW1
Ampicillin	90.83 ± 0.52		4.12 ± 13.30		74.48 ± 0.25		70.26 ± 3.40	
Kanamycin	82.91 ± 9.35		85.35 ± 0.61		89.05 ± 0.33		63.87 ± 5.23	

Note: *Xoo*, *Xc*, *Pc* and *Ar* represent *X. oryzae* pv. *oryzae*, *X. citri*, *P. carotovorum* and *A. rhizogenes* respectively.

Plant growth promoting rhizobacteria (PGPRs) include *B. subtilis, P. aeruginosa* and *P. fluorescens*, good bacteria in normal agricultural area, were also chosen for antibacterial testing. As mentioned, PGPRs should not be inhibited as well. Figure 4.3 – 4.4 showed no inhibitory potential was noticed on *B. subtilis*. It has been observed that only AW6 and IW4 showed high percentage inhibition against *P. aeruginosa* and *P. fluorescens*, others did not show any effective of inhibition at all. Furthermore, it was observed that hydrolysates from some samples supported growth of PGPRs as following results; *B. subtilis* growth was supported by hydrolysates from AW2 AW4 IW3 IW4 IW6 IW7 FW1 and FW2, *P. aeruginosa* growth was supported by hydrolysates from IW2 IW6 and FW1, lastly, *P. fluorescens* growth was supported by hydrolysates from AW2 AW4 IW3 IW4 IW 6 IW7 FW1 and FW2 as presented in table 4.3.

As described previously, AW6 showed the best antibacterial activity against plant pathogens, while IW4, IW3, and AW1 also expressed positive effects against those microbes. Although hydrolysates from AW6 and IW4 inhibited growth of *P. aeruginosa* and *P. fluorescens*, but *B. subtilis* still grew normally. Then after screening by broth dilution test, four effective samples, namely AW1, AW6, IW3 and IW4, were selected to purify.

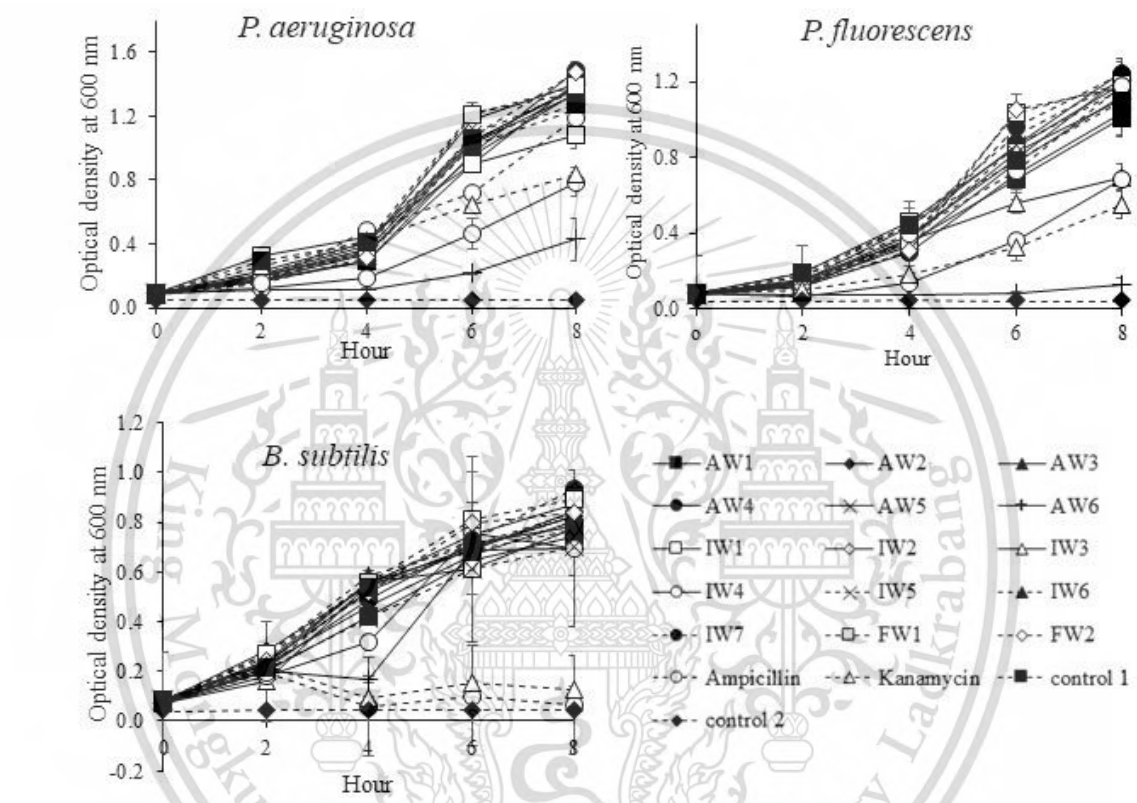

Figure 4.3 Antibacterial activity of 100 µg/ml protein hydrolysates from wastes against the PGPRs; *P. aeruginosa, P. fluorescens and B. subtilis.*

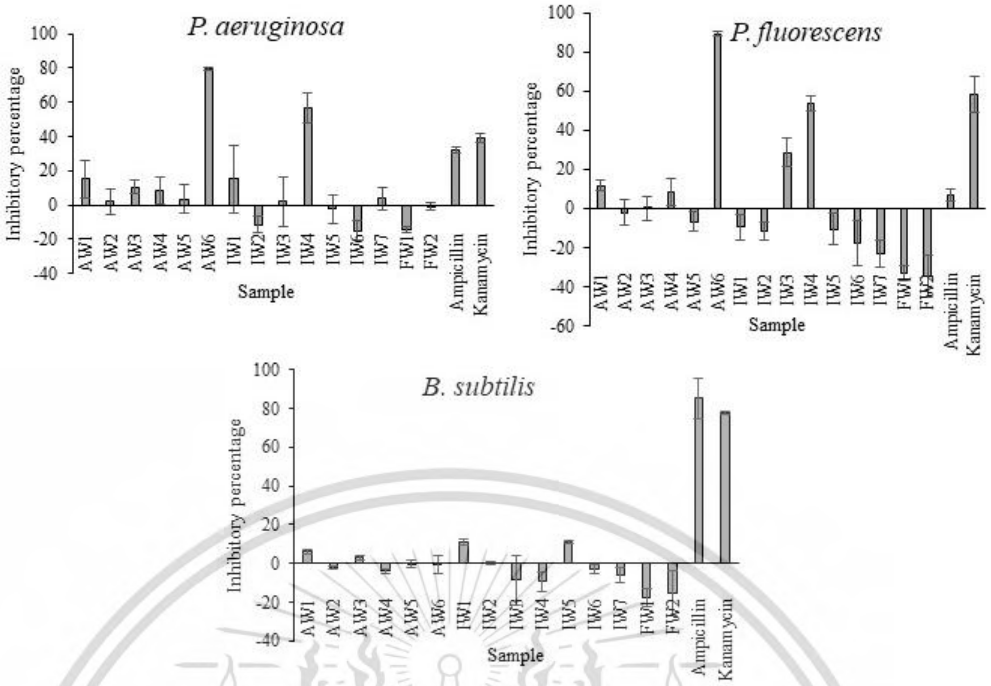

Figure 4.4 Inhibitory percentage at 6 h of protein hydrolysates from wastes against the PGPRs; *P. aeruginosa, P. fluorescens and B. subtilis.*

Table 4.3 Ranking of inhibitory percentage of protein hydrolysate samples against PGPRs growth.

Antibacterial activity ranking	Inhibitory percentage on target organism					
	Pa	**sample**	***Pf***	**sample**	***Bs***	**Sample**
1	79.49 ± 0.49	AW6	89.28 ± 0.85	AW6	11.13 ± 1.73	IW1
2	56.33 ± 8.79	IW4	53.31 ± 3.88	IW4	10.89 ± 0.95	IW5
3	15.03 ± 19.42	IW1	28.66 ± 7.01	IW3	6.10 ± 0.95	AW1
4	15.00 ± 11.23	AW1	11.66 ± 2.74	AW1	3.10 ± 1.05	AW3
5	10.24 ± 3.86	AW3	8.58 ± 6.78	AW4	0.10 ± 0.94	IW2
6	8.70 ± 7.83	AW4	0.08 ± 6.25	AW3	-0.29 ± 4.57	AW6
7	3.91 ± 6.58	IW7	-2.14 ± 6.54	AW2	-0.44 ± 2.03	AW5
8	3.21 ± 8.27	AW5	-6.63 ± 5.25	AW5	-2.18 ± 0.75	AW2
9	2.08 ± 14.40	IW3	-9.53 ± 6.49	IW1	-2.90 ± 2.55	IW6
10	2.05 ± 7.41	AW2	-10.68 ± 8.11	IW5	-3.97 ± 0.92	AW4
11	-0.95 ± 2.41	FW2	-11.41 ± 4.63	IW2	-5.90 ± 3.83	IW7
12	-2.46 ± 8.56	IW5	-17.86 ± 11.55	IW6	-8.66 ± 12.68	IW3
13	-11.50 ± 5.05	IW2	-23.16 ± 6.86	IW7	-9.39 ± 5.08	IW4
14	-14.49 ± 1.99	FW1	-32.94 ± 4.06	FW1	-15.34 ± 11.78	FW2
15	-15.03 ± 5.91	IW6	-34.56 ± 10.41	FW2	-17.56 ± 4.71	FW1
Kanamycin	39.00 ± 2.81		58.01 ± 9.34		77.60 ± 0.85	
Ampicillin	31.98 ± 1.59		6.66 ± 3.02		85.20 ± 10.57	

Note: *Pa*, *Pf* and *Bc* represent *P. aeruginosa*. *P. fluorescens* and *B. subtilis,* respectively.

4.3 Peptide purification

After antibacterial screening, four hydrolysate samples (AW1, AW6, IW3 and IW4) were selected to purify by three chromatographic steps: reverse-phase chromatography, cation exchange chromatography and off-gel fractionation.

Reverse-phase chromatography is a basic tool in the separation of peptides. This method using a Delta-Pak C18 column that is very hydrophobic surface. Hydrophobic compounds are retained by the adsorption of the surface because of hydrophobic effect. Therefore, hydrophilic fraction was early washed out by sterile water with 0.1% trifluoroacetic acid or TFA. This fraction was called unbound fraction of reverse-phase chromatography (Unbound of Reverse-phase; UBR). Hydrophobic fraction was eluted later by acetonitrile (ACN) with 0.1% TFA (bound fraction of reverse-phase chromatography, BR). In consequence of the method, each of four hydrolysate samples were separated into two fractions. Totally, eight samples were tested for their antibacterial activity. As presented in figure 4.5, UBR showed higher inhibitory potential than BR in every sample, it means that hydrophilic fraction gave a better result than hydrophobic fraction. In more detail, AW6 UBR showed the best inhibitory percentage around 60 percent in every bacteria sample (59.73, 59.04, 64.68, and 64.25 percent against *X. oryzae* pv. *oryzae*, *X. citri*, *P. carotovorum*, *A. rhizogenes,* respectively). While all of BR samples showed inhibitory percentage less than 15 in every repeats. Although AW1 UBR, IW3 UBR and IW4 UBR gave low percentage of inhibition against *P. carotovorum* and *A. rhizogenes*, they gave better results against *X. oryzae* pv. *oryzae* and *X. citri.* Then, samples that chosen to purify in the next step were AW1UBR, AW6UBR, IW3UBR and IW4 UBR.

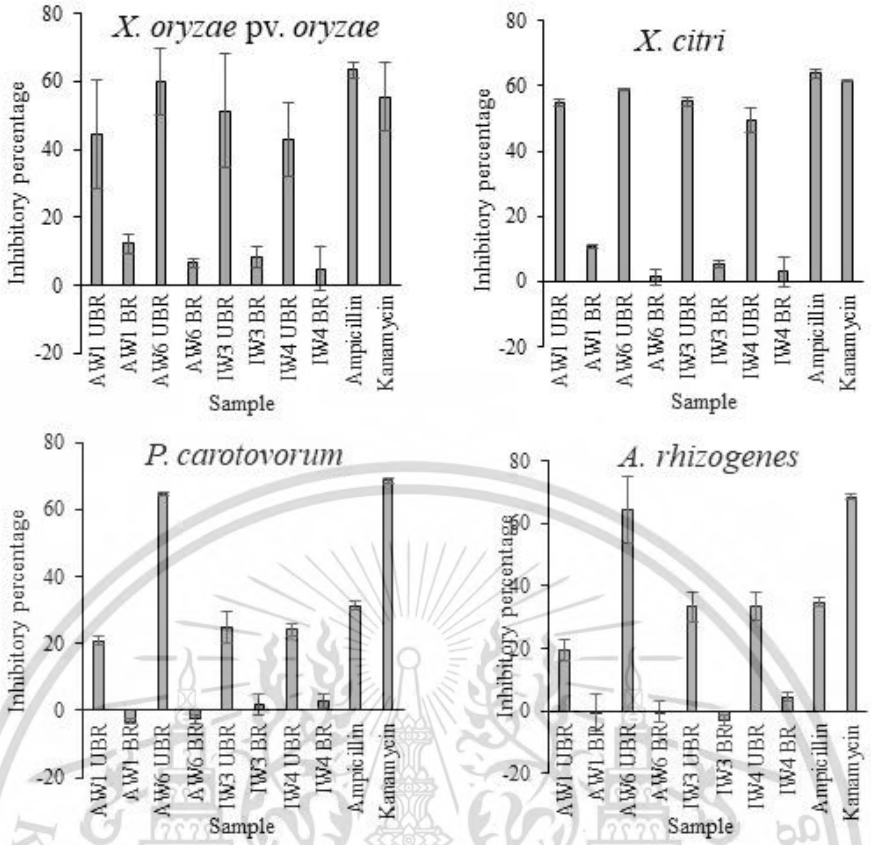

Figure 4.5 Inhibitory percentage against plant pathogens at 6 hour of protein hydrolysates from waste samples after purification by reverse-phase chromatography method using a Delta-Pak C18 column. UBR and BR represent unbound and bound fraction after reverse-phase chromatography, respectively.

Conductivity of all samples were measured before purification by cation exchanger. Also, it was found that conductivity or electric charge of all samples were too high to purify (33.6, 45.4, 38.9 and 43.7 mS/cm at 20°C in AW1 UBR, AW6 UBR, IW3 UBR and IW4 UBR, respectively). As a result, desalting column (Bio-Scale™ Mini Bio-Gel® P-6 desalting cartridges 50 ml, Biorad) was applied to remove salt and other small molecules by connected with AKTA™ start (GE Healthcare, Sweden). Subsequently, cation exchange chromatography was set through HiTrap column, SP Sepharose FF 1 ml (Cytiva) column. Most samples showed both of unbound (UBC) and bound peak (BC) of cation exchange chromatography (AW6UBC, AW6BC, IW3UBC, IW3BC, IW4UBC and IW4BC) except AW1 that only AW1UBC peak was shown as demonstrated

in Appendices. Subsequently, antibacterial activity testing was done and the results were shown in figure 4.6.

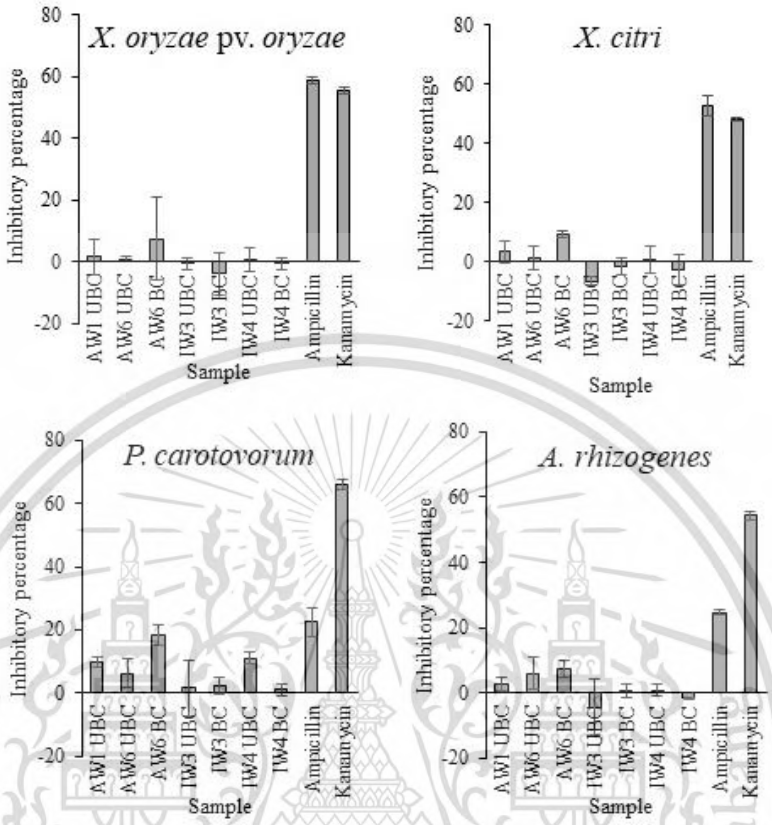

Figure 4.6 Inhibitory percentage against plant pathogens at 6 h of protein hydrolysates from waste samples after purification by cation exchange column. UBC and BC represent unbound and bound fractions, respectively after cation exchanger.

As shown in Figure 4.6, AW6 UBR-BC showed better inhibitory percentage than others in every bacteria sample (7.32, 9.21, 18.21 and 7.26 percent against *X. oryzae* pv. *oryzae*, *X. citri*, *P. carotovorum*, *A. rhizogenes,* respectively). Then, AW6 BC was chosen to purify in the next step, off-gel fractionation or pI-based peptide separation using the 3100 OFFGEL Fractionator with 18-well gel. The protein concentration before purification was 1.14 mg/ml then 200 µl or only 0.228 mg sample was loaded into each well equally. After a period of 7-day running, protein concentration was determined and the results were shown in table 4.4.

Table 4.4 Protein concentration of AW6BC after off-gel fractionation.

Well number	Protein concentration (mg/ml)	Well number	Protein concentration (mg/ml)
1	1.191	10	0.316
2	1.541	11	0.354
3	1.488	12	0.373
4	1.228	13	0.488
5	0.665	14	0.377
6	-0.111	15	0.647
7	-0.403	16	0.895
8	0.405	17	1.209
9	0.005	18	1.004

Due to the fact that protein concentration in each well was unequal and nearly no protein left in some wells, therefore concentration prepared for antibacterial activity testing was 0.3 mg/ml (the minimum concentration as shown in Table 4.4), while protein in well 6, 7 and 9 was used as much as it was. Then, 30 µl of each reaction was applied to antimicrobial assay, then the final concentration of sample in this step was 90 µg/ml and no replication. The sample volume was enough for only two strains of bacterial pathogens, which *X. oryzae* pv. *oryzae* and *P. carotovorum* were selected because of their good results in the last test. The results were shown in figure 4.7. Fraction 5, 6, 7 and 18 showed higher inhibitory percentage than others against *X. oryzae* pv. *oryzae* at 6.96, 9.76, 11.21 and 4.65 percent, respectively. While fraction 13 showed higher inhibitory percentage than others against *P. carotovorum* at 11.42 percent. Then, fraction 5, 6, 7, 13 and 18 were selected for further analysis by nanoLC-MS/MS.

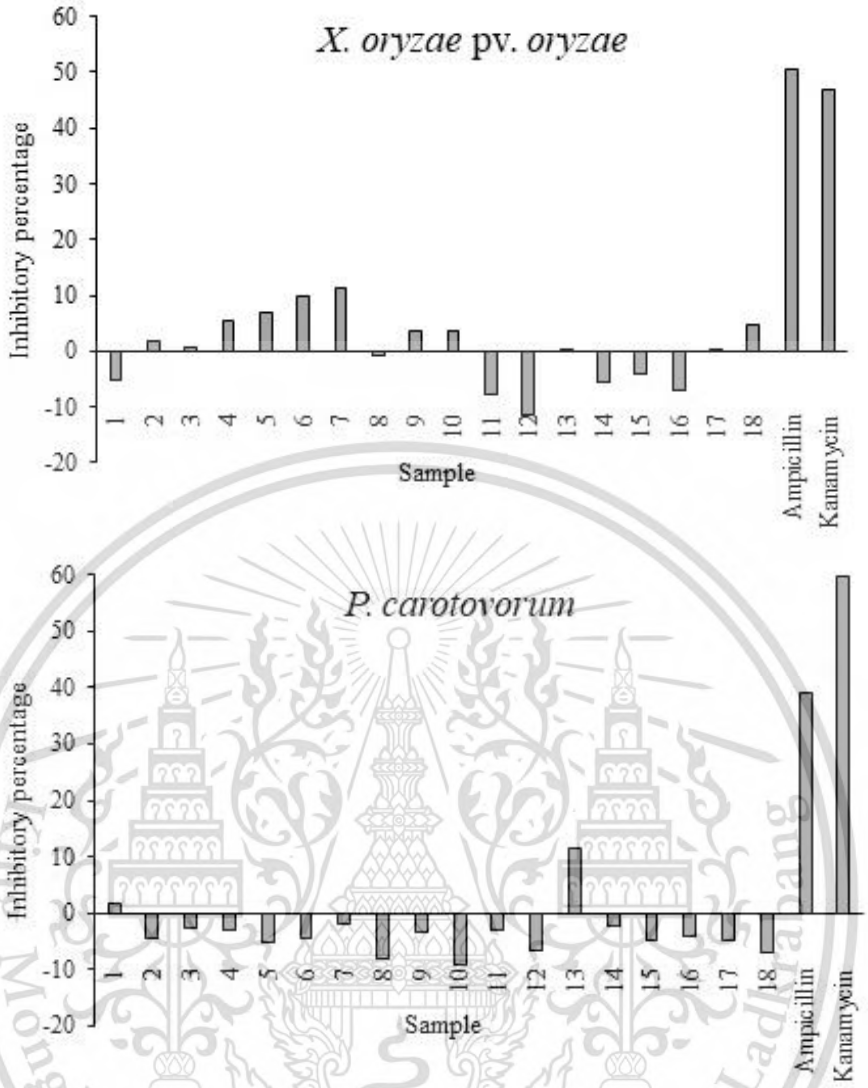

Figure 4.7 Inhibitory percentage against *X. oryzae* pv. *oryzae* and *P. carotovorum* at 6 h of protein hydrolysates from waste samples after purification by off-gel fractionation. Numbers in Y-axis represent numbers of 18 wells from off-gel fractionation.

4.4 Analysis by nanoLC-MS/MS and Peptide synthesis

The raw spectrum of LC-MS/MS data was used to search against suitable database using Mascot software, lots of peptide data of *Saccharum sp*. was obtained. Around 300-400 peptide sequences were found in each fraction. Then peptides with high peptide scores found in fraction that showed antibacterial activity against each bacterial pathogen (*X. oryzae* pv. *oryzae* and *P.*

carotovorum) with small size were selected and shown in table 4.5 – 4.6. All 13 peptides were synthesised for further analytical step.

Table 4.5 Peptides found in only fractions that showed antibacterial activity against *X. oryzae* pv. *oryzae* (fraction 5, 6, 7 and 18) with high peptide scores and not found in fraction 13.

Peptide no.	Accession no.	Protein names	Peptide sequence
1	A0A059Q0V8	Uncharacterized protein	PQLAVF
2	A0A5N5XU21	ATP-binding cassette domain-containing protein	VQLMNSL
3	A0A678TAJ2	Tr-type G domain-containing protein (Fragment)	TAMPRL
4	A0A1B2URG1	NAD(P)H-quinone oxidoreductase subunit 2, chloroplastic (EC 7.1.1.-) (NAD(P)H dehydrogenase, subunit 2) (NADH-plastoquinone oxidoreductase subunit 2)	ISSTSL
5	A0A3P3YVC5	Photosystem II CP47 reaction center protein (PSII 47 kDa protein) (Protein CP-47)	GAFHVTGL
6	A0A059PYU1	RNA helicase (EC 3.6.4.13)	VLSSWGDESTL
7	A0A678TPX2	LRR receptor-like serine/threonine-protein kinase GSO2	NLWSNEINQDMAEF

Table 4.6 Peptides found only in fractions that showed antibacterial activity against *P. carotovorum* (fraction 13) with high peptide scores and not found in fraction 5, 6, 7, and 18.

Peptide no.	Accession no.	Protein names	Peptide sequence
8	A0A059Q010	3-ketoacyl-CoA synthase (EC 2.3.1.-)	VSNCL
9	A0A059PZS2	Chitinase	WDTDNLSPDAVAAIKAAH PNVAVMAGL
10	A0A2H4YIU1	Expansin	MDRFL
11	Q8GT31	Phytocalpain (EC 3.4.22.17) (Fragment)	RVTGRDAL
12	A0A059Q1W5	Cation/H (+) antiporter	SIAGVTSYL
13	A0A6B9MSZ0 _9POAL	Tetraspanin-18	VMAAGL

4.5 Antimicrobial activity test of synthetic peptides

After peptide synthesis using Fmoc Solid-Phase Peptide Synthesis method (Hansen and Oddo, 2015), each of 13 peptides was tested for their antibacterial activity against plant pathogens and PGPRs, and the results were shown in table 4.7. It was observed that peptide no. 2 presented the highest activity against *P. carotovorum* and *A. rhizogenes* at 12.78 and 16.92 percent, respectively, while showed no effect to PGPRs (*B. sutilis* and *P. fluorescens*). Peptide no. 1 and 10 also showed the highest activity against *X. oryzae* pv. *oryzae* with inhibitory rate at 6.66 and 7.29 percent, respectively. The peptide no. 2 was selected for analysis of antibacterial mechanism against *P. carotovorum* and *A. rhizogenes*, while peptide no. 1 and 10 were studied in *X. oryzae* pv. *oryzae* using shotgun proteomic technique.

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Table 4.7 Antibacterial activity of synthesized peptides against plant pathogens and PGPRs.

Peptide no.	Peptide sequence	Accession no.	Protein name	Inhibitory percentage						
				Plant pathogen				PGPRs		
				Xoo	*Xc*	*Pc*	*Ar*	*Bs*	*Pa*	*Pf*
1	PQLAVF	A0A059Q0V8	Uncharacterized protein	6.66	4.86	-1.80	1.70	7.60	24.26	7.44
2	VQLMNSL	A0A5N5XU21	ATP-binding cassette domain-containing protein	-13.28	-9.09	12.78	16.92	-3.96	24.69	0.43
3	TAMPRL	A0A678TAJ2	Tr-type G domain-containing protein (Fragment)	3.27	9.87	6.18	4.29	8.92	25.81	-10.14
4	ISSTSL	A0A1B2URG1	NAD(P)H-quinone oxidoreductase subunit 2, chloroplastic (EC 7.1.1.-) (NAD(P)H dehydrogenase, subunit 2) (NADH-plastoquinone oxidoreduc5tase subunit 2)	1.07	6.63	6.91	2.73	9.00	26.03	-7.22
5	GAFHVTGL	A0A3P3YVC5	Photosystem II CP47 reaction center protein (PSII 47 kDa protein) (Protein CP-47)	5.03	8.23	4.01	0.21	3.82	21.74	2.08
6	VLSSWGDESTL	A0A059PYU1	RNA helicase (EC 3.6.4.13)	-2.11	6.42	3.86	-0.90	4.45	23.61	-9.56
7	NLWSNEINQDMAEF	A0A678TPX2	LRR receptor-like serine/threonine-protein kinase GSO2	2.93	10.02	6.75	3.00	11.33	24.87	-2.18
8	VSNCL	A0A059Q010	3-ketoacyl-CoA synthase	-1.29	4.49	3.16	0.85	10.39	24.70	-6.84
9	WDTDNLSPDAVAAIKAAHPNVAVMAGL	A0A059PZS2	Chitinase	3.87	6.01	3.61	-0.56	5.97	30.34	0.72
10	MDRFL	A0A2H4YIU1	Expansin	7.29	4.57	0.81	-2.47	5.54	26.22	6.30
11	RVTGRDAL	Q8GT31	Phytocalpain (EC 3.4.22.17) (Fragment)	3.62	10.72	6.33	0.22	10.63	29.08	-4.03
12	SIAGVTSYL	A0A059Q1W5	Cation/H (+) antiporter	-2.13	-0.59	0.59	-4.25	9.42	33.13	-7.00
13	VMAAGL	A0A6B9MSZ0_9POAL	Tetraspanin-18	-2.96	1.07	0.27	-4.15	5.46	29.73	-5.10

Note: Xoo, Xc, Pc, Ar, Bs, Pa and Pf represent *X. oryzae* pv. *oryzae*, *X. citri*, *P. carotovorum*, *A. rhizogenes*, *B. subtilis*, *P. aeruginosa and P. fluorescens* respectively.

4.6 Study of peptide-microbe interaction mechanism

From the antibacterial results above, the peptide-microbe interaction mechanism could be studied as shown in table 4.8. While untreated microbes were used as negative control and antibiotics were used as positive control.

Table 4.8 Selected peptides and bacterial plant pathogens used for study the antibacterial mechanism by shotgun proteomics.

Peptide no.	Peptide sequence	Bacterial plant pathogen
1	PQLAVF	*X. oryzae* pv. *oryzae*
10	MDRFL	*X. oryzae* pv. *oryzae*
2	VQLMNSL	*P. carotovorum*
2	VQLMNSL	*A. rhizogenes*

After treated *X. oryzae* pv. *oryzae, P. carotovorum* and *A. rhizogenes* with selected peptides for 6 h, all treated and untreated microbe samples were tryptic digested and injected into LC-MS/MS. After analysis by DecyderMS software, the MS/MS data of peptides were exported and further identified by Mascot software using protein databases available in the NCBI. Thousands of differentially expressed identified proteins from each sample were collected. However, all obtained data were simply illustrated the set of relationship by Venn diagram as shown in figure 4.8.

In this case, Venn diagram is a representation that used geometric forms like circles or triangles to show the groups of proteins from each treatment (control, peptides, antibiotics). Circles that overlap show same kinds of proteins while circles that do not overlap do not share those proteins. Then Venn diagram help to visually represent the similarities and differences between two or more treatments. As shown in figure 4.8, 1445 proteins were found in *X. oryzae* pv. *oryzae* treated with peptide no. 1 and 1688 proteins were found in *X. oryzae* pv. *oryzae* treated with peptide no. 10. In this amount, there were 304 and 452 proteins found only in *X. oryzae* pv. *oryzae* treated with peptide no. 1 and 10, respectively, and small number of proteins was overlapped with ampicillin, kanamycin and oxycline. About *P. carotovorum*, there were 1837 proteins found in *P. carotovorum* treated with peptide no. 2. Among these proteins, there was 733 proteins found in *P. carotovorum* treated with peptide no. 2 while 214 proteins overlapped between peptide no. 2 and

oxycline and 145 proteins overlapped between peptide no. 2 and kanamycin. For the last pathogen, there were 983 proteins found in *A. rhizogenes* treated with peptide no. 2. Among these proteins, there was 447 proteins found in *A. rhizogenes* treated with peptide no. 2 while 110 proteins overlapped between peptide no. 2 and oxycline and 79 proteins overlapped between peptide no. 2 and kanamycin.

From Venn diagram in Figure 4.8, it was observed that major proteins found in these bacterial plant pathogens was unique (not be much overlapped with proteins in circles of antibiotics). Then it could be concluded that peptide no. 1, 2 and 10 have the unique mechanisms in its own way. By this, the clearer explanation of using results of data from UniProt can be described.

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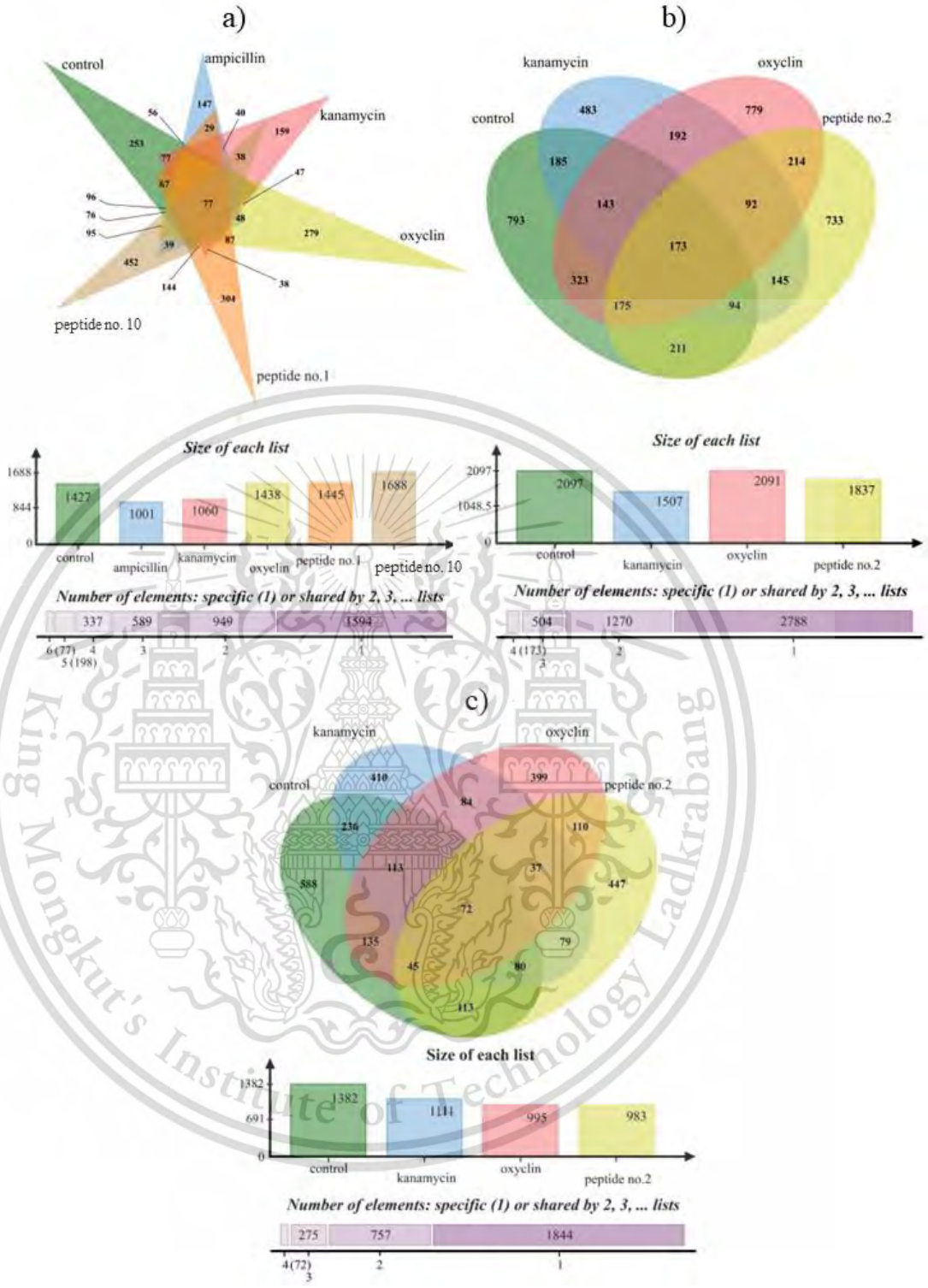

Figure 4.8 Venn diagram summary of proteins found in bacterial plant pathogens after treated with peptides and antibiotics a) *X. oryzae* pv. *oryzae* treated with peptide no.1, peptide no. 10, ampicillin, kanamycin and oxyclin, b) *P. carotovorum* treated with peptide no. 2, kanamycin and oxycline and c) *A. rhizogenes* treated with peptide no.2, kanamycin and oxycline.

Then, lots of data was further analyzed by UniProt, an approachable database of protein sequence and functional information, to find out gene ontology or function in peptide-treated pathogen cells. The results were shown in table 4.9-4.12. Gene ontology analysis of identified proteins of *X. oryzae* pv. *oryzae* after treated with peptide no.1 (PQLAVF) and peptide no. 10 (MDRFL) revealed that most were related to cell wall organization, integral component of membrane and DNA binding, while that of identified proteins of *P. carotovorum* after treated with peptide no. 2 (VQLMNSL) were most related to biological metabolism.

Table 4.9 Gene ontology of identified proteins of *X. oryzae* pv. *oryzae* after treated with peptide no.1 (PQLAVF).

Accession no.	Protein name	Gene name	Gene ontology
A0A4P6YKW0_XANOO	DNA-3-methyladenine glycosylase 2 family protein	EBA04_06140	base-excision repair
A0A411XWF8_XANOO	UvrABC system protein A (UvrA protein) (Excinuclease ABC subunit A)	uvrA EBA04_17010	nucleotide-excision repair
A0A2K9IQQ1_XANOO	Holliday junction ATP-dependent DNA helicase RuvA (EC 3.6.4.12)	ruvA EBA04_16430	DNA recombination; DNA repair; SOS response
RUVA_XANOR	Holliday junction ATP-dependent DNA helicase RuvA (EC 3.6.4.12)	ruvA XOO1661	DNA recombination; DNA repair; SOS response
A0A411XVK0_XANOO	Molecular chaperone	EBA04_15020	cell wall organization
A0A2K9IY96_XANOO	Biosynthetic peptidoglycan transglycosylase (EC 2.4.1.129) (Glycan polymerase) (Peptidoglycan glycosyltransferase MtgA) (PGT)	mtgA EBA04_15820	cell wall organization
Q5GYJ8_XANOR	DNA topoisomerase 4 subunit B (EC 5.6.2.2) (Topoisomerase IV subunit B)	parE XOO2969	chromosome segregation
Q5H323_XANOR	IS1404 transposase	XOO1394	DNA integration

Accession no.	Protein name	Gene name	Gene ontology
A0A0K0GJY9_XANOP	Transposase	PXO_00374	DNA integration
Q5GYY0_XANOR	Transposase	Tra8 XOO2837	DNA integration
Q5GZC0_XANOR	Transposase	XOO2697	DNA integration
A0A4P6YLY2_XANOO	Methyltransferase (EC 2.1.1.-)	EBA04_08545	DNA methylation
Q5GVS5_XANOR	N6_N4_Mtase domain-containing protein	XOO3944	DNA methylation
A0A0K0GKC5_XANOP	Phage replication protein	PXO_00564	DNA replication
Q5GTZ9_XANOR	DNA helicase (EC 3.6.4.12)	uvrD XOO4570	DNA replication
A0A411Y3S6_XANOO	Sigma-70 family RNA polymerase sigma factor	EBA04_09165	DNA-templated transcription,
Q5GZS3_XANOR	Outer-membrane lipoprotein carrier protein	lolA XOO2544	lipoprotein localization to outer membrane
A0A2K9IKP9_XANOO	Outer-membrane lipoprotein LolB	lolB EBA04_04720	lipoprotein localization to outer membrane
A0A2K9J067_XANOO	Protein TonB	EBA04_20940	protein transport
Q5H5X6_XANOR	Protein TonB	TonB XOO0390	protein transport
A0A0K0GJT0_XANOP	MotA protein	motA PXO_00026	protein transport
Q5H6W2_XANOR	Transposase	tnp XOO0054	DNA binding
Q5H608_XANOR	Possible transposase	XOO0358	DNA binding
A0A0K0GLG5_XANOP	Putative ISXoo4 transposase	PXO_01802	DNA binding
Q5H4G7_XANOR	Transposase and inactivated derivatives	XOO0900	DNA binding
MSCL_XANOR	Large-conductance mechanosensitive channel	mscL XOO3381	integral component of membrane
A0A2K9IU46_XANOO	DUF2244 domain-containing protein	EBA04_20155	integral component of membrane
A0A0K0GF48_XANOP	Proline-betaine transporter	PXO_03444	integral component of membrane
Q5GW22_XANOR	Potassium-transporting ATPase ATP-binding subunit (EC 7.2.2.6) (ATP phosphohydrolase [potassium-transporting] B chain) (Potassium-binding	kdpB XOO3845	integral component of membrane

Accession no.	Protein name	Gene name	Gene ontology
	and translocating subunit B) (Potassium-translocating ATPase B chain)		
A0A4P6YLP1_XANOO	Efflux RND transporter periplasmic adaptor subunit	EBA04_07560	integral component of membrane
Q06DY8_XANOO	Uncharacterized protein	Q06DY8_XANOO	integral component of membrane
A0A4V1AHY8_XANOO	Glucose/quinate/shikimate family membrane-bound PQQ-dependent dehydrogenase	EBA04_17110	integral component of membrane
A0A2K9J1X1_XANOO	Uncharacterized protein	EBA04_19630	integral component of membrane
A0A411XPL3_XANOO	1-acyl-sn-glycerol-3-phosphate acyltransferase	EBA04_00410	integral component of membrane
Q5GV14_XANOR	Arabinose efflux permease	AraJ XOO4205	integral component of membrane
A0A0K0GJL7_XANOP	TonB-dependent receptor	PXO_00017	integral component of membrane
A0A411Y586_XANOO	HlyD family secretion protein	EBA04_13115	integral component of membrane
A0A2K9IY11_XANOO	Rhomboid family intramembrane serine protease	EBA04_22770	integral component of membrane
A0A0K0GRD2_XANOP	L-fucose:H+ symporter permease	fucP PXO_02756	integral component of membrane
Q5H2L4_XANOR	Uncharacterized protein	XOO1553	integral component of membrane
Q5VLI0_XANOO	Putative inner membrane protein (Fragment)	Q5VLI0_XANOO	integral component of membrane
A0A411XYA0_XANOO	MFS transporter	EBA04_21860	integral component of membrane
A0A411XQX1_XANOO	MFS transporter	EBA04_03235	integral component of membrane

Accession no.	Protein name	Gene name	Gene ontology
Q5H1I4_XANOR	Uncharacterized protein	XOO1933	integral component of membrane
A0A4P6YM44_XANOO	Uncharacterized protein	EBA04_11400	integral component of membrane

Table 4.10 Gene ontology of identified proteins of *X. oryzae* pv. *oryzae* after treated with peptide no. 10 (MDRFL).

Accession no.	Protein name	Gene name	Gene ontology
A0A0K0GHT5_XANOP	Putative secreted protein	PXO_04681	carbohydrate metabolic process
A0A0K0GM60_XANOP	Uncharacterized protein	PXO_01454	carbohydrate metabolic process
A0A0K0GFJ5_XANOP	Maltokinase (EC 2.7.1.175) (EC 5.4.99.16) (Maltose alpha-D-glucosyltransferase) (Maltose-1-phosphate synthase)	PXO_03668	carbohydrate metabolic process
A0A0K0GRJ8_XANOP	Polysaccharide deacetylase	PXO_03488	carbohydrate metabolic process
A0A0K0GRG9_XANOP	Endoglucanase	PXO_03298	carbohydrate metabolic process
Q5GUS0_XANOR	Cell division protein FtsX	ftsX XOO4299	cell division
Q5H2Y2_XANOR	UDP-N-acetyl-alpha-D-muramoyl-L-alanyl-L-glutamate epimerase (EC 5.1.1.23) (UDP-MurNAc-L-Ala-L-Glu epimerase)	murL XOO1435	cell division; cell wall organization
Q5GUS0_XANOR	Cell division protein FtsX	ftsX XOO4299	cell division
Q5H218_XANOR	Pilus biogenesis protein	pilJ XOO1749	integral component of membrane; chemotaxis

Accession no.	Protein name	Gene name	Gene ontology
A0A411Y6F9_XANOO	Methyl-accepting chemotaxis protein	EBA04_16165	integral component of membrane; chemotaxis
A0A4P6YNL2_XANOO	Outer membrane protein assembly factor BamA	bamA EBA04_15090	integral component of membrane
A0A2K9IRQ3_XANOO	CDP-diacylglycerol--serine O-phosphatidyltransferase (EC 2.7.8.8) (Phosphatidylserine synthase)	pssA EBA04_18850	integral component of membrane
Q5H0H6_XANOR	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase	pgsA XOO2291	integral component of membrane
A0A4P6YPC0_XANOO	Biopolymer transporter TolI	EBA04_18095	integral component of membrane; DNA binding
Q5GZK5_XANOR	Flagellar biosynthesis	fliQ XOO2612	integral component of membrane
Q5GUR3_XANOR	Uncharacterized protein	XOO4306	integral component of membrane
A0A411Y0B8_XANOO	Type 3 secretion system secretin (T3SS secretin)	sctC EBA04_01780	integral component of membrane
Q5H587_XANOR	Uncharacterized protein	XOO0629	integral component of membrane
A0A4P6YMU8_XANOO	Zot domain-containing protein	EBA04_11455	integral component of membrane
A0A0K0GQ94_XANOP	Integral membrane protein DUF6	duf6 PXO_03092	integral component of membrane
A0A2K9IQY2_XANOO	Uncharacterized protein	EBA04_19450	integral component of membrane
Q5H276_XANOR	Phage-related tail protein	T XOO1691	integral component of membrane
A0A0K0GQB0_XANOP	Integral membrane protein	PXO_02894	integral component of membrane

Accession no.	Protein name	Gene name	Gene ontology
Q7WYV5_9XANT	Putative integral membrane protein (Fragment)	Q7WYV5_9XANT	integral component of membrane
Q5H1W9_XANOR	ATP/ADP translocase	XOO1798	integral component of membrane
A0A4P6YM46_XANOO	Chloride channel protein	EBA04_11465	integral component of membrane
Q5H6Z3_XANOR	Uncharacterized protein	STE14 XOO0023	integral component of membrane
A0A0K0GJV4_XANOP	Uncharacterized protein	PXO_00492	integral component of membrane
A0A4P6YMN2_XANOO	Diguanylate cyclase	EBA04_10620	integral component of membrane
Q5GXA1_XANOR	Uncharacterized protein	XOO3416	integral component of membrane
A0A2K9IZF9_XANOO	MCE family protein	EBA04_18885	integral component of membrane
Q5H5P6_XANOR	Magnesium transporter MgtE	mgtE XOO0470	integral component of membrane
A0A411Y596_XANOO	DUF4105 domain-containing protein	EBA04_13070	integral component of membrane
Q5GZK3_XANOR	GGDEF family protein	XOO2614	integral component of membrane
Q7WYW0_XANOO	Putative ABC-transporter permease (Fragment)	Wzm	integral component of membrane
A0A411Y681_XANOO	Cation transporter	EBA04_15390	integral component of membrane
A0A411Y684_XANOO	DUF4845 domain-containing protein	EBA04_15605	integral component of membrane
A0A2K9ISK1_XANOO	Cytochrome c-type biogenesis protein	EBA04_09125	integral component of membrane
A0A2K9ISB5_XANOO	DUF3426 domain-containing protein	EBA04_02315	integral component of membrane
Q5H3P7_XANOR	Mg-chelatase subunit ChlD	ChlD XOO1170	integral component of membrane

Accession no.	Protein name	Gene name	Gene ontology
A0A4P6YNV1_XANOO	DUF802 domain-containing protein	EBA04_19790	integral component of membrane
Q5H0M8_XANOR	DUF4190 domain-containing protein	XOO2239	integral component of membrane
Q5H501_XANOR	Lipoprotein	NlpA XOO0715	integral component of membrane
A0A0K0GPK8_XANOP	Outer membrane protein Slp	PXO_02439	integral component of membrane
Q5GXA7_XANOR	Uncharacterized protein	STE14 XOO3410	integral component of membrane
Q5H2K2_XANOR	ABC transporter ATP-binding protein	bapA XOO1565	integral component of membrane
A0A411Y0N1_XANOO	Uncharacterized protein	EBA04_02715	integral component of membrane
A0A411XUC4_XANOO	Transmembrane repetitive protein	EBA04_11750	integral component of membrane
A0A411XRD2_XANOO	YgcG family protein	EBA04_04260	integral component of membrane
A0A0K0GIE7_XANOP	ImcF-related family	imcF PXO_04696	integral component of membrane
Q5GVF5_XANOR	NADH dehydrogenase subunit 5	ndhF XOO4064	integral component of membrane
A0A411Y3F1_XANOO	YdcF family protein	EBA04_09340	integral component of membrane
Q5GW23_XANOR	Potassium-transporting ATPase KdpC subunit (ATP phosphohydrolase [potassium-transporting] C chain) (Potassium-binding and translocating subunit C) (Potassium-translocating ATPase C chain)	kdpC XOO3844	
A0A0K0GKB5_XANOP	Transposase	PXO_00579	nucleic acid binding
A0A0K0GN41_XANOP	Transposase	PXO_01240	nucleic acid binding

Accession no.	Protein name	Gene name	Gene ontology
A0A0K0GHK6_XANOP	ISXoo3 transposase ORF B	PXO_04310	nucleic acid binding
A0A411XZ08_XANOO	Site-specific integrase	EBA04_09040	DNA binding
A0A411Y4A3_XANOO	IS3 family transposase	EBA04_10525	DNA binding
A0A4P6YQD8_XANOO	IS30 family transposase	EBA04_20925	DNA binding
RUVC_XANOR	Crossover junction endodeoxyribonuclease RuvC (EC 3.1.21.10) (Holliday junction nuclease RuvC) (Holliday junction resolvase RuvC)	ruvC XOO1660	nucleic acid binding
A0A2K9IM24_XANOO	RNA polymerase sigma factor FliA (RNA polymerase sigma factor for flagellar operon) (Sigma F) (Sigma-28)	fliA EBA04_10585	DNA binding
Q5GWN6_XANOR	DNA-directed RNA polymerase specialized sigma subunit, sigma24 homolog	RpoE XOO3631	DNA binding
NUSB_XANOR	Transcription antitermination protein NusB (Antitermination factor NusB)	nusB XOO3851	RNA binding
A0A411XXQ5_XANOO	DNA-binding response regulator	EBA04_19650	DNA binding
A0A0K0GQF6_XANOP	Putative two-component response regulator	PXO_03063	DNA binding
Q5H3K9_XANOR	Two-component system regulatory protein	colR XOO1208	DNA binding
A0A2K9INZ8_XANOO	HlyD family efflux transporter periplasmic adaptor subunit	EBA04_14920	DNA binding
Q5GTV8_XANOR	IS1478 transposase	XOO4611	DNA binding

Accession no.	Protein name	Gene name	Gene ontology
A0A0K0GP24_XANOP	ISXoo3 transposase ORF A	PXO_02555	DNA binding
Q5GV50_XANOR	IS1478 transposase	XOO4169	DNA binding
A0A0K0GJ15_XANOP	Mutator family transposase	PXO_00238	DNA binding
Q5H4P9_XANOR	Putative transposase	XOO0817	DNA binding
Q5H6V2_XANOR	Putative transposase	XOO0064	DNA binding
A0A4P6YKK6_XANOO	IS5 family transposase	EBA04_03070	DNA binding
Q9AG81_XANOO	Putative transposase		DNA binding
Q5H6U5_XANOR	Putative transposase	XOO0071	DNA binding
A0A0K0GIR5_XANOP	Transposase	PXO_00237	DNA binding
Q5GW91_XANOR	Transposase and inactivated derivatives	XOO3776	DNA binding
Q5GXY7_XANOR	Predicted transcriptional regulators	SoxR XOO3180	DNA binding; regulation of transcription
Q5GVV0_XANOR	Transcriptional regulators	PurR XOO3918	DNA binding; regulation of transcription
Q5H5G8_XANOR	DNA-binding protein	fis XOO0548	DNA binding; regulation of transcription
Q5H4Q8_XANOR	DNA-binding protein H-NS	Hns XOO0808	DNA binding; regulation of transcription
RL16_XANOR	50S ribosomal protein L16	RL16_XANOR	translation
RS4_XANOR	30S ribosomal protein S4	RS4_XANOR	translation
RL35_XANOR	50S ribosomal protein L35	RL35_XANOR	translation
A0A2K9IMW6_XANOO	Glutamyl-Q tRNA(Asp) synthetase (Glu-Q-RSs) (EC 6.1.1.-)	A0A2K9IMW6_XANOO	translation

Table 4.11 Gene ontology of identified proteins of *P. carotovorum* after treated with peptide no. 2 (VQLMNSL).

Accession no.	Protein name	Gene name	Gene ontology
A0A419AGG5_PECCA	Flagellar hook-length control protein FliK	D5081_15245	bacterial-type flagellum assembly
C6D9A3_PECCP	Flagella basal body P-ring formation protein FlgA	PC1_2599	bacterial-type flagellum assembly
A0A419AGG4_PECCA	Flagellar biosynthetic protein FliP	fliP D5081_15220	bacterial-type flagellum organization
C6D997_PECCP	Flagellar basal-body rod protein FlgG (Distal rod protein)	PC1_2593	bacterial-type flagellum-dependent cell motility
A0A518VNY4_PECCC	Thioesterase	EGD00_19180	biosynthetic process
C6DJM4_PECCP	Homoserine O-acetyltransferase (EC 2.3.1.31)	PC1_0379	biosynthetic process
A0A419AUG1_PECCA	UTP--glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) (UDP-glucose pyrophosphorylase)	galU D5071_14080 D5081_09960	biosynthetic process
Q6QGY5_PECCC	BglB	bglB	carbohydrate metabolic process
A0A419AYY3_PECCA	6-phospho-beta-glucosidase (EC 3.2.1.86)	D5071_06090	carbohydrate metabolic process
Q9S5V7_PECCA	Peh	Peh	carbohydrate metabolic process
C6DBZ3_PECCP	Phosphoglucomutase (alpha-D-glucose-1,6-bisphosphate-dependent) (EC 5.4.2.2)	PC1_1212	carbohydrate metabolic process
A0A419AXA4_PECCA	Exo-poly-alpha-D-galacturonosidase	D5071_08610 D5081_12620	carbohydrate metabolic process
C6DFH1_PECCP	Carbohydrate kinase FGGY	PC1_3695	carbohydrate metabolic process

Accession no.	Protein name	Gene name	Gene ontology
C6D947_PECCP	Glycoside hydrolase family 43	PC1_0700	carbohydrate metabolic process
GLMM_PECCP	Phosphoglucosamine mutase (EC 5.4.2.10)	glmM PC1_0568	carbohydrate metabolic process
Q5UAU4_PECCC	BglA	bglA	carbohydrate metabolic process
A0A419AWP8_PECCA	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase (EC 6.3.2.13) (Meso-A2pm-adding enzyme) (Meso-diaminopimelate-adding enzyme) (UDP-MurNAc-L-Ala-D-Glu:meso-diaminopimelate ligase) (UDP-MurNAc-tripeptide synthetase) (UDP-N-acetylmuramyl-tripeptide synthetase)	murE D5071_09715 D5081_17465	cell cycle; cell division; cell wall organization; regulation of cell shape
MURA_PECCP	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7) (Enoylpyruvate transferase) (UDP-N-acetylglucosamine enolpyruvyl transferase) (EPT)	murA PC1_0287	cell cycle; cell division; cell wall organization; regulation of cell shape
C6DKY0_PECCP	Macrodomain Ter protein	matP PC1_2550	cell cycle; cell division; cell wall organization; regulation of cell shape
A0A518VIC2_PECCC	Membrane-bound lytic murein transglycosylase F (EC 4.2.2.n1) (Murein lyase F)	mltF EGD00_05940	cell wall macromolecule catabolic process; cell wall organization;

Accession no.	Protein name	Gene name	Gene ontology
			peptidoglycan metabolic process
A0A7V8PRL9_PECCA	Pectinesterase (EC 3.1.1.11)	H0258_03525	cell wall modification
A0A518VN93_PECCC	Molecular chaperone	EGD00_17485	cell wall organization; chaperone-mediated protein folding
A0A419AGX1_PECCA	Endolytic murein transglycosylase (EC 4.2.2.-) (Peptidoglycan polymerization terminase)	mltG D5071_05025 D5081_15655	cell wall organization; peptidoglycan biosynthetic process
C6DF30_PECCP	Methyl-accepting chemotaxis sensory transducer	PC1_3679	Chemotaxis; signal transduction
A0A419ARM6_PECCA	Methyl-accepting chemotaxis protein	D5071_19335	Chemotaxis; signal transduction
A0A7V8PS64_PECCA	MCP four helix bundle domain-containing protein	H0258_04875	Chemotaxis; signal transduction
A0A518VHN6_PECCC	HAMP domain-containing protein	EGD00_04170	Chemotaxis; signal transduction
C6DGT7_PECCP	Methyl-accepting chemotaxis sensory transducer	PC1_0112	Chemotaxis; signal transduction
C6DAX7_PECCP	N5-carboxyaminoimidazole ribonucleotide synthase (N5-CAIR synthase) (EC 6.3.4.18) (5-(carboxyamino)imidazole ribonucleotide synthetase)	purK PC1_2938	'de novo' IMP biosynthetic process
PUR7_PECCP	Phosphoribosylaminoimidazole-succinocarboxamide synthase (EC 6.3.2.6) (SAICAR synthetase)	purC PC1_1141	'de novo' IMP biosynthetic process
A0A0N9NSA3_PECCA	ISYps1 transposase	A0A0N9NSA3_PECCA	DNA integration

Accession no.	Protein name	Gene name	Gene ontology
A0A0N9MZK3_PECCA	Transposase OrfB	A0A0N9MZK3_PECCA	DNA integration
A0A419AQK3_PECCA	DUF4102 domain-containing protein (Fragment)	D5071_20950	DNA integration; recombination
A0A518VN30_PECCC	Site-specific integrase	EGD00_16945	DNA integration; recombination
C6DCX2_PECCP	Integrase family protein	PC1_3281	DNA integration; recombination
A0A7V8PW32_PECCA	Tyrosine-type recombinase/integrase	H0258_15495	DNA integration; recombination
A0A7V8THT4_PECCA	Site-specific integrase	H0258_16720	DNA integration; recombination
A0A419AIF1_PECCA	Terminase small subunit	D5081_11520	DNA packaging
RUVC_PECCP	Crossover junction endodeoxyribonuclease RuvC (EC 3.1.21.10) (Holliday junction nuclease RuvC) (Holliday junction resolvase RuvC)	ruvC PC1_1818	DNA recombination; DNA repair
RECR_PECCP	Recombination protein RecR	recR PC1_1078	DNA recombination; DNA repair
A0A518VGH5_PECCC	DNA helicase (EC 3.6.4.12)	recQ EGD00_01405	DNA recombination; DNA repair
A0A2S1TYB6_PECCC	Protein RecA (Recombinase A) (Fragment)	recA	DNA recombination; DNA repair
IHFA_PECCP	Integration host factor subunit alpha (IHF-alpha)	ihfA himA PC1_1894	DNA recombination; regulation of transcription, DNA-templated; regulation of translation

Accession no.	Protein name	Gene name	Gene ontology
A0A419AID3_PECCA	MGMT family protein	D5081_12470	DNA repair
LIGB_PECCP	DNA ligase B (EC 6.5.1.2) (Polydeoxyribonucleotide synthase [NAD(+)] B)	ligB PC1_4208	DNA repair; DNA replication
LEXA_PECCP	LexA repressor (EC 3.4.21.88)	lexA PC1_0514	DNA repair; DNA replication
C6DBW6_PECCP	DNA polymerase III subunit delta (EC 2.7.7.7)	PC1_1185	DNA replication
C6DF53_PECCP	Replication-associated recombination protein A	PC1_1721	DNA replication
A0A0K0MQ11_PECCA	RepFIB replication protein A	repB pA_00094	DNA replication
A0A7V8PUX7_PECCA	DNA replication terminus site-binding protein	tus H0258_12570	DNA replication
C6DAR9_PECCP	Site-specific DNA-methyltransferase (adenine-specific) (EC 2.1.1.72)	PC1_2876	DNA restriction-modification system
A0A419AID6_PECCA	DNA (cytosine-5-)-methyltransferase (EC 2.1.1.37)	D5081_11615	DNA restriction-modification system
C6D935_PECCP	RNA polymerase, sigma-24 subunit, ECF subfamily	PC1_0688	regulation of transcription
FABA_PECCP	3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.59) (3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabA) (Beta-hydroxydecanoyl thioester dehydrase) (Trans-2-decenoyl-[acyl-carrier-protein] isomerase) (EC 5.3.3.14)	fabA PC1_2552	fatty acid biosynthetic process
A0A419AH47_PECCA	3-oxoacyl-[acyl-carrier-protein] synthase 2 (EC 2.3.1.179)	D5081_15645	fatty acid biosynthetic process
C6DB24_PECCP	Acyl carrier protein phosphodiesterase (ACP	acpH PC1_1017	fatty acid biosynthetic process

Accession no.	Protein name	Gene name	Gene ontology
	phosphodiesterase) (EC 3.1.4.14)		
C6DA72_PECCP	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (ACCase subunit beta) (Acetyl-CoA carboxylase carboxyltransferase subunit beta) (EC 2.1.3.15)	accD PC1_2798	fatty acid biosynthetic process
A0A518VHG4_PECCC	Acyl-CoA dehydrogenase	EGD00_03705	fatty acid biosynthetic process
A0A419AWT7_PECCA	LPS-assembly protein LptD	lptD D5071_09890	Gram-negative-bacterium-type cell outer membrane assembly; lipopolysaccharide transport
C6DEY3_PECCP	LPS-assembly protein LptD	lptD PC1_3632	Gram-negative-bacterium-type cell outer membrane assembly; lipopolysaccharide transport
C6DC73_PECCP	Outer membrane protein assembly factor BamD	bamD PC1_3144	Gram-negative-bacterium-type cell outer membrane assembly; protein insertion into membrane
A0A419AKP3_PECCA	Ferric-rhodotorulic acid/ferric-coprogen receptor FhuE	fhuE D5081_08850	iron ion homeostasis
SUFE_PECCP	Cysteine desulfuration protein SufE	sufE PC1_2445	iron-sulfur cluster assembly

Accession no.	Protein name	Gene name	Gene ontology
A0A7V8PU42_PECCA	Chaperone protein HscA (Hsc66)	hscA H0258_10445	iron-sulfur cluster assembly; protein folding
A0A419AEI2_PECCA	Chaperone protein HscA (Hsc66)	hscA D5081_19300	iron-sulfur cluster assembly; protein folding
LPXK_PECCP	Tetraacyldisaccharide 4'-kinase (EC 2.7.1.130) (Lipid A 4'-kinase)	lpxK PC1_1770	lipid biosynthetic process
ARNA_PECCP	Bifunctional polymyxin resistance protein ArnA [Includes: UDP-4-amino-4-deoxy-L-arabinose formyltransferase (EC 2.1.2.13) (ArnAFT) (UDP-L-Ara4N formyltransferase); UDP-glucuronic acid oxidase, UDP-4-keto-hexauronic acid decarboxylating (EC 1.1.1.305) (ArnADH) (UDP-GlcUA decarboxylase) (UDP-glucuronic acid dehydrogenase)]	arnA PC1_2926	lipid biosynthetic process; lipopolysaccharide biosynthetic process
A0A419AVH6_PECCA	Bifunctional polymyxin resistance protein ArnA [Includes: UDP-4-amino-4-deoxy-L-arabinose formyltransferase (EC 2.1.2.13) (ArnAFT) (UDP-L-Ara4N formyltransferase); UDP-glucuronic acid oxidase, UDP-4-keto-hexauronic acid decarboxylating (EC 1.1.1.305) (ArnADH) (UDP-GlcUA decarboxylase) (UDP-	arnA D5071_12315	lipid biosynthetic process; lipopolysaccharide biosynthetic process

Accession no.	Protein name	Gene name	Gene ontology
	glucuronic acid dehydrogenase)]		
A0A518VP09_PECCC	Fatty acid hydroxylase family protein	EGD00_19210	lipid biosynthetic process
C6DHW7_PECCP	Cyclopropane-fatty-acyl-phospholipid synthase (EC 2.1.1.79)	PC1_2100	lipid biosynthetic process
A0A419APH3_PECCA	Acyl-CoA desaturase	D5081_02225	lipid metabolic process
A0A518VR03_PECCC	Acyl-CoA desaturase	EGD00_21630	lipid metabolic process
LEP4_PECCC	Prepilin leader peptidase/N-methyltransferase (Pectic enzymes secretion protein OutO) [Includes: Leader peptidase (EC 3.4.23.43) (Prepilin peptidase); N-methyltransferase (EC 2.1.1.-)]	outO	methylation
A0A7T5QNA7_PECCA	23S rRNA (guanosine-2'-O-)-methyltransferase RlmB (EC 2.1.1.185) (23S rRNA (guanosine2251 2'-O)-methyltransferase) (23S rRNA Gm2251 2'-O-methyltransferase)	rlmB JFY74_17365	methylation
A0A837DHS1_PECCC	DNA methyltransferase	RC98_12750	methylation
A0A7T5QM50_PECCA	tRNA 5-methylaminomethyl-2-thiouridine biosynthesis bifunctional protein MnmC (tRNA mnm(5)s(2)U biosynthesis bifunctional protein) [Includes: tRNA (mnm(5)s(2)U34)-methyltransferase (EC 2.1.1.61); FAD-dependent	mnmC JFY74_14190	methylation

Accession no.	Protein name	Gene name	Gene ontology
	cmnm(5)s(2)U34 oxidoreductase (EC 1.5.-.-)]		
A0A837DSG7_PECCC	Methyltransferase type 12	RC98_04535	methylation
A0A419AW25_PECCA	tRNA (N6-threonylcarbamoyladenosine(37)-N6)-methyltransferase TrmO	tsaA D5071_10720	methylation
A0A7T5QQE5_PECCA	Site-specific DNA-methyltransferase (adenine-specific) (EC 2.1.1.72)	JFY74_02635	methylation
C6DFQ1_PECCP	Methionine synthase (EC 2.1.1.13) (5-methyltetrahydrofolate--homocysteine methyltransferase)	PC1_3775	methylation
A0A7V8PVX1_PECCA	L-aspartate oxidase (EC 1.4.3.16)	nadB H0258_15080	NAD biosynthetic process
PNCB_PECCP	Nicotinate phosphoribosyltransferase (NAPRTase) (EC 6.3.4.21)	pncB PC1_1786	NAD biosynthetic process
C6DGE1_PECCP	Penicillin-binding protein 1A (EC 2.4.1.129) (EC 3.4.16.4)	PC1_3889	peptidoglycan catabolic process
A0A419AWW3_PECCA	1,6-anhydro-N-acetylmuramyl-L-alanine amidase AmpD	ampD D5071_09590	peptidoglycan catabolic process
A0A7V8PQG5_PECCA	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	amiB H0258_00285	peptidoglycan catabolic process
A0A419AKI5_PECCA	PTS trehalose transporter subunit IIBC (EC 2.7.1.201)	treB D5081_07430	phosphoenolpyruvate-dependent sugar phosphotransferase system
C6DKS5_PECCP	EIICB-Glc (EC 2.7.1.199) (PTS system glucose-specific EIICB component)	PC1_2495	phosphoenolpyruvate-dependent sugar phosphotransferase system

Accession no.	Protein name	Gene name	Gene ontology
A0A419B1B8_PECCA	PTS sugar transporter	D5071_00985	phosphoenolpyruvate-dependent sugar phosphotransferase system
A0A518VJU4_PECCC	DNA-binding response regulator	EGD00_09400	regulation of transcription
A0A518VIR6_PECCC	UDP-glucose 6-dehydrogenase (EC 1.1.1.22)	EGD00_06505	polysaccharide biosynthetic process
A0A419AVE2_PECCA	UDP-glucose 6-dehydrogenase (EC 1.1.1.22)	D5071_12330	polysaccharide biosynthetic process
A0A419ANV3_PECCA	Pectate lyase	D5071_21605	polysaccharide biosynthetic process
Q84CM2_PECCC	Glucanase (EC 3.2.1.-)	celC	polysaccharide biosynthetic process
A0A518VLY2_PECCC	Glycoside hydrolase	EGD00_13890	polysaccharide biosynthetic process
C6DAQ8_PECCP	Pectate lyase/Amb allergen	PC1_2863	polysaccharide biosynthetic process
Q4FF07_PECCC	Pel2	pel2	polysaccharide biosynthetic process
A0A7V8TEU3_PECCA	Prepilin peptidase-dependent protein	H0258_02270	protein transport
C6DAF0_PECCP	Uncharacterized protein	PC1_0908	protein transport
Q6R8K9_PECCC	Acyl-homoserine-lactone synthase (EC 2.3.1.184) (Autoinducer synthesis protein)	expI RC98_18970	quorum sensing
A0A518VND9_PECCC	DNA-binding response regulator	EGD00_17790	regulation of transcription
A0A419AKE7_PECCA	CadC family transcriptional regulator	D5081_08895	regulation of transcription
A0A518VNL0_PECCC	DNA-binding response regulator	EGD00_18235	regulation of transcription
C6DF22_PECCP	Two component transcriptional regulator, winged helix family	PC1_3671	regulation of transcription

Accession no.	Protein name	Gene name	Gene ontology
C6DGI4_PECCP	Transcriptional regulator, LacI family	PC1_0008	regulation of transcription
C6DGX3_PECCP	DNA-binding protein	PC1_1981	regulation of transcription
A0A419AQI6_PECCA	LacI family DNA-binding transcriptional regulator	D5081_00290	regulation of transcription
A0A518VJT2_PECCC	Crp/Fnr family transcriptional regulator	EGD00_09360	regulation of transcription
C6DJL8_PECCP	Putative PAS/PAC sensor protein	PC1_0372	regulation of transcription
C6DIX0_PECCP	Sigma54 specific transcriptional regulator, Fis family	PC1_2201	regulation of transcription
A0A419AT46_PECCA	PRD domain-containing protein	D5071_16510	regulation of transcription
A0A518VN85_PECCC	DUF1778 domain-containing protein	EGD00_17430	regulation of transcription
Y1817_PECCP	Probable transcriptional regulatory protein PC1_1817	PC1_1817	regulation of transcription
RS6_PECCP	30S ribosomal protein S6	rpsF PC1_3435	translation
A0A518VQ07_PECCC	50S ribosomal protein L33	rpmG EGD00_21435 RC98_19320	translation
A0A419ARU0_PECCA	Iron ABC transporter permease	D5071_18700	transmembrane transport
C6DIZ8_PECCP	Binding-protein-dependent transport systems inner membrane component	PC1_2229	transmembrane transport
C6DHI7_PECCP	Extracellular solute-binding protein family 1	PC1_0125	transmembrane transport
C6DAV8_PECCP	Extracellular solute-binding protein family 1	PC1_2919	transmembrane transport

Table 4.12 Gene ontology of identified proteins of *A. rhizogenes* after treated with peptide no. 2 (VQLMNSL).

Accession no.	Protein name	Gene name	Gene ontology
A0A546XIK3_AGRRH	Flagellar basal-body rod protein FlgF	flgF EXN68_12750	bacterial-type flagellum-dependent cell motility
A0A546XII0_AGRRH	Flagellar motor switch protein FliG	fliG EXN68_12775	bacterial-type flagellum-dependent cell motility
A0A546ZDF2_AGRRH	Flagellar motor switch protein FliN	fliN EXN70_14900	bacterial-type flagellum-dependent cell motility
A0A546ZBL8_AGRRH	Alpha-galactosidase	EXN70_17295	Carbohydrate metabolic process
A0A546ZEC7_AGRRH	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	nagZ EXN70_09215	Carbohydrate metabolic process
A0A546YIC8_AGRRH	Alpha-1,4 glucan phosphorylase (EC 2.4.1.1)	EXN70_20765	Carbohydrate metabolic process
A0A7S4ZSI2_AGRRH	ABC transporter family protein	pC6.5b_348	carbohydrate transport
A0A546XEB4_AGRRH	ABC transporter ATP-binding protein	EXN68_16355	carbohydrate transport
A0A546X9G6_AGRRH	ABC transporter ATP-binding protein	EXN68_23540	carbohydrate transport
A0A547CKT0_AGRRH	ABC transporter ATP-binding protein	EXN70_04040	carbohydrate transport
A0A546X9L1_AGRRH	sn-glycerol-3-phosphate ABC transporter ATP-binding protein UgpC	ugpC EXN68_23735	carbohydrate transport
A0A546ZBI3_AGRRH	sn-glycerol-3-phosphate ABC transporter ATP-binding protein UgpC	ugpC EXN70_17300	carbohydrate transport
A0A546YMQ2_AGRRH	Dihydrofolate synthase/folylpolyglutamate synthase (EC 6.3.2.12) (Folylpoly-gamma-glutamate synthetase-dihydrofolate synthetase)	EXN70_02090	cell cycle; cell wall organization; peptidoglycan biosynthetic process

Accession no.	Protein name	Gene name	Gene ontology
	(Folylpolyglutamate synthetase)		
A0A546ZJ22_AGRRH	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7) (Enoylpyruvate transferase) (UDP-N-acetylglucosamine enolpyruvyl transferase) (EPT)	murA EXN70_08085	cell cycle; cell wall organization; peptidoglycan biosynthetic process
A0A546ZEG8_AGRRH	Trigger factor (TF) (EC 5.2.1.8) (PPIase)	tig EXN70_09105	cell cycle; cell wall organization; peptidoglycan biosynthetic process
A0A546ZLK9_AGRRH	Glycoside hydrolase	EXN70_03170	Cell wall organisation
A0A546XM29_AGRRH	D-alanine--D-alanine ligase (EC 6.3.2.4) (D-Ala-D-Ala ligase) (D-alanylalanine synthetase)	ddl EXN68_07580	Cell wall organisation
A8W0J5_AGRRH	Rcorf140 (Type IV secretion system protein VirD4)	EXN68_25460	conjugation
Q9F5E3_AGRRH	Riorf109 protein	riorf109	conjugation
A0A546YWP8_AGRRH	Ti-type conjugative transfer system protein TraG	traG EXN70_31440	conjugation
Q9F5D9_AGRRH	Riorf113 protein	riorf113	conjugation
A0A546X6F6_AGRRH	IS21 family transposase	EXN68_24530	DNA integration; DNA recombination
A0A546X6A0_AGRRH	Integrase	EXN68_24180	DNA integration; DNA recombination
A0A546XGK9_AGRRH	Site-specific integrase	EXN68_15350	DNA integration; DNA recombination
A0A546XNL0_AGRRH	DNA ligase (ATP) (EC 6.5.1.1)	EXN68_01135	DNA repair
A0A546XBL9_AGRRH	Methylated-DNA--[protein]-cysteine S-methyltransferase (EC 2.1.1.63)	EXN68_21320	DNA repair

Accession no.	Protein name	Gene name	Gene ontology
A0A546YIM9_AGRRH	Methylated-DNA--[protein]-cysteine S-methyltransferase (EC 2.1.1.63)	EXN70_20275	DNA repair
A0A546XNK9_AGRRH	Site-specific DNA-methyltransferase (adenine-specific) (EC 2.1.1.72)	EXN68_00920	DNA replication
A0A546ZCC2_AGRRH	DNA polymerase I (EC 2.7.7.7)	polA EXN70_12465	DNA replication
A0A546XQ90_AGRRH	Beta-ketoacyl synthase	EXN68_04310	fatty acid biosynthetic process
A0A546Z011_AGRRH	3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.59) (3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabA) (Beta-hydroxydecanoyl thioester dehydrase) (Trans-2-decenoyl-[acyl-carrier-protein] isomerase) (EC 5.3.3.14)	fabA EXN70_29900	fatty acid biosynthetic process
A0A546Z5R7_AGRRH	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha (ACCase subunit alpha) (Acetyl-CoA carboxylase carboxyltransferase subunit alpha) (EC 2.1.3.15)	accA EXN70_25140	fatty acid biosynthetic process
A0A547BVQ5_AGRRH	Mannonate dehydratase (EC 4.2.1.8)	EXN70_05030	glucuronate catabolic process
A0A546ZBN0_AGRRH	FMN-binding glutamate synthase family protein	EXN70_17475	glutamate biosynthetic process
A0A546XG55_AGRRH	Glutamine amidotransferase	EXN68_14835	glutamine metabolic process
A0A546Y5T1_AGRRH	Glutamine amidotransferase	EXN70_24700	glutamine metabolic process

Accession no.	Protein name	Gene name	Gene ontology
A0A546Z7X2_AGRRH	Histidinol-phosphatase (EC 3.1.3.15) (Histidinol-phosphate phosphatase)	hisN EXN70_20835	histidine biosynthetic process
A8VZW2_AGRRH	Urocanate hydratase (Urocanase) (EC 4.2.1.49) (Imidazolonepropionate hydrolase)	hutU EXN68_27050	histidine catabolic process to glutamate and formamide
A0A546YYU0_AGRRH	Histidine utilization repressor	hutC EXN70_30570	histidine metabolic process
A0A546XN67_AGRRH	Isoleucine--tRNA ligase (EC 6.1.1.5) (Isoleucyl-tRNA synthetase) (IleRS)	ileS EXN68_00230	isoleucyl-tRNA aminoacylation
A0A546Z6L1_AGRRH	Acetoacetate--CoA ligase (EC 6.2.1.16)	EXN70_21890	lipid metabolic process
A0A547ARG0_AGRRH	Alpha/beta hydrolase	EXN70_24235	lipid metabolic process
A0A546Z039_AGRRH	Patatin-like phospholipase family protein	EXN70_30055	lipid metabolic process
A0A546XK12_AGRRH	Phosphodiester glycosidase family protein	EXN68_05950	metabolic process
A0A546ZH40_AGRRH	FAD-binding dehydrogenase	EXN70_05450	metabolic process
A0A546ZFN8_AGRRH	Bifunctional glutamine synthetase adenylyltransferase/adenylyl-removing enzyme (ATP:glutamine synthetase adenylyltransferase) (ATase) [Includes: Glutamine synthetase adenylyl-L-tyrosine phosphorylase (EC 2.7.7.89) (Adenylyl removase) (AR) (AT-N); Glutamine synthetase adenylyl transferase (EC 2.7.7.42) (Adenylyl transferase) (AT) (AT-C)]	glnE EXN70_11595	metabolic process

Accession no.	Protein name	Gene name	Gene ontology
A0A546XF05_AGRRH	ABC transporter ATP-binding protein	EXN68_17050	peptide transport
A0A546XC66_AGRRH	ABC transporter ATP-binding protein	EXN68_19500	peptide transport
A0A546XB97_AGRRH	ABC transporter ATP-binding protein	EXN68_20670	peptide transport
A0A546ZHW0_AGRRH	ABC transporter ATP-binding protein	EXN70_06785	peptide transport
A0A546XDN9_AGRRH	L,D-transpeptidase	EXN68_18120	peptidoglycan biosynthetic process
A0A546ZF69_AGRRH	Murein L,D-transpeptidase	EXN70_10675	peptidoglycan biosynthetic process
A0A546Z6P1_AGRRH	L,D-transpeptidase	EXN70_22165	peptidoglycan biosynthetic process
A0A546Z548_AGRRH	Uncharacterized protein	EXN70_24365	peptidoglycan biosynthetic process
A0A546XNX7_AGRRH	Peptidoglycan glycosyltransferase (EC 2.4.1.129)	EXN68_01590	peptidoglycan biosynthetic process
A0A546ZIZ5_AGRRH	Type III secretion system protein	EXN70_08705	protein secretion
A0A546XBF0_AGRRH	Type I secretion system permease/ATPase	EXN68_20945	protein secretion
VIRC2_AGRRH	Protein virC2	virC2	regulation of transcription
A0A7S4ZSX0_AGRRH	Uncharacterized protein	pC5.8d_693	regulation of transcription
A0A546Z675_AGRRH	Antitoxin	EXN70_24130	regulation of transcription
A0A546XM07_AGRRH	Helix-turn-helix transcriptional regulator	EXN68_09950	regulation of transcription
A0A546YL24_AGRRH	MerR family transcriptional regulator	EXN70_09500	regulation of transcription
A0A546ZIS0_AGRRH	Winged helix-turn-helix transcriptional regulator	EXN70_08480	regulation of transcription

Accession no.	Protein name	Gene name	Gene ontology
A0A546ZEK2_AGRRH	50S ribosomal protein L1	rplA EXN70_09835	regulation of translation
signal transduction	signal transduction	signal transduction	signal transduction
signal transduction	signal transduction	signal transduction	signal transduction
A0A546ZHQ4_AGRRH	ABC transporter permease	EXN70_06490	transmembrane transport
A0A546ZHG5_AGRRH	ABC transporter substrate-binding protein	EXN70_06015	transmembrane transport
A0A546Z8S0_AGRRH	Sugar ABC transporter permease	EXN70_17680	transmembrane transport
A0A546ZIJ5_AGRRH	ABC transporter permease	EXN70_08060	transmembrane transport
A0A546X9K6_AGRRH	Carbohydrate ABC transporter permease	EXN68_23525	transmembrane transport
A0A546ZL82_AGRRH	Mechanosensitive ion channel family protein	EXN70_02460	transmembrane transport
A0A546X3B4_AGRRH	ABC transporter permease	EXN68_25615	transmembrane transport
A0A546ZII0_AGRRH	Proline/glycine betaine ABC transporter permease	EXN70_07835	transmembrane transport
A0A546Z9L9_AGRRH	ABC transporter substrate-binding protein	EXN70_19315	transmembrane transport
A0A546Z9J6_AGRRH	ABC transporter permease	EXN70_19130	transmembrane transport
A0A546XFW8_AGRRH	tRNA(Ile)-lysidine synthase (EC 6.3.4.19) (tRNA(Ile)-2-lysyl-cytidine synthase) (tRNA(Ile)-lysidine synthetase)	tilS EXN68_14260	tRNA processing
A0A546Z312_AGRRH	RtcB family protein	EXN70_28985	tRNA processing
A0A546XCU6_AGRRH	t(6)A37 threonylcarbamoyladenosine biosynthesis protein TsaE (tRNA threonylcarbamoyladenosine biosynthesis protein TsaE)	tsaE EXN68_17630	tRNA processing

Data of gene ontology from UniProt program can be analyzed by organized into groups of functions as shown in figure 4.9 to 4.12.

According to Figure 4.9, it was noticed that most of proteins found in *X. oryzae* pv. *oryzae* treated with peptide no. 1 were related to integral component of membrane and small number of proteins related to DNA biological process such as DNA repair, DNA integration, DNA methylation, DNA replication, DNA/RNA binding and transcription. After percentage calculating, 53.33% cell membrane and cell wall proteins, 11.11% DNA/RNA binding and transcription proteins, 8.89% DNA repair proteins, 8.89% DNA integration proteins, 6.67% DNA replication proteins, 6.67% protein transport proteins and 4.44% DNA methylation proteins were noted. These means antimicrobial mechanism of peptide no.1- *X. oryzae* pv. *oryzae* was related to cell membrane and cell wall in majority part. It's possible that form of peptide no.1 caused *X. oryzae* pv. *oryzae* cell leak and some part of peptide affected DNA biological process in bacterial cells.

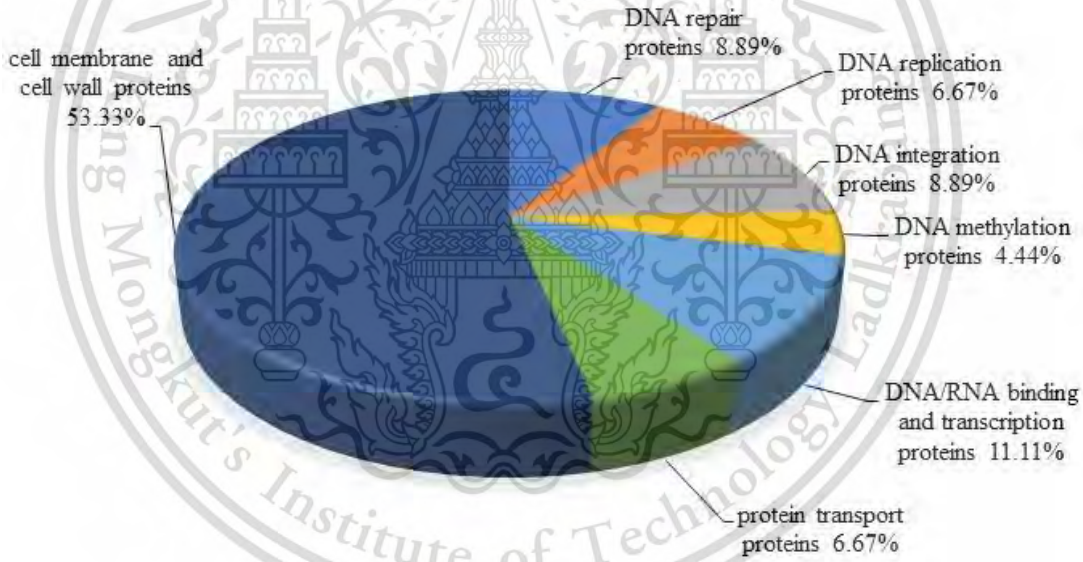

Figure 4.9 Functional classification percentage of identified proteins of *X. oryzae* pv. *oryzae* after treated with peptide no.1.

Functional classification percentage of identified proteins of *X. oryzae* pv. *oryzae* after treated with peptide no.10 was demonstrated in figure 4.10. It was noticed that proteins related to cell membrane and cell wall were majority found at 53.49% including cell wall organization proteins and proteins that were integral component of membrane. DNA/RNA binding proteins and transcription proteins were found 33.72%, while carbohydrate metabolic proteins, translation proteins and cell division proteins were found 5.81, 4.65 and 2.33% respectively. The functional

percentage of identified proteins found in *X. oryzae* pv. *oryzae* after treated with peptide no. 1 and 10 were similar. These means peptide no.1 and no. 10 are mainly interacting with cell membrane directly and some part of these peptide interacting with intracellular targets like DNA, RNA and proteins. Then peptide no.1 and no. 10 can be called membrane-active AMPs.

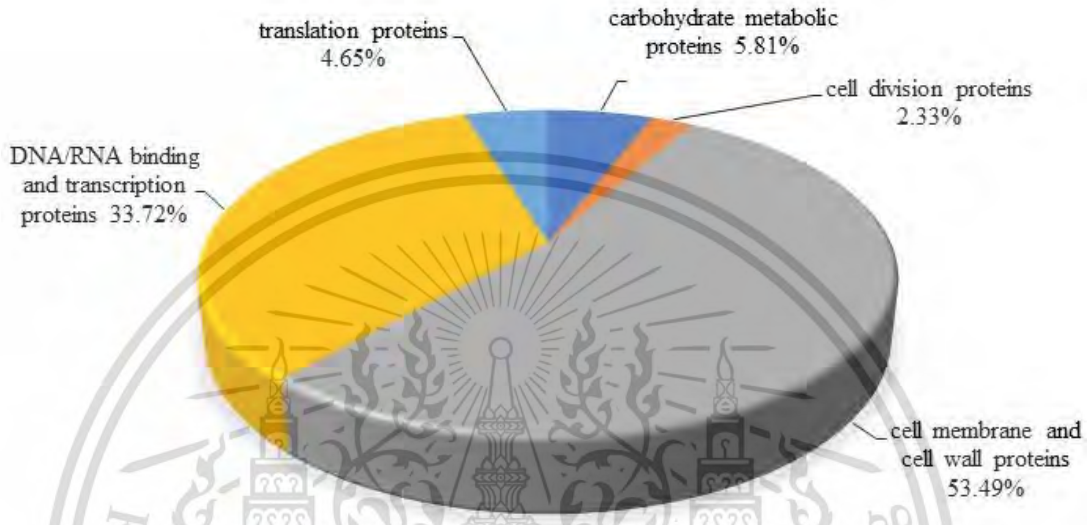

Figure 4.10 Functional classification percentage of identified proteins of *X. oryzae* pv. *oryzae* after treated with peptide no.10.

According to figure 4.11, it was noticed that most of proteins found in *P. carotovorum* treated with peptide no. 2 were related to biosynthetic process at 34.75% including general biosynthetic process, peptidoglycan catabolic process, phosphopyruvate-dependent sugar phosphotransferase system and polysaccharide biosynthetic process proteins. Moreover, we found regulation of transcription proteins 13.56%, cell membrane and cell wall proteins 9.32%, DNA modification and packaging proteins 9.32%, DNA integration and recombination proteins 5.93%, DNA repair proteins 5.93%, chemotaxis and signal transduction proteins 4.24%, bacterium-type flagellum assembly and motility proteins 3.39%, DNA replication proteins 3.39%, iron assembly and homeostasis proteins 3.39%, cell division proteins 2.54%, protein transport proteins 1.69%, translation proteins 1.69% and quorum sensing proteins 0.85%. These percentages imply that peptide no. 2 was mostly interact with targets inside the cells including biosynthetic process, DNA transcription and translation, DNA repair, signal transduction, etc. While some parts of peptides (9.32%) interact with cell membrane and cell wall of *P. carotovorum*.

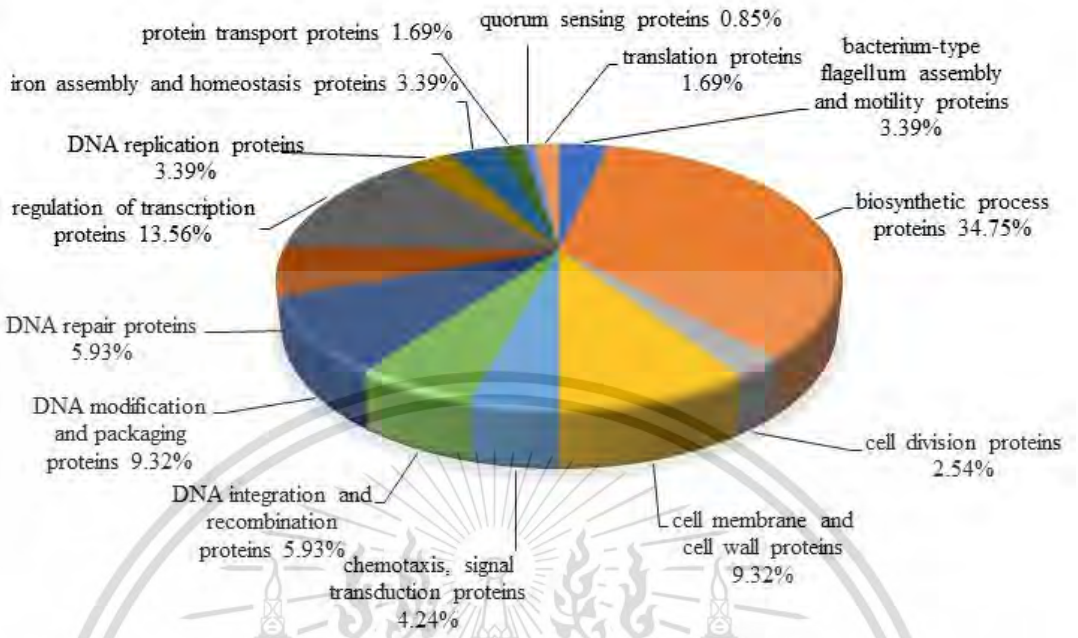

Figure 4.11 Functional classification percentage of identified proteins of *P. carotovorum* after treated with peptide no.2.

Finally, functional classification percentage of identified proteins of *A. rhizogenes* after treated with peptide no.2 was demonstrated in figure 4.12. It was noticed that half of functional identified proteins was related to biosynthetic process including carbohydrate metabolic process and transport proteins, fatty acid and lipid biosysthesis proteins, amino acid metabolic proteins and peptidoglycan biosynthetic proteins at 50.63%. The proteins were grouped in cell membrane and cell wall proteins 18.99%, regulation of transcription proteins 8.86%, conjugation proteins 5.06%, bacterium-type flagellum motility proteins 3.80%, DNA integration proteins 3.80%, DNA repair proteins 3.80%, DNA replication proteins 2.53% and signal transduction proteins 2.53%. The functional classification percentage in *P. carotovorum* and *A. rhizogenes* after treated with peptide no. 2 are similar that most of proteins related to metabolic process. Then peptide no. 2 could be called intracellular-active AMPs.

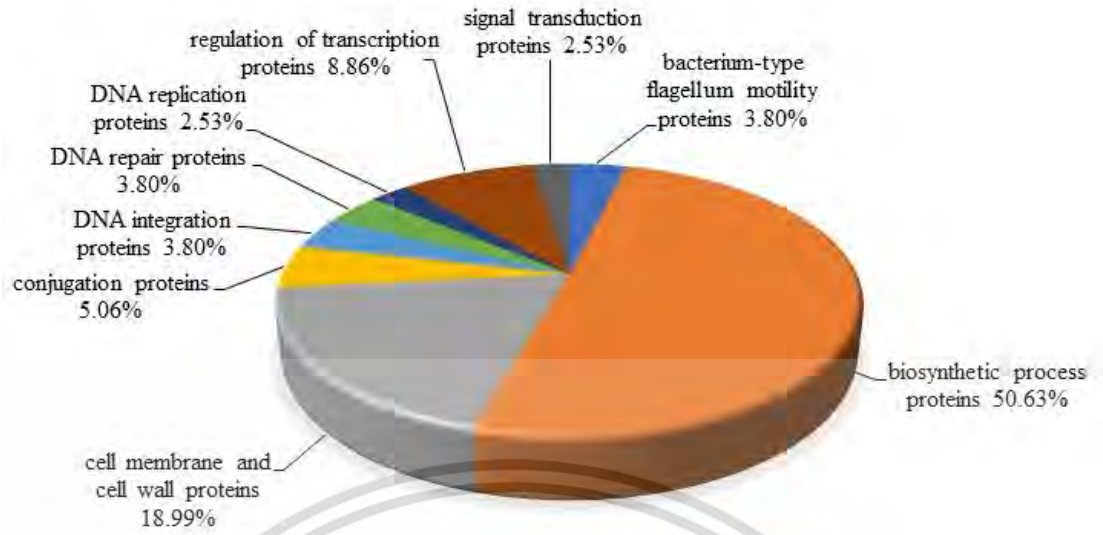

Figure 4.12 Functional classification percentage of identified proteins of *A. rhizogenes* after treated with peptide no.2.

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Chapter 5

Discussions

5.1 Protein concentration in agricultural wastes

In recent years, scientists have focused on the research of plant extracts, essential oil, secondary metabolites and new synthesized molecules as effective antimicrobial agents (Mabona et al., 2013; Nazzaro et al., 2013; Runyoro et al., 2006). The agricultural wastes were selected to be sources of AMPs because it was considered that peptides or proteins obtained from inexpensive samples, and agricultural products should be saved for human and animals. Before hydrolysis with pepsin, protein concentration of all agricultural waste samples was determined by Lowry assay. This method is reliable, but it requires a lot of micro-pipetting work and buffer preparation (Larson et al., 1986; Legler et al., 1985; Markwell et al., 1987). Moreover, color development reaches maximum in 20-30 min, after which there is a gradual loss of signal around 1% per hour (Peterson, 1977). For this reason, preparation of all steps in this method was done in the same day to lessen errors.

According to table 4.1, average of protein by weight of 15 agricultural wastes was 3.59%. Similar to the observations of Peripolli et al. (2016) and Shen et al. (1998) which reported obtained crude protein 4-4.7% and 3-6% in rice straw, respectively. de Almeida et al. (2018) found 18.2% protein in bagasse, while Velazquez-Martinez et al. (2021) detected 2.2 %. In 2010, Akinfemi reported that 3.89% of crude protein was found in corn cob.

5.2 Screening of antibacterial property

Several methods can be used to screen the *in vitro* antimicrobial activity of extract compound, such as disk diffusion, broth or agar dilution, time-kill test and cytofluorometric methods. Dilution methods are one of the most proper techniques for determination of antimicrobial activity. Either agar or broth dilution method can be used to quantitatively evaluate the *in vitro* antimicrobial activity against bacterial and fungi (Balouiri et al., 2016). The most recognised standards are provided by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Pfaller et al., 2004).

Broth dilution method or broth micro-dilution method is one of the simple antimicrobial activity testing methods. The diluted antimicrobial agent in a liquid growth medium was dispensed in 96-well microplate. It is normally expressed in µg/ml. Each well is inoculated with a microbial inoculum set up in the same medium after dilution of standardized microbial suspension to 0.5 McFarland turbidity scale. After thoroughly mixing, the 96-well plates are incubated with agitation under suitable conditions.

From ranking of inhibitory percentage of protein hydrolysate samples against plant pathogenic bacterial growth as shown in Table 4.2, AW6 or protein hydrolysate from bagasse ranked first in every targeted pathogen (*X. oryzae* pv. *oryzae*, *X. citri*, *P. carotovorum* and *A. rhizogenes*). These results conform to report by Velazquez-Martinez et al. (2021) that sugarcane bagasse with 2.2% crude protein showed high antimicrobial properties against *E. coli*, *B. cereus*, *S. aureus*, and the modified yeast SGS1. The ranked second of inhibition rate, coconut residue, also conformed to report that coconut AMPs (Cn-AMPs) showed extremely efficient of antimicrobial activity against multiple Gram-positive and Gram-negative pathogenic bacteria including *E. coli*, *B. subtilis*, *S. aureus* and *P. aeruginosa*. Moreover, antimicrobial result of rice straw also conformed to work of Park et al. (2006), where the growth of the bloom-forming cyanobacterium *Microcystis aeruginosa* was inhibited by rice straw extract.

From this foremost step, we have four effective samples (AW1, AW6. IW3 and IW4) for protein purification in the further step.

5.3 Peptide purification

Conventional separation of peptides requires some purification techniques. Scott et al. (2007) explained that some mixtures of proteins from waste samples should be separated with polar side groups and nonpolar side groups, while some, basic amino acids and acidic amino acids may be accomplished. Moreover, a complex mixture of amino acids can also be separated using chromatographic purification techniques. Ion exchange chromatography, a purification technique based on ions and their affinity to the ion exchanger, is important to develop in the separation of peptides from complex mixtures as well.

As mentioned above, this research began the purification steps with reversed-phase chromatography that separate hydrophobic and hydrophilic fractions apart, followed by cation exchange chromatography and pI-based purification (off-gel fractionation), then antibacterial activity of all fractions was assayed. In each step in the order of purification process, the further

narrow scope of bioactive peptides was obtained. As shown in figure 5.1, 4 samples of UBR after reverse-phase chromatography were obtained. Then after cation exchange chromatography, only AW6-BC showed antibacterial activity. After last step, pI-based purification, 5 samples from well 5, 6, 7, 13 and 18 were selected for further analysis by LC-MS/MS.

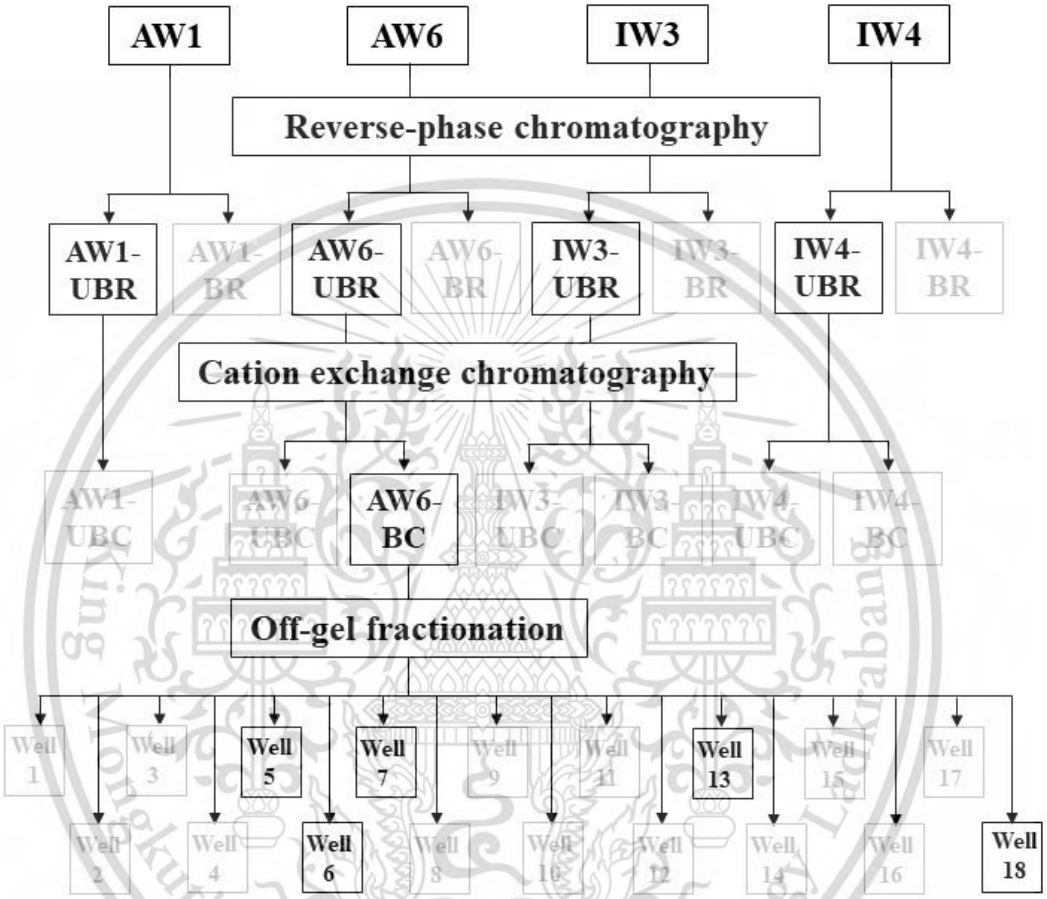

Figure 5.1 Effective samples obtained after applied to each purification steps include AW1UBR, AW6UBR, IW3UBR and IW4UBR obtained after reverse-phase chromatography, AW6BC obtained after cation exchange chromatography and AW6BC well 5, 6, 7, 13 and 18 obtained after off-gel fractionation; UBR and BR represent unbound and bound fraction of reverse-phase chromatography, UBC and BC represent unbound and bound fraction of cation exchange chromatography.

5.4 Analysis by nanoLC-MS/MS and Peptide synthesis

After three steps of peptide purification followed by nanoLC-MS/MS and mascot software analysis, an enormous amount of peptide data of AW6 or *Saccharum sp*. was demonstrated, more than 300 peptide sequences were found in each fraction. Then peptides with high peptide scores with small size were selected as shown in table 4.5-4.6. These 13 peptides were selected and sent out for synthesis. Small AMPs could be proper alternative because the cost of synthesis is much lower than the long ones while still maintaining most of the advantages of native AMPs (Choi et al., 2017). Moreover, Liu et al (2007) revealed that although the longer chains had more antibacterial potential, they stimulated increased lysis of host cells. Then, peptide by peptide scores and size around 6-8 amino acids mostly selected. The 13 peptides were synthesized and studied for the antimicrobial mechanisms against plant pathogen cells.

5.5 Peptide-microbe interaction mechanism

Among 13 peptides, 3 AMPs were found to effectively inhibit growth of *X. oryzae* pv. *oryzae*, *P. carotovorum* and *A. rhizogenes*. Peptide no. 1 and no. 10 were membrane-active AMPs, while peptide no. 2 was intracellularly active AMP. Otvos (2005) reported that AMPs' killing effect can be both membrane interruption and inhibition of functional proteins or metabolic process in target cells (Figure 5.2). Thus, there are two modes of action that are membrane active and intracellular active. Most membrane-active AMPs are amphipathic that initial electrostatic interaction with the negatively charged cell membrane and the insertion into cell membrane (Sani and Separovic, 2017). Gram-negative bacteria contain inner and outer membranes. The outer membrane is mostly consisted of lipopolysaccharides, whereas the inner membrane is abundant in saturated cyclopropane phosphatidylethanolamine and a significant amount of anionic phosphatidylglycerol (Horvath and Daum, 2013).

In the case of *X. oryzae* pv. *oryzae* after treated with peptide no. 1 and 10, the most proteins were related to integral component of membrane and lipoprotein localization to outer membrane. This could be implied that peptide no.1 and 10 disrupt cell membrane directly especially in outer membrane. As shown in figure 4.8, although ampicillin is one of beta lactam antibiotics that induce a mortal malfunction of bacterial cell wall synthesis but protein profile of *X. oryzae* pv. *oryzae* treated with peptides and ampicillin were considerably different.

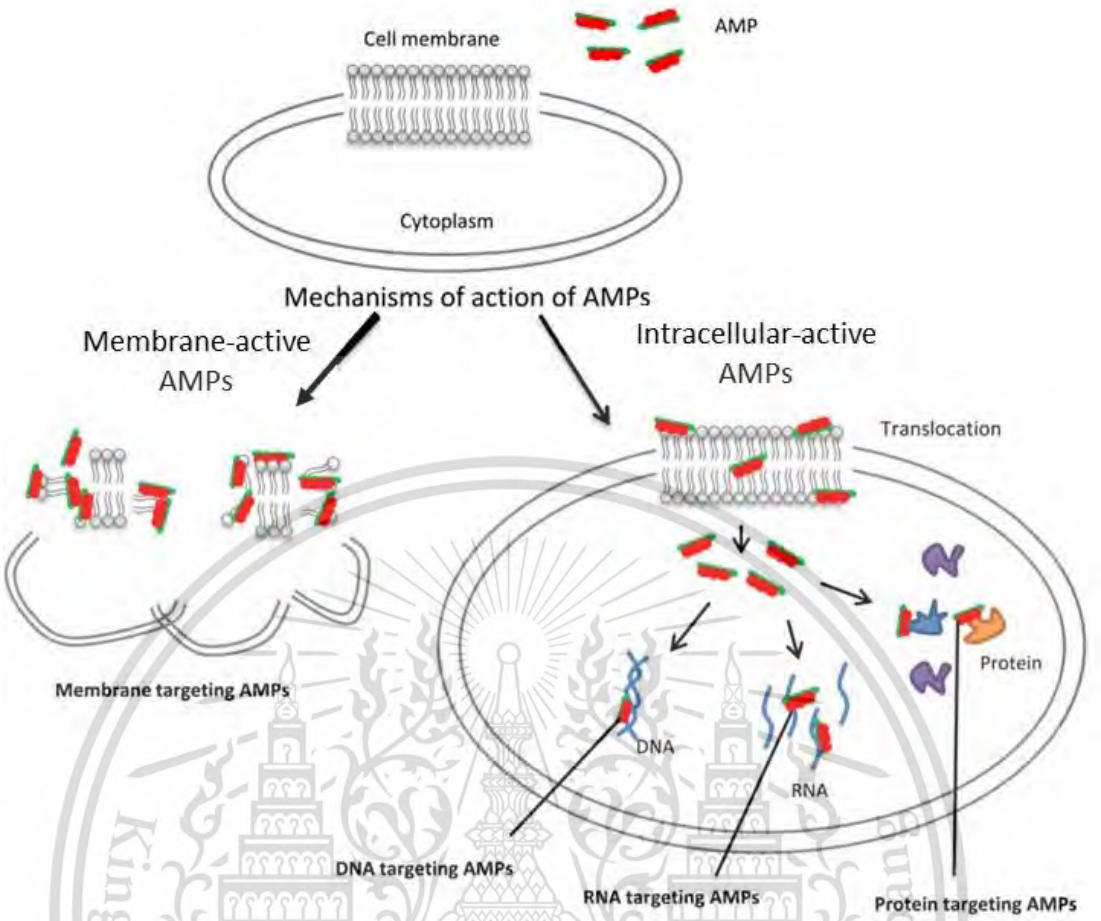

Figure 5.2 Mechanisms of action of membrane-active and intracellular-active AMPs (Shah et al., 2016).

Moreover, membrane-active AMPs are usually unformed in aqueous solution, while form an α-helical structure in the presence of a lipid membrane. In the proper condition, membrane-active AMPs create transmembrane pores or channel that cause leakage of intracellular molecules, eventually leading to cell death (Yeaman and Yount, 2003). Various modes of action have been proposed on the basis of arrangement of AMPs on membrane: the barrel-stave pore, toroidal pore, or carpet mechanism, as well as a number of less disruptive mechanisms as shown in Figure 5.3. Furthermore, the peptide-microbe interaction is dependent on lipid membrane composition, peptide structure, peptide concentration, temperature, and pH (Sani and Separovic, 2017).

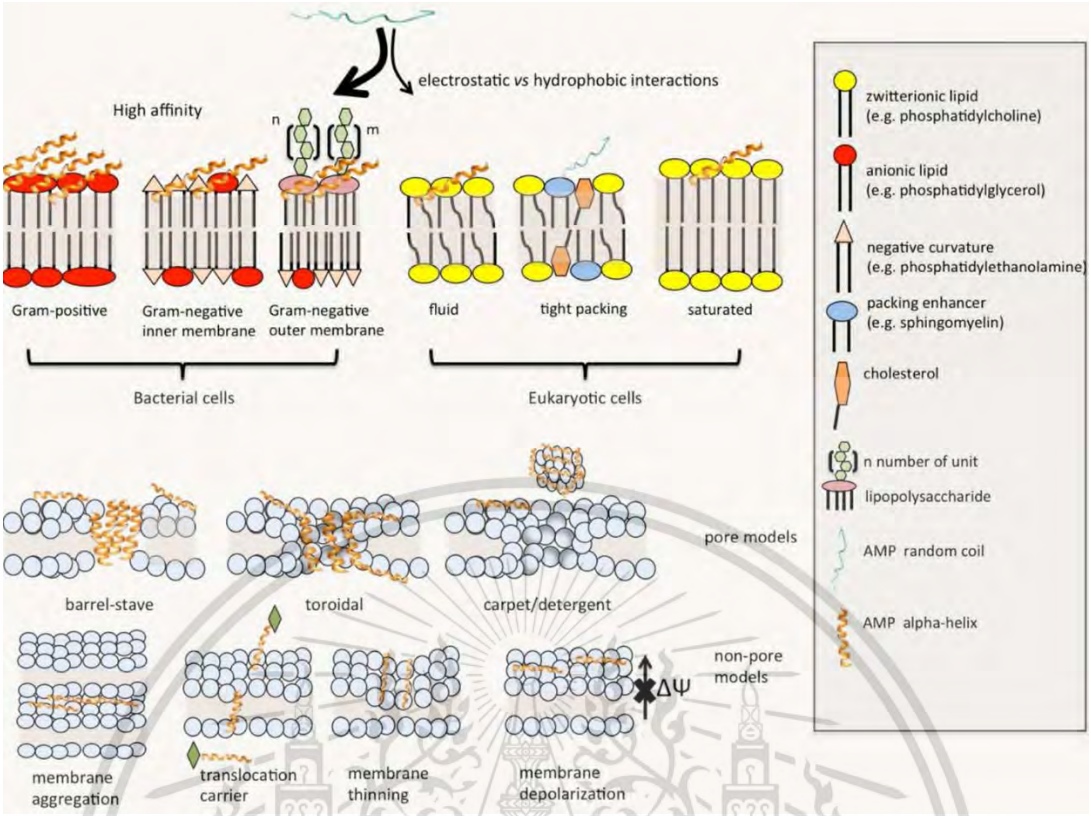

Figure 5.3 Peptide-microbe interaction in membrane model (Sani and Separovic, 2017).

Simultaneously, intracellularly active AMPs can inhibit or kill microbial cells without causing membrane disruption. This kind of AMPs interact with targets inside the cells; DNA, RNA or proteins (Otvos, 2005).

Several AMPs are reported to have only one mode of action under any concentration as an example, apidaecin expresses nonmembrane lytic mode of action in every concentration and condition (Casteels et al., 1989). However, dual mechanisms are reported for many AMPs which are depending on peptide concentration. Typically, AMPs at higher than MIC concentration lead to membrane lysis as detergent, whereas concentration lower than MIC cause membrane penetration and target on different macromolecules in cells like DNA, RNA or protein (Cudic and Otvos, 2002). This causes inhibition of the metabolic processes and further lead to death of bacteria. From table 4.11-4.12 and figure 4.11-4.12, mechanism of peptide no.2 related to nucleic acids (DNA/RNA), lipids and proteins. These results conformed to several reports previously as described below.

Some positively charged AMPs bind to negatively charged molecules like DNA or RNA. For examples, a host defense tridecapeptide indolicidin directly bind to DNA and inhibit DNA

biosynthesis (Marchand et al., 2006; Tsai et al., 2018), buforin II bind to both DNA and RNA (Kobayashi et al., 2004) and tachyplesin I bind to the minor groove of double strand DNA, leads to apoptosis-like death in *E. coli* (Kwun and Lee, 2021). In the cases of lipid targets, some AMPs are reported to target lipids in cell wall or cell membrane by inhibiting the conversion of lipid II to the polymeric peptidoglycan and blocking the cell wall synthesis. Few examples of lipid-targeting AMPs are human neutrophil defensin-1 (HNP-1) (de Leeuw et al., 2010), nisin (Wiedemann et al., 2001), mutacin (Hasper et al., 2006), copsin (Essig et al., 2014) and plectasin (Schneider et al., 2010). While various AMPs are reported to targets proteins associated with several metabolic processes in cell, for examples; PR-39, Bac-7 and Apidaecin are AMPs that binding proteins, inhibit protein synthesis and inhibit protein folding (Li et al., 2006; Mardirossian et al., 2014; Runti et al., 2017).

Interestingly, although peptide no.1 and no. 10 have main part as membrane-active AMPs, there were some proteins related to metabolic process. Simultaneously, peptide no.2 has main part working like intracellular-active AMPs while some parts also affected to cell wall and cell membrane. These results are similar to some reports in previous years. Shi et al. (2016) reported that melittin, AMP from *Apis mellifera*, was investigated by examining its consequences on cell membranes, energy metabolism, and nucleic acid synthesis. Melittin disrupts membrane by making holes resulting in the leakage of cytoplasm. In addition, melittin may inhibit biosynthesis in both DNA and proteins. Moreover, melittin, bactericidal peptide named indolicidin also showed more than one mechanism of antimicrobial action. The indolicidin structure in aqueous solution was an amphipathic and globular conformation, in opposite to the structure adopted in lipid, and these two structures were suggested to have different functions. In the study of gel retardation and fluorescence quenching experiments explained that indolicidin showed DNA-binding mechanism and interacting with lipid bilayers at different concentrations (Hsu et al., 2005).

Chapter 6

Conclusions and suggestions

6.1 Conclusions

The objectives of this study were aimed to determine the antibacterial activity of protein hydrolysates from three kinds of agricultural wastes against bacterial plant pathogens, to purify bioactive peptides from protein hydrolysates and to study antibacterial mechanism of active peptides against plant pathogens using shotgun proteomics technique. By this, the present study can be concluded in many aspects as showed below:

1. At high level of confidence (R^2=0.99) when determined protein concentration of all fifteen samples according to the procedure of Lowry, protein concentration (percent protein by weight) of all samples was 3.59 in average, protein concentration from rice straw and corn leaves were highest among all samples (10.10 and 9.87%, respectively) while corn cob leaves showed lowest protein concentration at 1.14%.

2. Some protein hydrolysates from agricultural wastes can be used to inhibit growth of bacterial plant pathogens. Antibacterial investigation by broth dilution assay showed some samples as potential sources with antibacterial protein hydrolysates at higher than 50% of inhibition level after being incubated for 6 h. Four out of from 15 samples including AW1 (rice straw), AW6 (bagasse), IW3 (peanut seed coat) and IW4 (coconut residue) showed obviously higher potential than others, AW6 ranked first in all pathogens (*X. oryzae* pv. *oryzae*, *X. citri*, *P. carotovorum* and *A. rhizogenes*).

3. Concernong PGPRs, any inhibitory potential was unnoticed on *B. subtilis*, however, AW6 and IW4 showed high percentage inhibition against *P. aeruginosa* and *P. fluorescens*, while other protein hydrolysate samples showed no inhibitory effect at all. In addition, it was noticed that hydrolysates from some samples supported growth of PGPRs as followings; *B. subtilis* growth was supported by hydrolysates from AW2 AW4 IW3 IW4 IW6 IW7 FW1 and FW2, *P. aeruginosa* growth was supported by hydrolysates from IW2 IW6 and FW1, lastly, *P. fluorescens* growth was supported by hydrolysates from AW2 AW4 IW3 IW4 IW 6 IW7 FW1 and FW2.

4. After purification for three steps (reversed-phase chromatography, cation exchange chromatography and off-gel fractionation), 5 samples from well 5, 6, 7, 13 and 18 of hydrophilic-cation AW6 were selected for further analysis by LC-MS/MS to find out effective peptides.

5. Thirteen peptides with high peptide scores were selected to synthesized and only 3 peptides (peptide no. 1, 2 and 10) among them showed antibacterial activity against *Xoo*, *P. carotovorum* and *A. rhizogenes*.

6. From Venn diagram, mostly proteins found in bacterial plant pathogens treated with peptides were unique from treated with antibiotics. Then it could be concluded that these peptides have the unique mechanisms on their own natural process.

7. Data of gene ontology from UniProt database showed that most of proteins found in *X. oryzae* pv. *oryzae* treated with peptide no. 1 and no. 10 were related to cell membrane and cell wall more than 50%, other proteins related to transcription, DNA repair, DNA integration, DNA replication and protein transport. These means mechanism of peptide no.1- *X. oryzae* pv. *oryzae* was mostly related to cell membrane and cell wall. Possibly, peptide no.1 caused *X. oryzae* pv. *oryzae* cell leak and some part of peptide affected DNA biological process in cells. While most of proteins found in *P. carotovorum* and *A. rhizogenes* treated with peptide no. 2 related to biological processes in cell.

6.2 Suggestions

1. Protein hydrolysates from AW1 (rice straw), AW6 (bagasse), IW3 (peanut seed coat) and IW4 (coconut residue) might be used to apply in agricultural areas that is dispersal of plant pathogens.

2. In this research methodology, some bacterial plant pathogens may be added to find more effective of antibacterial property.

3. Waste samples should be prepared in bigger scale for loss samples protection that might be happened in purification steps.

4. From peptide-microbe mechanism results, scanning electron microscope should be applied for clearer results and more understandable about mechanisms in bacterial cells.

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APPENDICES

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Appendix A

Lowry assay

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Lowry assay

Cu+ ions from the Biuret reaction form an unstable blue complex with the Folin-Ciocalteau reagent. This complex serves as a measure of the protein concentration.

Stock reagent of Lowry assay

1. CTC solution (0.2 g $CuSO_4.7H_2O$ + 0.4 g Tartalic acid + steriled water to 100 ml)
2. 0.8 N NaOH (1.6 g NaOH + steriled water to 50 ml)
3. 5% SDS (2.5 g SDS steriled water to 50 ml)
4. 20% Na_2CO_3 (10 g Na_2CO_3 steriled water to 50 ml)

Reagent A	CTC solution	5	ml
	0.8 N NaOH	10	ml
	5% SDS	20	ml
	20% Na_2CO_3	5	ml
Reagent B	Folin- Ciocalteau reagent	1	ml
	Steriled water	5	ml

Assay

1. 5 µl protein sample into 96-well plates (4 replications).
2. Add reagent A 200 µl, then incubate for 30 min at RT.
3. Add reagent B 50 µl, then incubate for 30 min at RT.
4. Measure absorbance at 750 nm.

BSA standard

Prepare 0, 2, 4, 6, 8, 10 µg/µl, then measure absorbance at 750 nm and calculate y= mx+c from the standard curve.

Appendix B

Desalting results

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Chromatogram of desalting chromatography peak with 50 ml P-6 Desalting column.

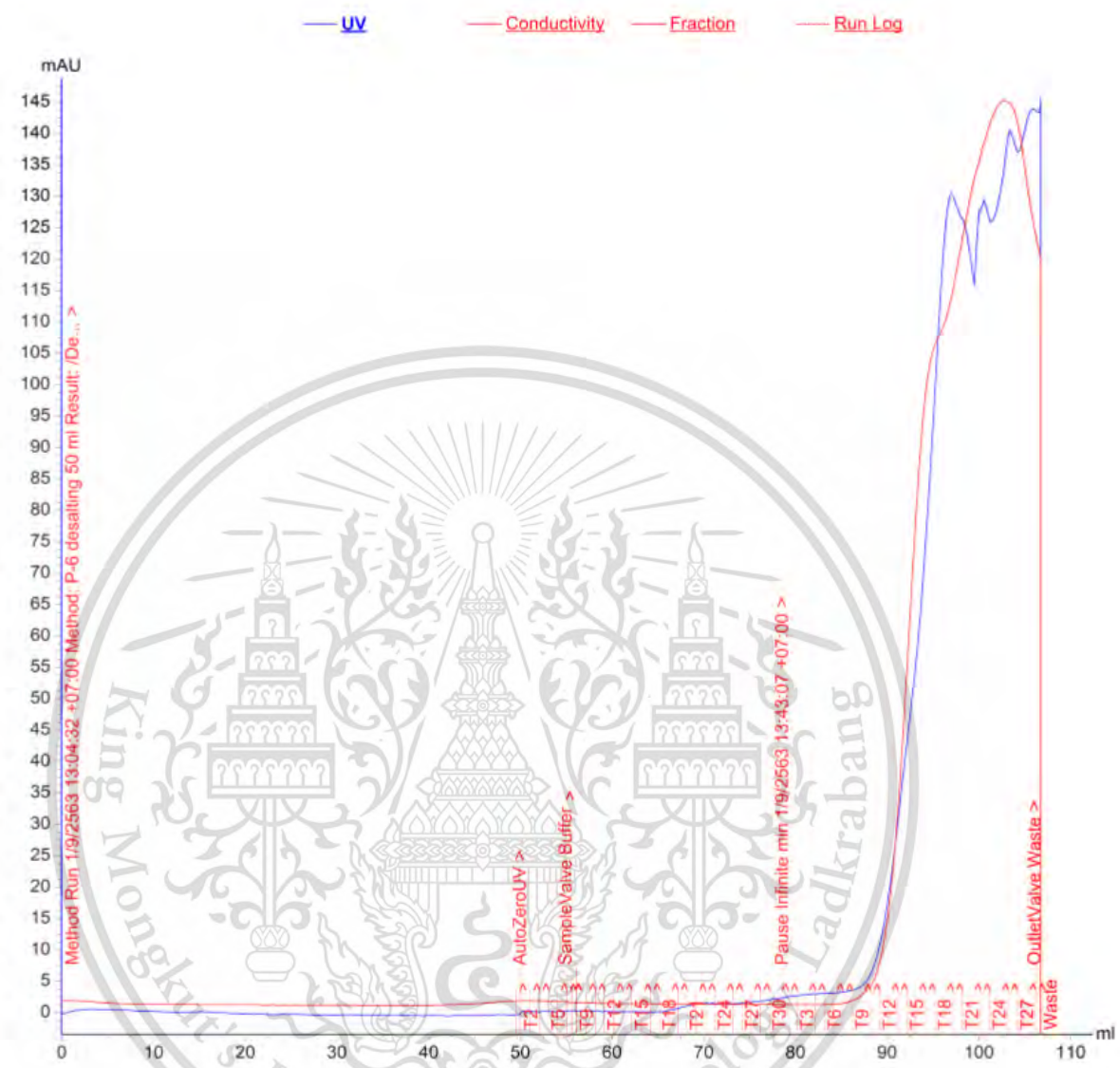

Figure B.1 Chromatogram of AW1 after desalting.

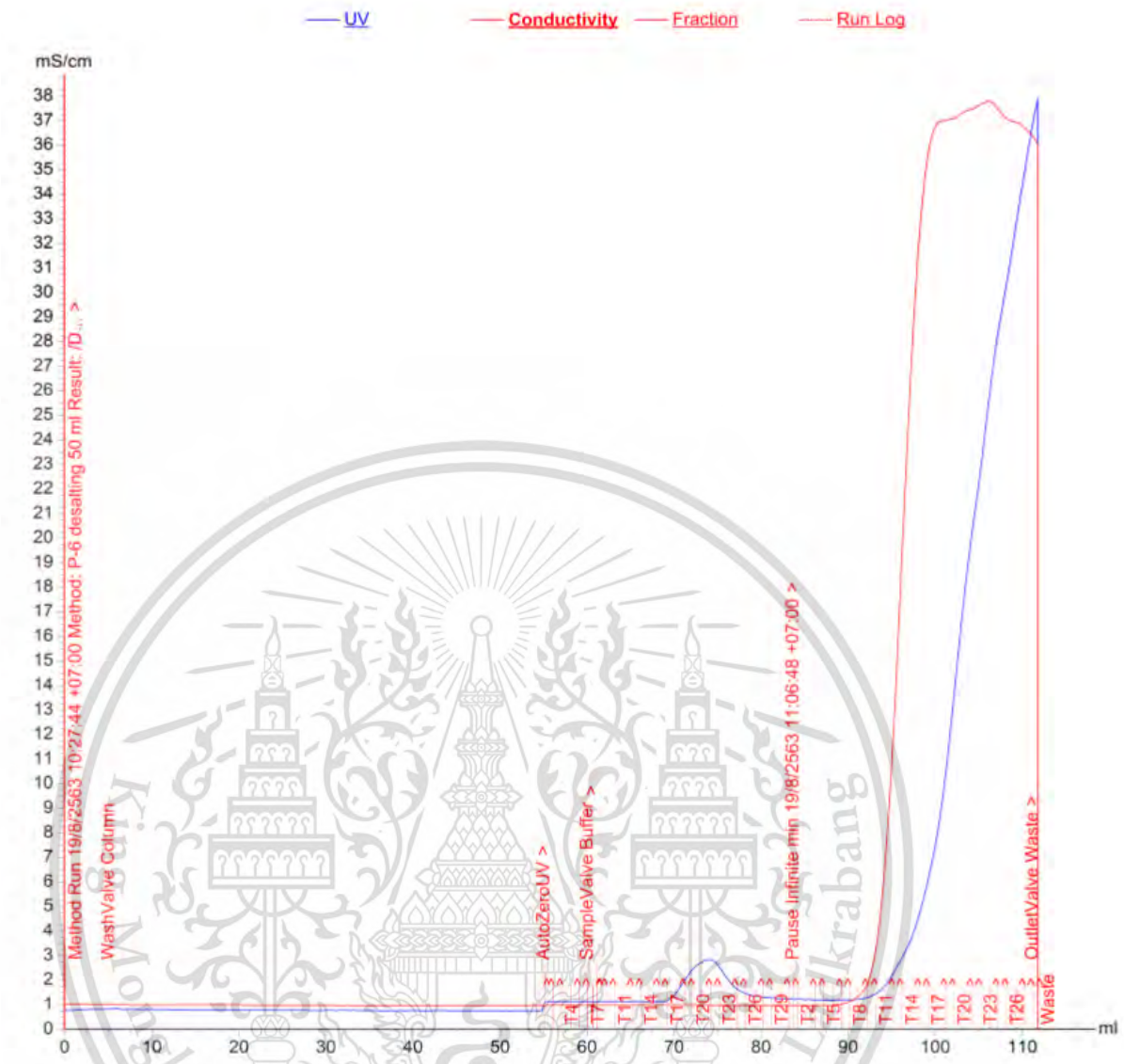

Figure B.2 Chromatogram of AW6 after desalting.

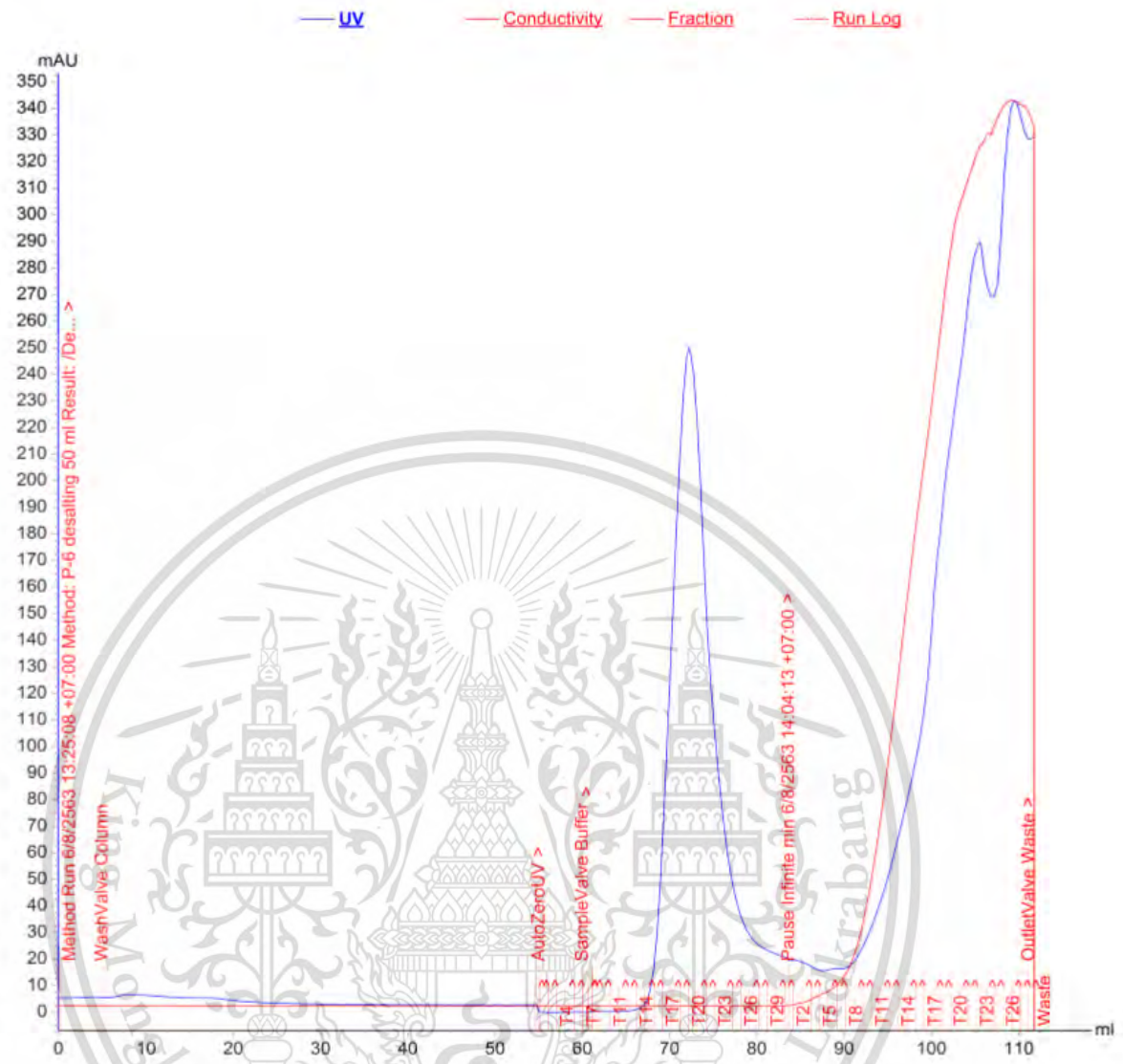

Figure B.3 Chromatogram of IW3 after desalting.

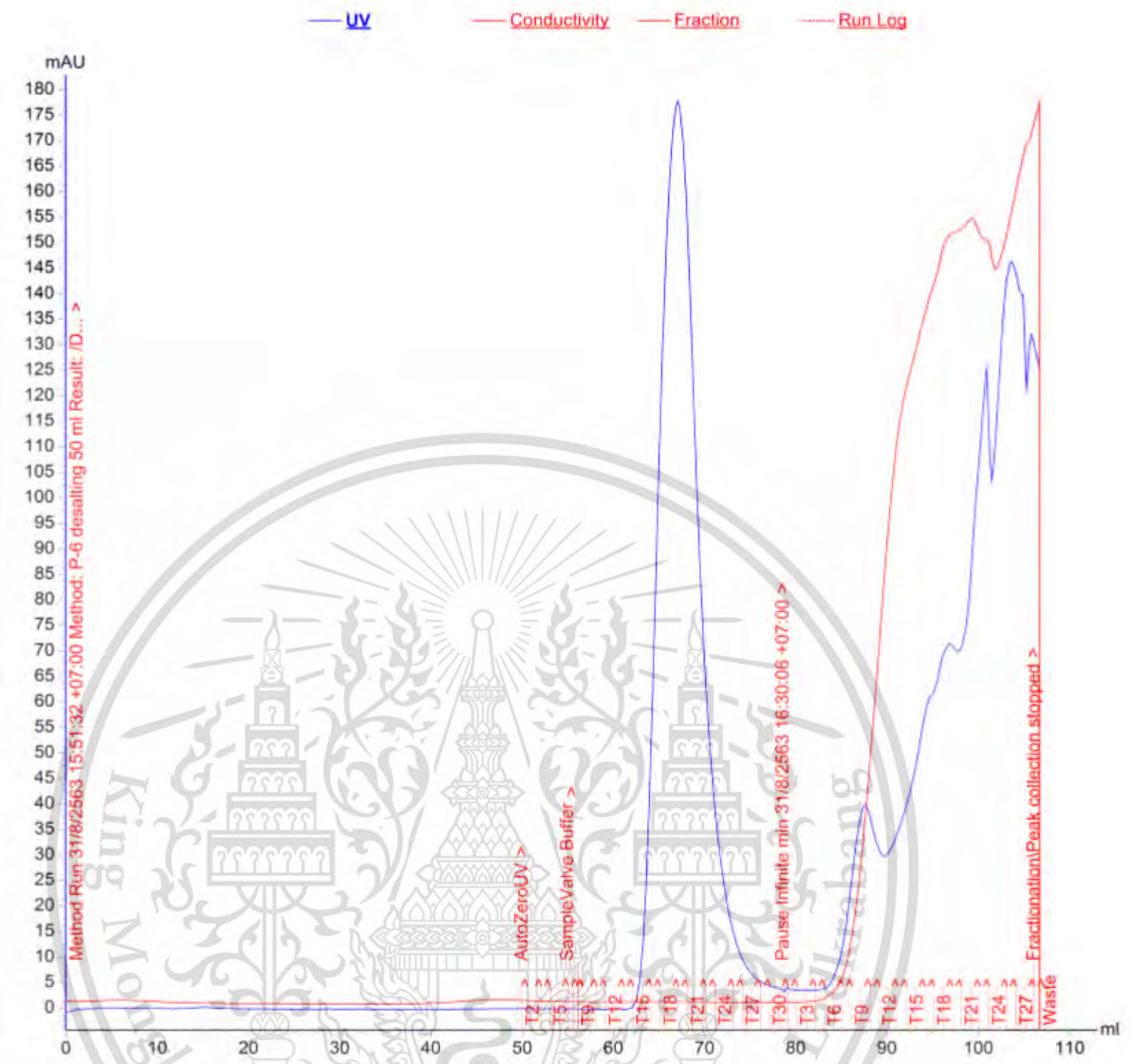

Figure B.4 Chromatogram of IW4 after desalting.

Appendix C

Cation exchange chromatography results

King Mongkut's Institute of Technology Ladkrabang

Chromatogram of cation exchange chromatography peak with 1 ml SPFF column

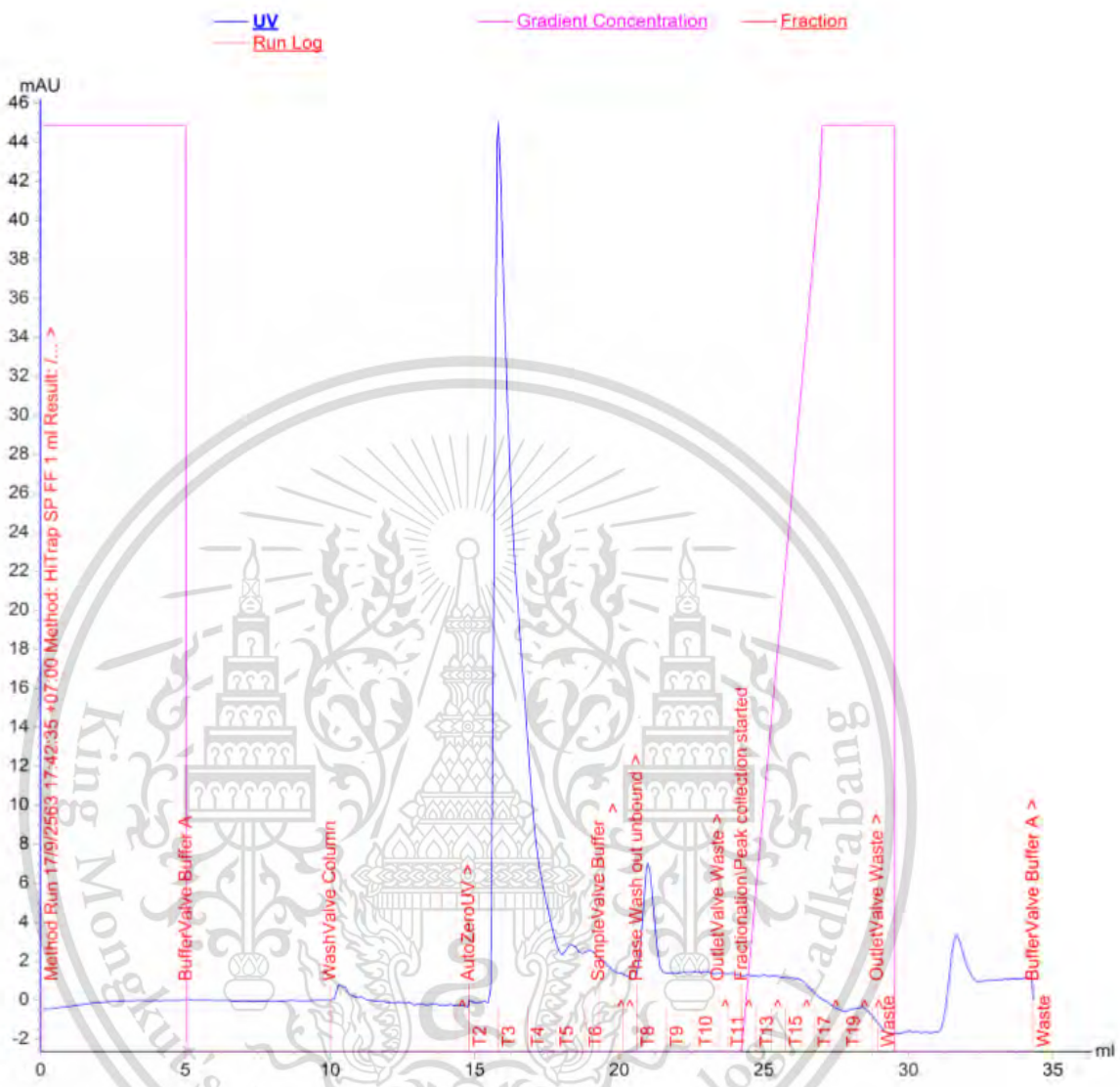

Figure C.1 Chromatogram of AW1 after cation exchange chromatography.

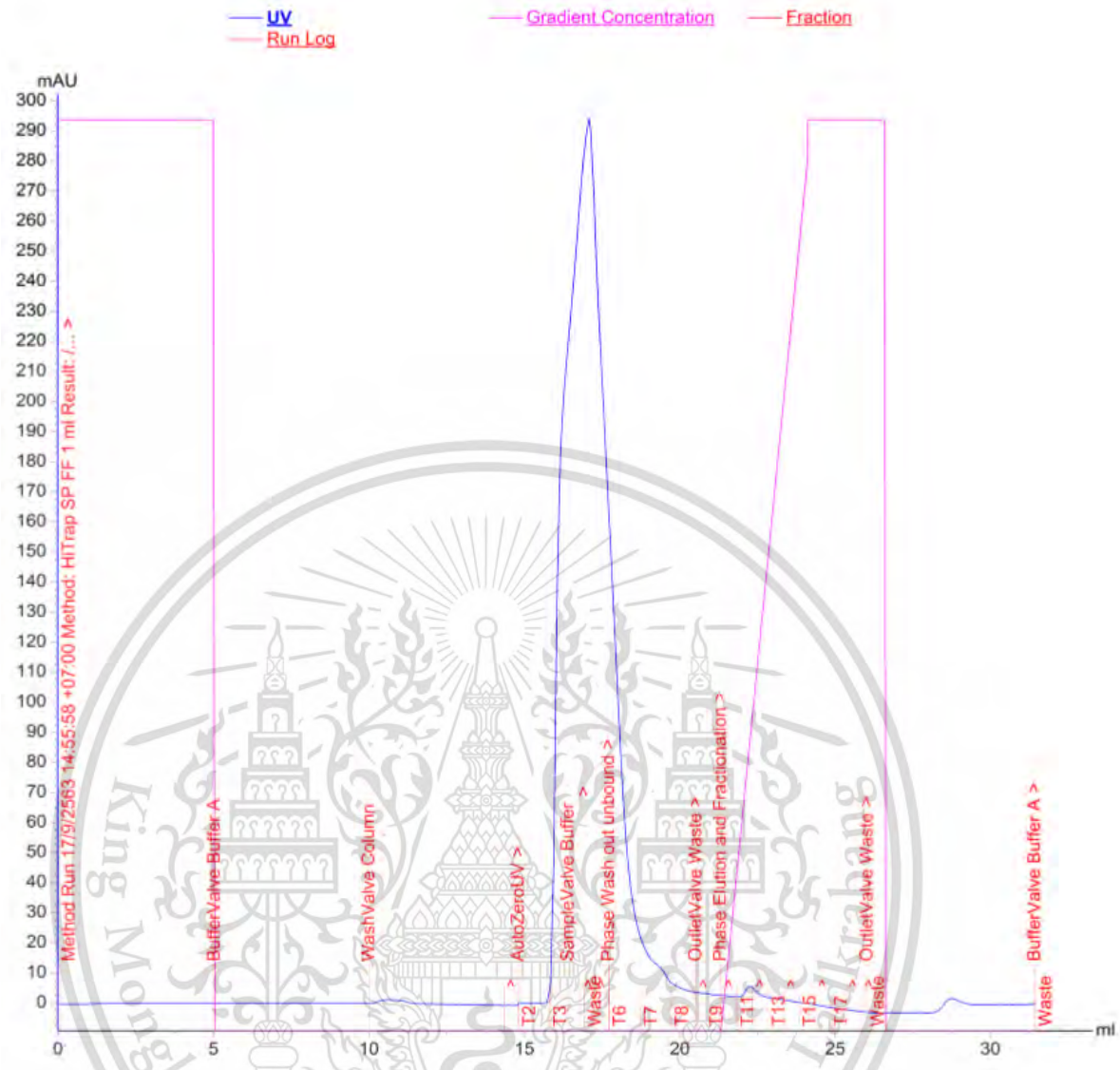

Figure C.2 Chromatogram of AW6 after cation exchange chromatography.

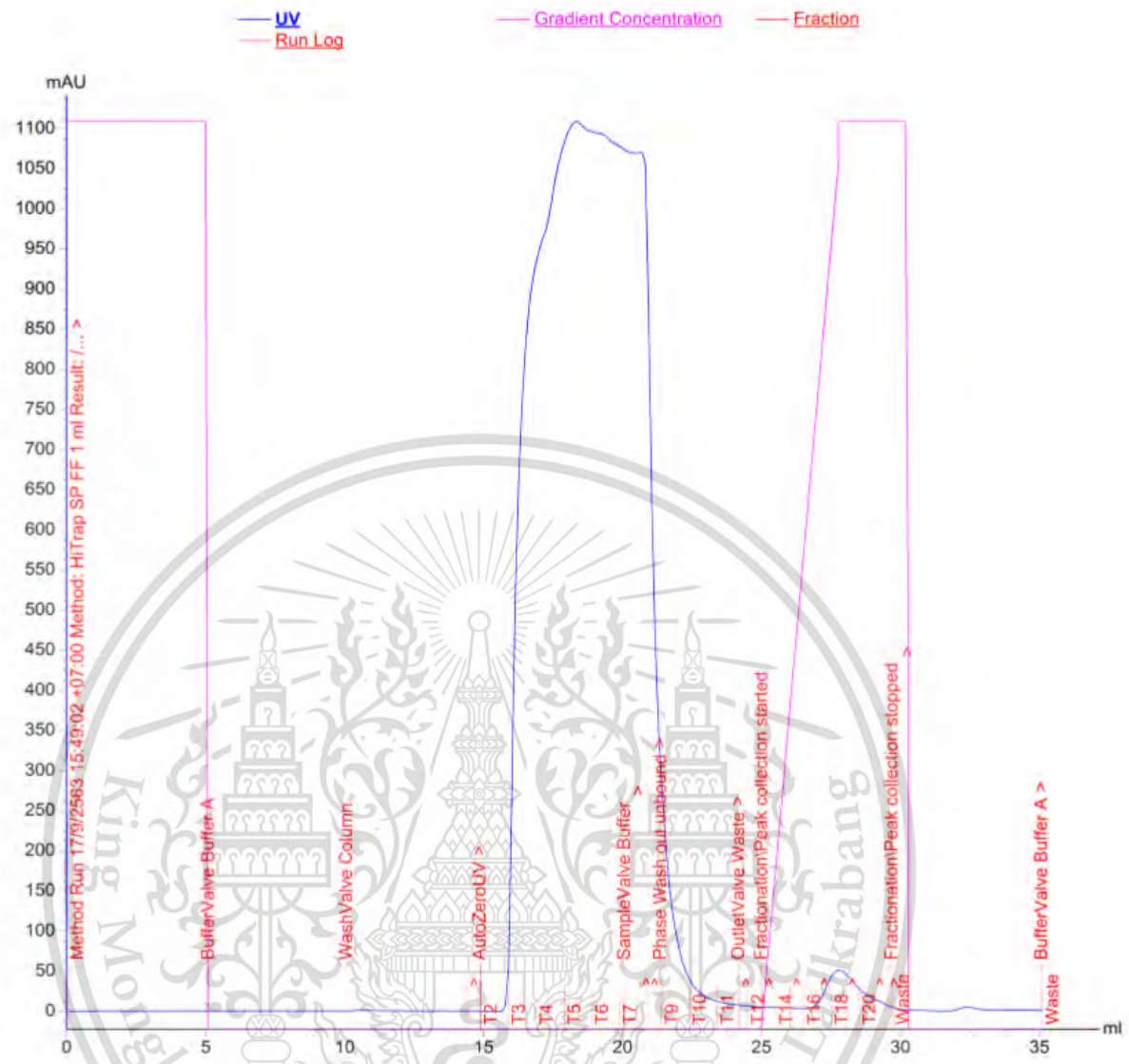

Figure C.3 Chromatogram of IW3 after cation exchange chromatography.

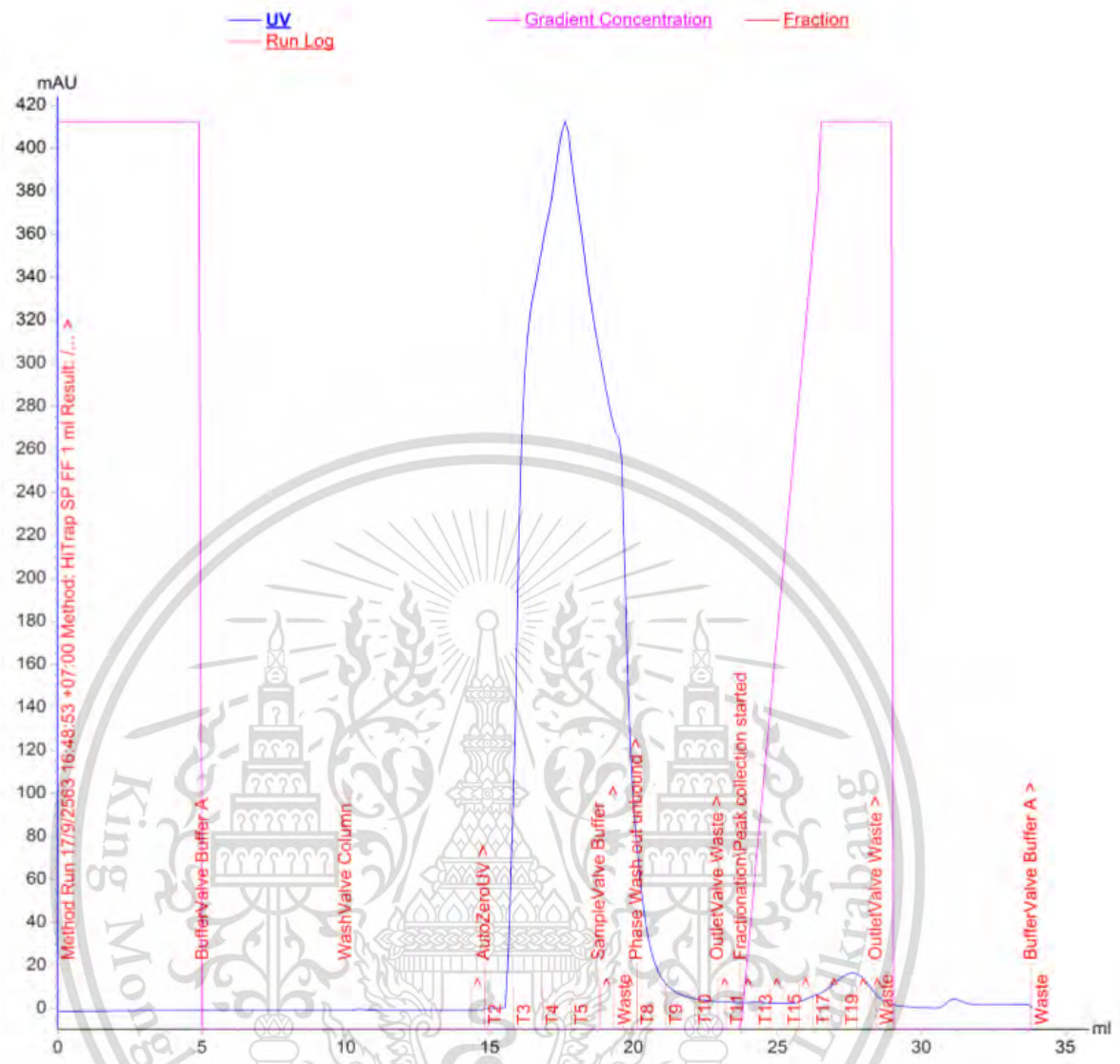

Figure C.4 Chromatogram of IW4 after cation exchange chromatography.

Appendix D

Academic publications

King Mongkut's Institute of Technology Ladkrabang

1. Protein hydrolysates from agricultural wastes for plant bacterial disease control

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Protein hydrolysates from agricultural wastes for plant bacterial disease control

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Ditsawanon, T., Parinthawong, N., Phaonakrob, N. and Roytrakul, S. (2022). Protein hydrolysates from agricultural wastes for plant bacterial disease control. International Journal of Agricultural Technology 18(2):479-488.

Abstract Agricultural wastes, agro-industrial wastes and fishery wastes were collected and the protein hydrolysates were obtained with pepsin. Antibacterial activity of smaller than 3 kDa protein hydrolysates was determined against the plant pathogenic bacteria; *Xanthomonas citri*, *Ralstonia solanacearum*, *Burkholderia cepacia* and also against plant growth promoting rhizobacteria (PGPRs); *Bacillus subtilis*, *Pseudomonas aeruginosa* and *P. fluorescens*. Coconut residues (agro-industrial waste from coconut milk production), peanut seed coat (from peanut-based snack production) and rice straw (waste from rice farms) showed antimicrobial activity against *X. citri*, *R. solanacearum* and *B. cepacia* with higher than 74% inhibition. Coconut residue also increased growth of PGPRs, *B. subtilis* and *P. fluorescens*. Further protein hydrolysates from Nile tilapia (Oreochr*omis niloticus*) and snake-head fish (*Clarias batrachus*) fin increased growth of all PGPRs.

Keywords: Agricultural wastes, Antimicrobial activity, Protein hydrolysates, Plant pathogenic bacteria

Introduction

The losses caused by phytopathogens have been estimated to be up to 16% in cultivated areas worldwide (Oerke, 2006). Bacterial phytopathogens cause several diseases and lead to abnormal growths, rots, spots and wilts. Many bacterial pathogens use secreted proteins to destroy cell walls and intrude into host cells causing necrosis. Several of these pathogens cause diseases in economically important plants – wilt is but one example. A consequence of these disease outbreaks was a large reduction in plant and animal diversity in ecosystems globally.

Most research in bacterial plant pathology targets control of disease outbreaks, which decrease yield in agricultural products directly or indirectly.

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There are three strategies to control these diseases; chemical application, biological control and genetic resistance. A common approach uses chemicals, because these are easy to use and highly effective. However, they can lead to long-term soil pollution and some are carcinogens for living organisms, restricting their future use (Daoubi *et al.,* 2005). Chemical approaches include antibiotics, but use of the same antibiotics for a long time has limited the permitted antibiotics, as they will develop antibiotic resistance. Thus, effective methods, that are environmentally friendly and benefit consumers and farmers, must be found.

Antimicrobial peptides (AMPs) are good guards for protection against attacking pathogens and have several functions that lead to innate immunity (Park *et al.,* 2004) This is better for the environment and consumers. Over 1,500 AMPs have been found in many living things, both eukaryotes and prokaryotes (Wang and Wang, 2004). Usually, AMPs show a broad activity to kill fungi, bacteria, parasites and viruses, in consequence, AMPs are grouped in general as antifungal, antibacterial, antiparasitic and antiviral, respectively (Zhang and Gallo, 2016). The antibacterial activity derives from the amphiphilic characteristics and a high density of positive charges within the peptide structure. This lets peptide attachment and insertion into the bacterial cell membrane, forming pores and causing cell lysis and cytoplasm leakage (Powers *et al.*, 2004; Lee *et al.,* 2016).

Presently, many types of AMPs against bacterial plant pathogens were reported, for example, Tantong *et al.* (2016) examined plant AMP defensins for antibacterial activity against pathogenic bacteria *Xanthomonas oryzae* pv. *oryzae* (causes leaf blight), *X. oryzae* pv. *oryzicola* (causes leaf streak) and *Pectobacterium carotovorum* (causes soft rot disease); they found that some defensin peptides exhibited inhibitory action with minimum inhibitory concentration (MIC) from 0.6 to 63 μg/ml. Shi *et al.* (2016) chose *X. oryzae* pv. *oryzae* for antibacterial testing, using melittin, an AMP from honeybee venom, they showed that melittin performed well against *X. oryzae* with IC_{50} 9-10 μM. Moreover, images from scanning electron microscopy revealed that melittin strongly disrupted bacterial cell membranes, makes holes in the cell membrane and inhibits DNA and protein synthesis leading to bacterial cell death. Citrus canker caused by *X. citri* decreased citrus fruit product quality significantly, so copper and streptomycin have been used to control the disease. However, appearance of resistant *X. citri* led to a reduction of disease control. Three hexapeptides, from totally fourteen new small synthetic AMPs, showed bactericidal activities against several *X. citri* strains at 10 μg/ml and disease development was suppressed significantly, when these AMPs were applied to citrus leaves in the present of pathogens (Choi *et al.,* 2017). Moreover, Morais

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et al. (2019) reported that eight alpha helical cationic peptides, originating from plant protein, targeted outer membrane proteins in gram negative bacteria. In addition, magainin II, a 23 amino acid peptide exhibited significant bactericidal activity for *B. cepacia* with IC_{50} at 128 µg/ml.

Plant growth promoting rhizobacteria (PGPRs) - a group of bacteria that grow around plant root systems, due to release of plant root exudates – have various benefits to on growth, also used in disease control (Gray and Smith, 2015). Plant growth is promoted directly by biosynthesis of growth promoting compounds, for example, phytohormones, vitamins and enzymes. In the case of indirectly promotion, PGPRs inhibit phytopathogens by synthesis of antagonistic substances and lead to resistance against pathogens (Glick, 2012). PGPR genera, include *Bacillus* and *Pseudomonas* (Bhattacharyya and Jha, 2012), and *B. subtilis*, *P. aeruginosa* and *P. fluorescens* were chosen to study in this work.

However, no effective AMPs from wastes has been reported to inhibit bacterial plant pathogens and increase PGPR growth. Then the objective of this project was to determine the antibacterial activity of protein hydrolysates, smaller than 3 kDa, from three groups of wastes (agricultural wastes, agro-industrial wastes and fishery wastes), against plant pathogenic bacteria and PGPRs.

Materials and methods

Time and place of research

This research was conducted in year 2020-2021 at the Functional Proteomics Technology, Functional Ingredients and Food Innovation Research Group Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, in Pathumthani, Thailand.

Experimental design

The experimental design used a completely randomized design: 14 kinds of agricultural waste were tested, while experimental units were bacterial plant pathogens (*X. citri* DOA-BC902, *R. solanacearum* DOA-BC1954 and *B. cepacia* ATCC25416) and plant growth promoting rhizobacteria or PGPRs (*B. subtilis* ATCC6633, *P. aeruginosa* ATCC27853 and *P. fluorescens* TISTR2630), grown in tryptic soy broth (TSB) (Difco BBL, USA) at 28 ℃ in wells of 96-well plates. Experiments were run in triplicate.

Preparation of waste samples

Waste samples were collected and classified into three groups - agricultural, agro-industrial and fishery wastes - as shown in Table 1.

Table 1. Waste samples

Sample	Code	Details of waste source (all locations in Thailand)
Source: Agricultural waste		
Rice straw	AW1	Mueang Chachoengsao District Agricultural Extension Office (13.6690N, 101.0891E)
Corn cob	AW2	Corn farm, Sakaeo (13.5035N, 102.2872E)
Corn leaves	AW3	Corn farm, Sakaeo (13.5035N, 102.2872E)
Corn cob leaves	AW4	Corn farm, Sakaeo (13.5035N, 102.2872E)
Sugarcane leaves	AW5	Sugarcane plantation, Sakaeo (13.50181N, 102.2875E)
Source: Agro-industrial waste		
Fermented soybean	IW1	Residues, light soy sauce production, Hi-q Food Products Co., Ltd, Chachoengsao (13.7489N, 100.9518E)
Soybean pellet	IW2	Residues, soybean milk production, market, Chachoengsao (13.6924N, 101.0807E)
Peanut seed coat	IW3	Residues, peanut based snack production, Mae-Ruay Snack Food Factory Co Ltd, Bangkok (13.6557N, 100.4305E)
Coconut residue	IW4	Residues, coconut milk production, market, Chachoengsao (13.6924N, 101.0807E)
Coffee grounds	IW5	Arabica grounds. Rosetta Coffee Shop, Chachoengsao (13.6701N, 101.0562E)
Fish residue	IW6	Residues, fish sauce production, King Mongkut's University of Technology Thonburi (13.5790N, 100.4418E)
Fish residue (desalted)	IW7	Residues, fish sauce production, rinsed by water, King Mongkut's University of Technology Thonburi, (13.5790N, 100.4418E)
Source: Fishery waste		
Nile tilapia fish fin	FW1	Market, Chachoengsao (13.6623N, 101.0343E)
Clarias sp. fish fin	FW2	Market, Chachoengsao (13.6623N, 101.0343E)

Preparation of crude protein

Total protein from 50 g of samples was extracted using 0.05 M sodium acetate, pH 4.0 and mechanical shaking (25 ±2 ℃, 200 rpm, 1 h). followed by heating at 121 °C for 15 min. The total protein concentration of the supernatant was measured by Lowry assay (Lowry *et al.,* 1951). Bovine serum albumin (BSA) was used as a protein standard. Protein concentration was evaluated by measuring the absorbance at 750 nm (OD_{750}) and calculated from a calibration curve.

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Preparation of protein hydrolysates

The proteins were hydrolyzed with pepsin (Sigma–Aldrich, St. Luis, MO, USA) at a 1:25 (pepsin:sample) ratio in a shaker (37 ℃, 200 rpm, 12 h), and then boiled for 10 min. The crude hydrolysates were centrifuged (10,000×g, 10 min), then the supernatant was diluted five times with 0.5 M sodium acetate. The diluted hydrolysates were filtered through a semipermeable membrane (Vivaspin 20, 3 kDa MWCO, GE Healthcare, UK) and hydrolysates smaller than 3 kDa peptides were frozen at -20 ℃ until used.

Antibacterial activity determination

The antimicrobial activity of the protein hydrolysates was determined against three bacterial plant pathogens (*X. citri*, *R. solanacearum* and *B. cepacia*) and three PGPRs (*B. subtilis*, *P. aeruginosa* and *P. fluorescens*), using the broth dilution method in triplicate, following Sornwatana *et al.* (2013). Bacteria were grown for 24 h at 28 ℃ in tryptic soy agar (TSA) (Difco BBL, USA), then a single colony was selected to culture in TSB medium for 12-16 h until the OD_{600} reached ~0.05. Then smaller than 3 kDa hydrolysates were diluted to a final concentration of 100 µg/ml. Protein hydrolysates from each sample were filtered through a 0.2 µm membrane. The bacteria in TSB, phosphate buffered saline (PBS), and kanamycin antibiotic were used as controls. Samples were placed in 96-well plates and shaken at 200 rpm at 28 ℃: the OD_{600} was recorded after incubation for 0, 2, 4, 6 and 8 h in a microplate reader (Synergy H1 Hybrid Multi-Mode Reader, Biotek). Inhibition was calculated after incubation for 6 h.

Results

Antibacterial activity

Broth dilution assay was used to assess antibacterial activity against pathogens for the protein hydrolysates. The hydrolysates from AW1, IW3 and IW4 showed antibacterial activity to the pathogens (*X. citri*, *R. solanacearum* and *B. cepacia*) at 100 µg/ml - the same concentration used by Choi *et al.* (2017) - compared with the controls - see Figure 1.

Three antibiotics (kanamycin, ampicillin and oxytetracycline) were tested for antibacterial activity: kanamycin was the most effective in controlling pathogen growth. Then, kanamycin was used as an antibiotic control in the following antibacterial experiments. Note that bacterial growth in TSB, without kanamycin (control 1), was continuously grown from 0 to 8 h. This indicated

that the top three samples, showing antibacterial activity against pathogens, were AW1, IW3 and IW4. They showed an almost unchanged OD_{600}, throughout the experiment, compared with kanamycin and controls, so the OD_{600}, after 6 h, was selected to study the antibacterial activity (Table 2). Then, OD_{600} for the best three active samples was plotted versus time - see Figure 1.

Table 2. Antibacterial activity of 50 µg/ml protein hydrolysates from all samples against the pathogens after 6 hours

Sample	Optical density at 600 nm (Mean ±SD)		
	X. citri	*R. solanacearum*	*B. cepacia*
AW1	0.113 ±0.015 ^{cde}	0.064 ±0.001 ^{d}	0.102 ±0.006 ^{bcd}
AW2	0.205 ±0.040 ^{h}	0.074 ±0.002 ^{gh}	0.245 ±0.049 ^{e}
AW3	0.131 ±0.004 ^{ef}	0.070 ±0.002 ^{ef}	0.139 ±0.011 ^{d}
AW4	0.124 ±0.012 ^{def}	0.065 ±0.002 ^{d}	0.118 ±0.016 ^{cd}
AW5	0.144 ±0.011 ^{efg}	0.071 ±0.000 ^{fg}	0.118 ±0.011 ^{cd}
IW1	0.468 ±0.034 ^{j}	0.086 ±0.002 ^{jk}	0.314 ±0.038 ^{f}
IW2	0.209 ±0.010 ^{hi}	0.078 ±0.002 ^{h}	0.223 ±0.041 ^{e}
IW3	0.090 ±0.002 ^{bcd}	0.066 ±0.001 ^{de}	0.085 ±0.008 ^{abc}
IW4	0.077 ±0.003 ^{abc}	0.060 ±0.001 ^{c}	0.074 ±0.004 ^{abc}
IW5	0.178 ±0.007 ^{gh}	0.077 ±0.002 ^{h}	0.148 ±0.009 ^{d}
IW6	0.241 ±0.058 ^{i}	0.083 ±0.003 ^{ij}	0.220 ±0.061 ^{e}
IW7	0.148 ±0.008 ^{efg}	0.070 ±0.001 ^{ef}	0.136 ±0.030 ^{d}
FW1	0.174 ±0.007 ^{gh}	0.076 ±0.003 ^{h}	0.201 ±0.010 ^{e}
FW2	0.155 ±0.009 ^{fg}	0.068 ±0.003 ^{def}	0.138 ±0.009 ^{d}
Kanamycin	0.064 ±0.003 ^{ab}	0.055 ±0.001 ^{b}	0.280 ±0.017 ^{ab}
Control 1	0.437 ±0.017 ^{j}	0.089 ±0.006 ^{k}	0.054 ±0.002 ^{g}
Control 2	0.046 ±0.001 ^{a}	0.046 ±0.001 ^{a}	0.416 ±0.038 ^{a}

Note: Means marked with the same superscript letter in a column were not statistically different (p<0.05) using Duncan's Multiple Range Test.

Inhibition of bacterial growth

The broth dilution method showed eight samples were potential sources of antibacterial protein hydrolysates, with inhibitory levels, higher than 50%, to at least one targeted bacteria (except *R. solanacearum*), after incubation for 6 h. Among these samples, AW1, IW4 and IW3 showed clearly higher activity than the others - see Table 3. IW4 had the highest activity against all pathogens. IW3 showed lower activity against *X. citri* and *B. cepacia*, while AW1 ranked third against *X. citri*, second against *R. solanacearum* and fourth against *B. cepacia*.

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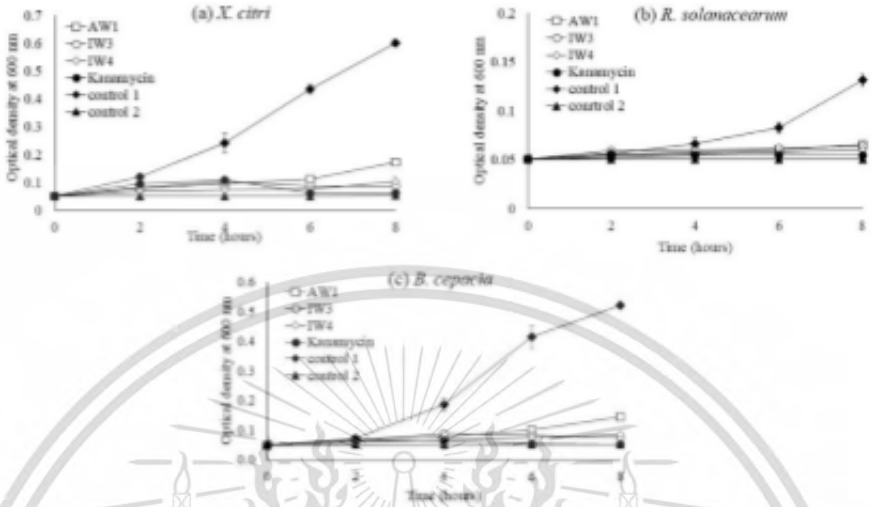

Figure 1. Antibacterial activity of 100 µg/ml protein hydrolysates from AW1, IW3 and IW4 against the pathogens (a) *X. citri*, (b) *R. solanacearum* and (c) *B. cepacia*. Control 1 was bacterial growth, without antibiotic, and control 2 was PBS, without microorganisms

Table 3. Top eight antibacterial activity against plant pathogenic bacteria

Antibacterial activity ranking	Inhibition of target organism		
	X. citri	*R. solanacearum*	*B. cepacia*
kanamycin	85.4%	37.8%	87.0%
1	82.3% (IW4)	32.2% (IW4)	82.1% (IW4)
2	79.4% (IW3)	27.7% (AW1)	79.6% (IW3)
3	74.1% (AW1)	-	75.6% (AW4)
4	71.6% (AW4)	-	75.4% (AW1)
5	70.0% (AW3)	-	71.7% (AW5)
6	67.1% (AW5)	-	67.4% (IW7)
7	66.1% (IW7)	-	66.8% (FW2)
8	64.5% (FW2)	-	66.6% (AW3)

For the PGPRs (*B. subtilis*, *P. aeruginosa* and *P. fluorescens*), no inhibitory activity was observed for *B. subtilis* and *P. fluorescens*. Only one sample from IW4 showed high inhibition against *P. aeruginosa*. Furthermore, hydrolysates from some samples promoted PGPR growth. *B. subtilis* growth was enhanced by hydrolysates from AW2, AW4, IW3, IW4, IW6, IW7, FW1 and FW2, while *P. aeruginosa* growth was increased by hydrolysates from IW2, IW6 and FW1, lastly, *P. fluorescens* growth was induced by hydrolysates from AW2, AW4, IW3, IW4, IW6, IW7, FW1 and FW2. Oxytetracycline was the best antibacterial agent (> 50%) against all three PGPRs - see Figure 2.

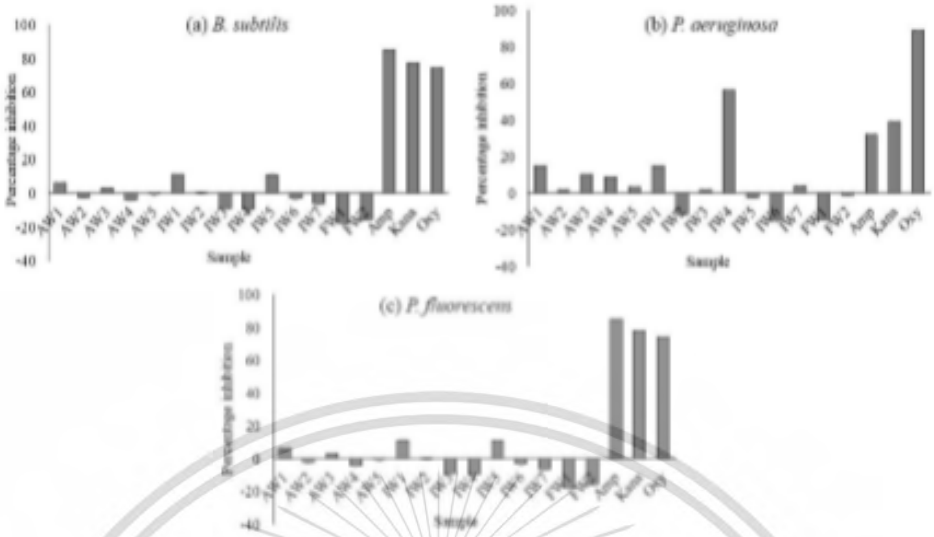

Figure 2. PGPR growth inhibition (a) *B. subtilis*, (b) *P. aeruginosa* and (c) *P. fluorescens*

Discussion

The results of antibacterial activity against bacterial plant pathogens were according with research in 2017, that fourteen new small synthetic antimicrobial peptides (AMPs) were developed by Choi *et al.* (2017) for controlling the citrus canker disease and were evaluated as an alternative to streptomycin. Interestingly, BHC10 (one of small synthetic AMPs) showed bactericidal activity especially on *X. citri* subsp. *citri* at 100 µg/ml, same concentration used in this work. Besides, Morais *et al.* (2019) reported that some AMPs from plant protein targeted outer membrane proteins in gram negative bacteria. Among them, magainin II showed considerable bactericidal activity for *B. cepacia*.

In our work, *R. solanacearum* was not significantly inhibited by both kanamycin and hydrolysate samples. A small number of antibiotics have shown antibacterial efficiency on different isolates of *R. solanacearum*, but some antibiotics were found to be ineffective in controlling this pathogen (Champoiseau *et al.,* 2010). Thus, it is necessary to seek more potent substances against *R. solanacearum*. Previously, methyl bromide fumigation was widely used, but it is not only expensive, but applying it to wide areas is also difficult. A few antibiotics have also been used to eliminate bacterial wilts. Streptomycin was commonly used in cultivation, but overuse induced bacterial resistance (Zhao *et al.,* 2011). Verma *et al.* (2017) found that antibiotics with various combinations of ambistryn and ceftriaxone at different proportions (1:1,

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1:3, 3:1) were effective against three isolates of *R. solanacearum* in small eggplant, capsicum, and tomato isolates. Thus, appropriate combinations of antibiotics were highly to moderately potent against *R. solanacearum*.

For PGPRs, these bacteria can increase nitrogen fixation, plant hormone production, solubilize insoluble compounds and induce systemic resistance (ISR) in the plants (Ghorbanpour *et al*., 2016; Chaudhary and Shukla, 2019). Then, developing PGPRs is one of the ways to enhance the yield of agricultural products (Glick, 2014). As the results, we found that fish residue (IW6) and *Nile tilapia* fish fin (FW1) were able to induce growth of all PGPRs (*B. subtilis*, *P. aeruginosa* and *P. fluorescens*). These can be useful for further studies to find effective peptides that promote PGPRs

Eight protein hydrolysate samples showed antibacterial activity against *X. citri*, *R. solanacearum* and *B. cepacia*. Coconut residue (IW4), peanut seed coat (IW3) and rice straw (AW1) showed outstanding antibacterial activity. While some samples enhanced PGPR growth (*B. subtilis*, *P. aeruginosa* and *P. fluorescens*). It was noted that coconut residue (IW4) strongly inhibited *X. citri*, *R. solanacearum* and *B. cepacia*, and increased growth of the PGPRs, *B. subtilis* and *P. fluorescens*. The protein hydrolysates from coconut residue have strong potential as biocontrols or fertilizers for protecting plants from bacterial diseases and promoting growth. In addition, protein hydrolysates can be purified to yield bioactive peptides.

Acknowledgements

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2. Novel small antimicrobial peptides extracted from agricultural wastes act against phytopathogens but not rhizobacteria

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Novel Small Antimicrobial Peptides Extracted from Agricultural Wastes Act against Phytopathogens but Not Rhizobacteria

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Abstract: Nonedible materials such as agricultural wastes can serve as sources of antimicrobial peptides (AMPs) effective against bacterial plant pathogens. In this study, thirteen agricultural samples were collected and their protein hydrolysates obtained using pepsin. Peptides smaller than 3 kDa were purified by reverse-phase chromatography, cation exchange chromatography, and pI-based fractionation and tested for activity against plant pathogenic bacteria at each step. Active peptides were then analyzed for putative mechanisms using nanoLC–MS/MS and the Mascot program. Ultimately, eight candidate peptides originating from bagasse were selected and chemically synthesized for a comparative study of growth inhibition in plant pathogenic bacteria and plant growth-promoting rhizobacteria (PGPRs). Three synthesized peptides exhibited a potent activity against plant pathogenic bacteria while also supporting the growth of PGPRs. Proteomics analysis revealed the peptides PQLAVF (Pro-Gln-Leu-Ala-Val-Phe) and MDEFL (Met-Asp-Arg-Phe-Leu) to act against *Xanthomonas oryzae* pv. *oryzae* via membrane-active mechanisms, while peptide VQLMNSL (Val-Gln-Leu-Met-Asn-Ser-Leu) acted against *Pectobacterium carotovorum* and *Agrobacterium rhizogenes* through intracellular-active mechanisms. Further study remains necessary to customize peptides by amino acid substitution not only for a higher effective activity against these and other critical pathogens, but also for a higher stability of peptides in critical condition when applied in industrial processes in the future.

Keywords: antimicrobial peptides; plant pathogens; waste samples; peptide–microbe mechanisms

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1. Introduction

Bacteria-caused diseases have been a crucial factor influencing agricultural plant production and food manufacturing for thousands of years. They still pose a considerable threat to the food supplies of many countries today [1]. Although advancements in science and technology have led to considerable decreases in the frequency and severity of disease outbreaks, 20–30% of actual production is still hampered each year because of plant diseases [2,3]. Bacterial plant diseases are less common than fungal or viral diseases, but the economic losses they induce are nonetheless devastating [4].

Currently, bacterial plant disease control relies mainly on chemical agents; however, the effects of such agents on long-term environmental pollution and as carcinogens in the food chain limit their future use [5]. Moreover, several strategies for plant disease control combined with many farm practices applied in modern cultivation have caused

unintended troubles, including environmental degradation [6], loss of biodiversity [7,8], and the creation of advantageous habitats for the contamination, reproduction, transmission, and rapid evolution of plant pathogens [9–11]. In addition, chemical antibiotics such as streptomycin and oxytetracycline are applied for the treatment of plant bacterial diseases, but this practice enables the development of antibiotic resistance. Once antibiotic resistance is found in a plant pathogen population, it speedily becomes widespread [12], which increases the negative impacts of plant diseases on food security and human society [13]. Ultimately, to achieve sustainable plant pathogen control, it is necessary to develop effective alternatives for combating resistant pathogens that are also environmentally friendly.

Antimicrobial peptides (AMPs) are natural guardians against pathogen invaders and function in innate immune systems [14]. Over 1500 AMPs have been found in many living things, both eukaryotes and prokaryotes [15]. Commonly, AMPs exhibit broad activity against many types of organisms, namely fungi, bacteria, parasites, and viruses; hence, they are categorized as antifungal, antibacterial, antiparasitic, and antiviral, respectively [16]. The antibacterial activity of peptides is a result of amphiphilic composition and a high degree of positive charge within their structure. This quality supports peptide attachment and insertion into the bacterial cell membrane to create a pore, thereby bringing about membrane disruption and cell lysis [17,18]. A number of AMP families have been reported in plants, such as defensins, thionins, snakins, lipid transfer proteins, cyclotides, and hevein-like proteins. Some plant AMPs have had their structures and activities reported in the PhytAMP database. Overall, peptides in PhytAMP most commonly exhibit antifungal activity (51%), followed by antibacterial (33%) and antiviral (10%) activities [19].

Peptide size is also an important factor determining the efficacy of agricultural antibacterial agents [20]. Small synthetic antimicrobial peptides (ssAMPs), usually less than ten amino acids in size, could provide an acceptable alternative because their synthesis cost is significantly lower than the cost of producing long peptides. Choi et al. [12] set out to create new antibacterial hexapeptide ssAMPs with efficacy against *Xanthomonas citri* subsp. *citri*, the citrus canker pathogen. Of fourteen hexapeptides tested, they found three that were able to kill various *X. citri* strains when applied at a concentration of 10 μg/mL. In addition, soaking citrus leaves with the hexapeptides alongside pathogens significantly suppressed disease progress. Importantly, ssAMP sequences can be selected quickly through screens using proteomic techniques, while still retaining most of the functional aspects of native AMPs.

In this work, we chose agricultural wastes as ssAMP sources. Agricultural production has increased by more than three times over the past five decades due to the growth of the world population. Agricultural wastes are mainly generated from farming activities pertaining to crop production, such as planting, pruning, and harvesting. A large amount of waste also comes from agro-industrial production, with an annual increase in this area of about 7.5% [21]. The negative influence of agricultural wastes on human health, animal health, and bio-pollution is significant. In many developing countries, agricultural wastes are randomly discarded or burnt in public areas; this constitutes the beginning of air and soil pollution, and residue from wastes may seep into a water source, thereby causing water pollution [22].

The objective of this study was to investigate the antibacterial activity of ssAMPs from agricultural wastes (both wastes from farming areas and agro-industrial wastes) obtained through protein hydrolysis with pepsin. Only peptides of less than 3 kDa were retained and purified with reverse-phase chromatography, cation exchange chromatography, and off-gel fractionation. Final fractions containing peptides active against bacterial plant pathogens were analyzed using LC–MS/MS and the Mascot program, and small peptides with high Mascot scores were selected for synthesis and experimental evaluation of activity. Three peptides that demonstrated efficacy against bacterial plant pathogens but not against plant growth-promoting rhizobacteria (PGPRs), which have important

roles in biocontrol, were finally selected for characterization of their antibacterial mechanisms via proteomic profiling of expressed bacterial proteins after exposure to peptides.

2. Materials and Methods

2.1. Time and Place of Research

This research was conducted during 2020–2022 at the Functional Proteomics Technology Laboratory, Functional Ingredients and Food Innovation Research Group Laboratory, National Center for Genetics Engineering and Biotechnology, and National Science and Technology Development Agency in Pathumthani, Thailand.

2.2. Sample Collection

Waste samples were collected and classified into two groups, agricultural wastes and agro-industrial wastes (Table 1 and Figure 1).

Table 1. Waste sample classification.

Sample	Code	Source	Location (Latitude, Longitude)
Agricultural wastes			
Rice straw	AW1	Rice farm	Chachoengsao, Thailand (13.6690° N, 101.0891° E)
Corn cobs	AW2	Corn farm	Sakaeo, Thailand (13.5035° N, 102.2872° E)
Corn leaves	AW3	Corn farm	Sakaeo, Thailand (13.5035° N, 102.2872° E)
Corn husks	AW4	Corn farm	Sakaeo, Thailand (13.5035° N, 102.2872° E)
Sugarcane leaves	AW5	Sugarcane farm	Sakaeo, Thailand (13.50181° N, 102.2875° E)
Bagasse	AW6	Sugarcane farm	Sakaeo, Thailand (13.50181 °N, 102.2875° E)
Agro-industrial wastes			
Fermented soybeans	IW1	Light soy sauce production	Hi-q Food Products Co., Ltd, Chachoengsao, Thailand (13.7489° N, 100.9518° E)
Soybean pellet	IW2	Soybean milk production	market in Chachoengsao, Thailand (13.6924° N, 101.0807° E)
Peanut seed coat	IW3	Peanut-based snack production	Mae-Ruay Snack Food Factory Co Ltd, Bangkok, Thailand (13.6557° N, 100.4305° E)
Coconut residue	IW4	Coconut milk production	market in Chachoengsao, Thailand (13.6924° N, 101.0807° E)
Coffee grounds	IW5	Arabica grounds, the primary coffee industry residue	Rosetta Coffee Shop, Chachoengsao, Thailand (13.6701° N, 101.0562° E)
Fish residue	IW6	Fish sauce production	King Mongkut's University of Technology Thonburi, Thailand (13.5790° N, 100.4418° E)
Fish residue (rinsed)	IW7	Fish sauce production (rinsed)	King Mongkut's University of Technology Thonburi, Thailand (13.5790° N, 100.4418° E)

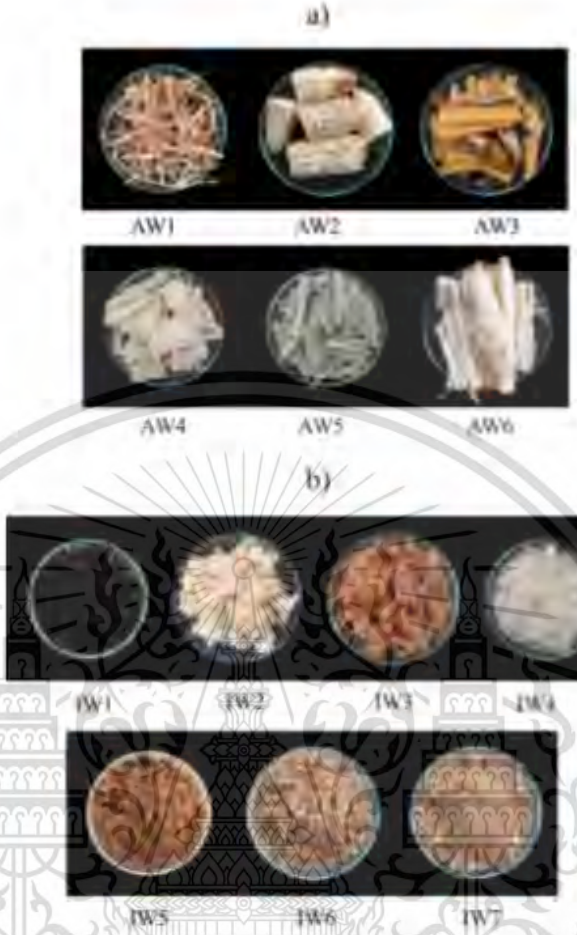

Figure 1. Images of the waste samples, by group: (a) agricultural wastes; (b) agro-industrial wastes.

2.3. Preparation of Protein Hydrolysates and Small Peptides (<3 kDa)

Crude proteins were prepared by extracting each waste sample using 0.05 M sodium acetate, pH 4.0 with mechanical shaking at 25 ± 2 °C for 1 h, followed by autoclaving at 121 °C for 15 min to select only heat-tolerance proteins that show high stability when applied in industrial condition, and the autoclave condition could also eliminate all microbes that might be contaminated in samples. The total protein concentration in the supernatant was measured by a Lowry assay [25] using bovine serum albumin (BSA) as the protein standard. The absorbance at 750 nm (OD750) was measured and the protein concentration calculated from a calibration curve. The crude proteins were then hydrolyzed with pepsin (Sigma–Aldrich, St. Luis, MO, USA) in a ratio of 1:25 (pepsin:sample), and incubated with shaking at 200 rpm for 12 h at 37 °C. Next, the reaction was terminated by boiling for 10 min. The supernatant of the crude hydrolysate was retained after centrifugation at 10,000 g for 10 min at RT. The resulting hydrolysates were collected and diluted by five times with 0.5 M sodium acetate (NaOAc), then filtrated (cut-off) through a semipermeable

membrane (Vivaspin 20, 3 kDa MWCO, GE Healthcare, Chicago, UK) to yield peptides smaller than 3 kDa, which were frozen at −20 °C until use.

2.4. Bacterial Plant Pathogens and Antimicrobial Activity Assays

Four plant pathogens were selected for investigation: *Xanthomonas oryzae* pv. *oryzae* (isolated from rice), *Xanthomonas citri* DOA-BC902, *Pectobacterium carotovorum* DOA-BC681, and *Agrobacterium rhizogenes* TISTR511. When assaying antibacterial activity, each pathogen was first prepared in tryptic soy agar (TSA) (Difco BBL, USA) and cultured for 24 h at 28 °C. Then, a single colony was picked and cultured in tryptic soy broth (TSB) (Difco BBL, Sparks, MD, USA) for 12–16 h to achieve an inoculum of 0.05 at OD600 (4×10^7 CFU/mL). The previously filtrated less-than-3 kDa peptides were then assayed for antibacterial activity against the pathogens in triplicate using the broth dilution method, for which bacteria in TSB, phosphate-buffered saline (PBS), and antibiotics (ampicillin and kanamycin) were used as controls. Antibiotics and hydrolysates/peptides were used at a final concentration of 100 μg/mL. OD600 values after incubation for 0, 2, 4, and 6 h were recorded using a microplate reader (Synergy H1 Hybrid Multi-Mode Reader, Biotek, Winusky, VT, USA). The inhibitory percentage was calculated from [(OD_{600} control-OD_{600} test)/OD_{600} control] × 100.

2.5. Experimental Design and Statistical Analysis

A completely randomized experimental design was used. Thirteen kinds of agricultural wastes were tested, and the experimental units were bacterial plant pathogens (*X. oryzae* pv. *oryzae*, *X. citri* DOA-BC902, *Pectobacterium carotovorum* DOA-BC681, and *A. rhizogenes* TISTR511) and plant growth-promoting rhizobacteria (PGPRs) (*Bacillus subtilis* ATCC6633, *Pseudomonas aeruginosa* ATCC27853, and *Pseudomonas fluorescens* TISTR2630), grown in tryptic soy broth (TSB) (Difco BBL, USA) at 28 °C in 96-well plates. Experiments were run in triplicate, and all results are presented as mean ± standard deviation.

2.6. Peptide Purification by Reverse-Phase Chromatography

The active peptides were initially purified by reverse-phase chromatography using a Delta-Pak C18 column (100 Å, 3.9 mm × 150 mm; Interlink Scientific Services Ltd., Kent, UK) previously equilibrated with 0.1% trifluoroacetic acid (TFA) in acetonitrile (ACN). The column was washed with 0.1% TFA in sterile water, after which a sample containing 0.1% TFA was loaded to bind the column. The hydrophilic fraction (coded UBR: unbound fraction of reverse-phase chromatography) was eluted from the column with 0.1% TFA in sterile water. Next, the hydrophobic fraction (coded BR: bound fraction of reverse-phase chromatography) was eluted stepwise with 0.1% TFA in ACN. All steps were carried out at an adjusted flow rate of 1 mL/min. Both UBR and BR fractions were further evaluated for antimicrobial activity.

2.7. Peptide Purification by Cation Exchange Chromatography

Prior to this purification step, the conductivity of all active fractions from the reverse-phase purification was examined. If conductivity was observed, contaminants were first eliminated using a P-6 desalting column (50 mL; Bio-Rad Laboratories, Inc., Heracles, CA, USA). Afterward, salt-removed samples were adjusted to pH 4. Then, salt-removed and nonconductive samples were ion-separated by cation exchange chromatography using AKTATM start (GE Healthcare, Chicago, IL, USA) through a HiTrap SP Sepharose FF (1 mL; Cytiva, Marlborough, MA, USA) cation chromatography exchange column with a flow rate of 1 mL/min and fraction volume of 1 mL. The column was then washed out with 50 mM NaOAc (pH 4) at 3 column volumes (CV) at a flow rate of 0.5 mL/min and fraction volume of 1 mL; this fraction was labeled UBC (the unbound fraction of cation exchange chromatography). Subsequently, the final fraction BC (bound fraction of cation exchange chromatography) was eluted with 1 M NaCl, a gradient of 0–100, linear 3 CV,

and fixed fraction volume of 0.5 mL. Each fraction was buffer-exchanged from 50 mM NaOAc, pH 4 with a gradient of 1 M NaCl to sterile water prior to antibacterial activity determination.

2.8. Peptide Purification by pI-Based Fractionation

Active fractions from cation exchange chromatography were separated according to their isoelectric points (pI). For pI-based peptide separation, the 3100 OFFGEL Fractionator was utilized with an 18-well (18 cm) setup and pH interval from 3 to 10 according to the supplier's protocol; details are listed in Table 2. The peptides in each resultant fraction with demonstrated bioactivity were further analyzed by nanoLC–MS/MS and the Mascot software (Matrix Science, London, UK) [24].

Table 2. Parameters used when running the 18 cm OFFGEL unit.

pH Interval	Step Voltage Mode	Voltage (V)	Duration (h:min)	kVh
3–10	1 Step and hold	500	1:00 (8:00)	0.5
	2 Gradient	1000	1:00	0.8
	3a Gradient	8000	3:00	13.5
	4a Step and hold	8000	0:46–1:30	6.2–12.2
	3b Gradient	10,000	3:00	16.5
	4b Step and hold	10,000	0:20–0:55	3.2–9.2
	Total			21.0–27.0

2.9. Peptide Synthesis and Determination of Antibacterial Activity

Peptides were synthesized following the solid-phase peptide synthesis method reported by Hansen and Oddo [25]. After synthesis, the peptide samples were prepared for antimicrobial activity testing.

2.10. Study of Peptide–Microbe Interaction Mechanisms

After treatment of the selected plant pathogenic microbes with active peptides for 6 h, all control and experimental samples were tryptic-digested and their protein profiles determined by LC–MS/MS using the Ultimate 3000 Nano/Capillary LC System (Thermo Scientific, Waltham, MA, USA) coupled to an ESI-Ion Trap MS HCT ultra PTM Discovery System (Bruker Daltonics GmbH, Billica, MA, USA) with electrospray. The obtained MS/MS spectra were analyzed with the DeCyder MS 2.0 differential analysis software (GE Healthcare, Chicago, IL, USA), and the resulting output was searched against the NCBI database using the Mascot software (Matrix Science, London, UK) [24]. Similarities and differences of protein expression profiles obtained under different treatments were visualized by a Venn diagram [26] and protein functionality annotations were retrieved from UniProt (https://www.uniprot.org/id-mapping accessed on 1 April 2022.).

3. Results

3.1. Antibacterial Activity of Peptides Less Than 3 kDa in Size

Out of the thirteen waste samples investigated here, four hydrolysates (AW6, IW4, IW3, and AW1) showed obviously superior potential, with inhibitory percentages of 50% or higher (Table 3). Notably, AW6 ranked first for every targeted bacterial pathogen, while IW4 ranked second for all targets except *X. oryzae* pv. *oryzae*. In addition, IW3 ranked second against *X. oryzae* pv. *oryzae* and third against *X. citri*, while AW1 ranked fourth for *X. citri*.

Table 3. Ranking of <3 kDa peptide samples according to inhibitory activity against plant pathogenic bacteria at 6 h after treatment.

Antibacterial Activity	Inhibitory Percentages Against Target Organisms							
Ranking	*X. oryzae* pv. *oryzae*		*X. citri*		*P. carotovorum*		*A. rhizogenes*	
1	84.90 ± 1.98 ^{a}	AW6	84.21 ± 0.40 ^{ab}	AW6	87.06 ± 0.33 ^{ab}	AW6	65.87 ± 1.58 ^{ab}	AW6
2	79.20 ± 8.73 ^{a}	IW3	82.30 ± 0.58 ^{abc}	IW4	81.95 ± 0.75 ^{b}	IW4	59.28 ± 2.16 ^{bc}	IW4
3	57.61 ± 5.48 ^{b}	IW4	79.41 ± 0.46 ^{abcd}	IW3	60.06 ± 0.86 ^{d}	IW7	57.09 ± 0.69 ^{c}	IW5
4	47.81 ± 12.29 ^{bc}	AW1	74.07 ± 3.35 ^{bcde}	AW1	55.93 ± 2.45 ^{de}	AW4	43.11 ± 2.40 ^{d}	AW1
5	44.50 ± 7.76 ^{bcd}	IW7	71.62 ± 2.86 ^{cde}	AW4	55.93 ± 0.25 ^{de}	AW1	39.92 ± 1.92 ^{d}	AW5
6	42.74 ± 11.70 ^{cde}	AW5	69.95 ± 0.95 ^{def}	AW3	54.66 ± 1.01 ^{de}	AW3	37.92 ± 3.01 ^{de}	AW4
7	40.40 ± 2.68 ^{cde}	AW4	67.12 ± 2.43 ^{ef}	AW5	52.74 ± 2.18 ^{e}	AW5	36.53 ± 2.74 ^{de}	AW3
8	39.20 ± 2.68 ^{cde}	AW3	66.06 ± 1.72 ^{ef}	IW7	51.10 ± 1.09 ^{e}	IW5	29.54 ± 8.50 ^{e}	IW7
9	31.68 ± 3.53 ^{def}	AW2	59.19 ± 1.66 ^{fg}	IW5	40.94 ± 0.56 ^{f}	AW2	14.57 ± 5.02 ^{f}	IW2
10	29.52 ± 2.94 ^{ef}	IW5	53.17 ± 9.22 ^{gh}	AW2	37.38 ± 2.89 ^{f}	IW2	14.37 ± 9.97 ^{f}	IW3
11	19.26 ± 2.11 ^{f}	IW2	52.10 ± 2.20 ^{gh}	IW2	28.00 ± 3.63 ^{g}	IW6	9.18 ± 2.83 ^{fg}	AW2
12	0.74 ± 3.17 ^{g}	IW6	44.85 ± 13.37 ^{h}	IW6	7.89 ± 1.48 ^{h}	IW3	5.39 ± 7.83 ^{gh}	IW6
13	−7.35 ± 17.30 ^{g}	IW1	−7.09 ± 7.73 ^{i}	IW1	1.85 ± 11.24 ^{i}	IW1	0.20 ± 2.42 ^{h}	IW1
kanamycin	82.91 ± 9.35 ^{a}		85.35 ± 0.61 ^{a}		89.05 ± 0.33 ^{a}		63.87 ± 5.23 ^{abc}	
ampicillin	90.83 ± 0.52 ^{a}		4.12 ± 13.30 ^{i}		74.48 ± 0.25 ^{g}		70.26 ± 3.40 ^{a}	

Inhibitory percentage = [(OD600 control-OD600 test)/OD600 control] × 100. Means marked with the same superscript letter in a column were not statistically different ($p < 0.05$) by Duncan's multiple range test.

3.2. Antibacterial Activity after Peptide Purification

After antibacterial screening, four peptide samples (AW1, AW6, IW3, and IW4) were selected for purification in three steps: reverse-phase chromatography, cation exchange chromatography, and pI-based fractionation. Bacterial growth inhibition was re-evaluated in triplicate after each purification step (Table 4).

Table 4. Observed inhibitory percentages of purified peptide samples against growth of plant pathogenic bacteria.

Peptide Samples from Each Purification Step	Inhibitory Percentage Against Bacterial Plant Pathogens			
	X. oryzae pv. *oryzae*	*X. citri*	*P. carotovorum*	*A. rhizogenes*
After reverse-phase chromatography				
AW1 UBR	**44.57 ± 16.00 ^{b}**	**54.91 ± 1.08 ^{b}**	**20.74 ± 1.38 ^{e}**	**19.22 ± 3.37 ^{c}**
AW1 BR	12.19 ± 3.01 ^{e}	10.81 ± 0.63 ^{e}	−3.24 ± 0.9 ^{g}	−0.26 ± 5.82 ^{d}
AW6 UBR	**59.73 ± 9.76 ^{ab}**	**59.04 ± 0.29 ^{ab}**	**64.68 ± 0.73 ^{b}**	**64.25 ± 10.47 ^{a}**
AW6 BR	6.60 ± 1.30 ^{e}	1.30 ± 2.47 ^{e}	−2.56 ± 1.47 ^{g}	−0.10 ± 3.47 ^{d}
IW3 UBR	**51.41 ± 16.88 ^{ab}**	**55.18 ± 1.53 ^{ab}**	**24.86 ± 4.65 ^{d}**	**33.31 ± 4.73 ^{b}**
IW3 BR	8.18 ± 3.13 ^{e}	5.21 ± 1.10 ^{e}	1.78 ± 3.12 ^{f}	−2.82 ± 1.65 ^{d}
IW4 UBR	**43.00 ± 10.76 ^{b}**	**49.53 ± 3.64 ^{b}**	**24.20 ± 1.75 ^{de}**	**33.48 ± 4.22 ^{b}**
IW4 BR	4.95 ± 6.25 ^{e}	2.96 ± 4.68 ^{e}	2.85 ± 2.26 ^{f}	4.34 ± 1.40 ^{d}
kanamycin	55.47 ± 10.21 ^{ab}	61.56 ± 0.19 ^{ab}	68.64 ± 0.83 ^{a}	68.33 ± 0.77 ^{a}
ampicillin	63.29 ± 2.51 ^{a}	63.75 ± 1.19 ^{a}	31.29 ± 1.39 ^{c}	34.72 ± 1.36 ^{b}
After cation exchange chromatography				
AW1 UBR-UBC	1.47 ± 5.63 ^{e}	3.26 ± 3.83 ^{e}	9.71 ± 1.54 ^{cde}	2.62 ± 2.05 ^{de}
AW6 UBR-UBC	0.65 ± 0.81 ^{cde}	1.03 ± 4.09 ^{de}	6.03 ± 4.54 ^{de}	5.83 ± 4.87 ^{cde}
AW6 UBR-BC	**7.32 ± 13.43 ^{b}**	**9.21 ± 0.97 ^{c}**	**18.21 ± 3.06 ^{b}**	**7.26 ± 2.48 ^{c}**
IW3 UBR-UBC	−0.61 ± 1.93 ^{cde}	−6.51 ± 1.22 ^{f}	1.61 ± 8.87 ^{ef}	−4.44 ± 8.72 ^{g}
IW3 UBR-BC	−3.84 ± 6.80 ^{d}	−1.66 ± 3.10 ^{e}	2.19 ± 2.49 ^{ef}	0.65 ± 2.08 ^{ef}

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IW4 UBR-UBC	−0.60 ± 3.88^{cd}	0.51 ± 4.69^{de}	11.05 ± 2.05^{c}	0.75 ± 1.76^{ef}
IW4 UBR-BC	−0.55 ± 1.86^{cd}	−2.81 ± 4.93^{ef}	0.97 ± 1.80^{f}	−1.62 ± 0.18^{fg}
kanamycin	55.32 ± 0.96^{a}	48.12 ± 0.47^{b}	66.01 ± 1.63^{a}	54.32 ± 1.28^{a}
ampicillin	58.54 ± 1.18^{a}	52.95 ± 3.43^{a}	22.40 ± 4.63^{b}	24.65 ± 0.62^{b}
		After off-gel fractionation		
AW6 UBR-BC well 1	−5.16	N/A	1.71	N/A
AW6 UBR-BC well 2	1.89	N/A	−4.47	N/A
AW6 UBR-BC well 3	0.61	N/A	−2.67	N/A
AW6 UBR-BC well 4	5.43	N/A	−3.08	N/A
AW6 UBR-BC well 5	**6.96**	**N/A**	**−5.20**	**N/A**
AW6 UBR-BC well 6	**9.76**	**N/A**	**−4.43**	**N/A**
AW6 UBR-BC well 7	**11.21**	**N/A**	**−1.75**	**N/A**
AW6 UBR-BC well 8	−0.73	N/A	−7.98	N/A
AW6 UBR-BC well 9	3.65	N/A	−3.41	N/A
AW6 UBR-BC well 10	3.50	N/A	−9.08	N/A
AW6 UBR-BC well 11	−7.64	N/A	−3.06	N/A
AW6 UBR-BC well 12	−11.35	N/A	−6.58	N/A
AW6 UBR-BC well 13	**0.08**	**N/A**	**11.42**	**N/A**
AW6 UBR-BC well 14	−5.50	N/A	−2.23	N/A
AW6 UBR-BC well 15	−4.13	N/A	−4.91	N/A
AW6 UBR-BC well 16	−7.09	N/A	−3.95	N/A
AW6 UBR-BC well 17	0.50	N/A	−4.70	N/A
AW6 UBR-BC well 18	**4.65**	**N/A**	**−6.96**	**N/A**
kanamycin	46.82	N/A	59.57	N/A
ampicillin	50.35	N/A	39.07	N/A

Inhibitory percentage = (Control - Test/Control) × 100. Means marked with the same superscript letter in a column were not statistically different ($p < 0.05$) by Duncan's multiple range test. The bolding signifies the chosen samples for further steps. Each activity assay was conducted in triplicate except those following the pI-based fractionation test, which had only enough material for one replicate. Negative values indicate higher growth (instead of inhibition) than untreated control.

The reverse-phase chromatography method yielded two fractions; hence, antibacterial activity was subsequently assayed in a total of eight samples. As indicated in Table 4, the UBR fraction exhibited a higher inhibitory activity than the corresponding BR fraction for every sample; that is, the hydrophilic fractions gave better results. While all BR samples showed inhibitory activity, values were consistently less than 15. The single most effective sample was AW6 UBR, which demonstrated the best inhibitory percentage (around 60%) for every bacterium tested. Meanwhile, the samples AW1 UBR, IW3 UBR, and IW4 UBR all showed a low percent inhibition against *Pectobacterium carotovorum* and *A. rhizogenes*, but better efficacy against *X. oryzae* pv. *oryzae* and *X. citri*. Ultimately, the samples chosen for the next purification step were AW1 UBR, AW6 UBR, IW3 UBR, and IW4 UBR.

The second purification employed cation exchange chromatography. All samples featured both unbound (coded-UBC) and bound (coded-BC) peaks except AW1 UBR, for which only an unbound peak was obtained (AW1 UBR-UBC). Evaluating the samples for antibacterial activity revealed AW6 BC to have the best inhibitory percentage for every bacterial pathogen (Table 4). Accordingly, AW6 BC was chosen for the third purification step, pI-based or off-gel fractionation. After the 7-day running time, the quantity of protein obtained in each well was only enough to assay antibacterial activity against two pathogens with one replicate each, the results of which comprise the last section of Table 4. The fractions from wells 5, 6, 7, and 18 all inhibited the growth of *X. oryzae* pv. *oryzae*,

while that from well 13 inhibited *P. carotovorum*. Consequently, AW6 UBR-BC wells 5, 6, 7, 13, and 18 were selected for peptide sequencing and further analysis using LC–MS/MS and the Mascot software.

3.3. Antibacterial Activity of Small Synthetic Peptides

In this study, thousands of peptides were analyzed and sequenced by Mascot; eight peptides having high peptide scores and fewer than ten amino acid residues were selected for determination of antibacterial activity (Table 5). The active peptides were selected from those more effective against plant pathogens and less effective against PGPRs. The results showed very obviously that peptides no. 1 (PQLAVF) and no. 5 (MDRFL) were most effective against *X. oryzae* pv. *oryzae*, while peptide no. 2 (VQLMNSL) exhibited the greatest efficacy against *Pectobacterium carotovorum* and *A. rhizogenes*. In addition, peptide no. 2 did not demonstrate any effect against PGPRs (*Bacillus subtilis* and *Pseudomonas fluorescens*). These three effective peptides were used in the subsequent determination of peptide–microbe mechanisms.

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Table 5. Inhibitory percentages of small synthetic peptides derived from purified protein hydrolysates of bagasse (*Saccharum sp.*; AW6).

Peptide no.	Protein Accession Number	Protein Name	Peptide Sequence	Inhibitory Percentage						
				Plant Pathogens				PGPRs		
				X. oryzae pv. oryzae	*X. citri*	*Pectobacterium carotovovorum*	*A. rhizogenes*	*B. subtilis*	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*
1	**A0A059Q0V8**	**Uncharacterized protein**	**PQLAVF**	**6.66 ± 0.24**	**4.86 ± 0.18**	**−1.80 ± 0.02**	**1.70 ± 0.02**	**7.60 ± 0.11**	**24.26 ± 0.37**	**7.44 ± 0.32**
2	**A0A5N5XU21**	**ATP-binding cassette domain-containing protein**	**VQLMNSL**	**−13.28 ± 1.21**	**−9.09 ± 0.71**	**12.78 ± 6.18**	**16.92 ± 3.54**	**−3.96 ± 0.50**	**24.69 ± 0.62**	**0.43 ± 0.03**
3	A0A678TAJ2	Tr-type G domain-containing protein (Fragment)	TAMPRL	3.27 ±0.08	9.87 ± 0.37	6.18 ± 0.13	4.29 ± 0.07	8.92 ± 0.31	25.81 ± 0.49	−10.14 ± 0.06
4	A0A1B2URG1	NAD(P)H-quinone oxidoreductase subunit 2, chloroplastic (EC 7.1.1.-)	ISSTSL	1.07 ± 0.02	6.63 ± 0.18	6.91 ± 0.08	2.73 ± 0.00	9.00 ± 0.23	26.03 ± 0.47	−7.22 ± 0.20
5	**A0A2H4YIU1**	**Expansin**	**MDRFL**	**7.29 ± 0.20**	**4.57 ± 0.19**	**0.81 ± 0.01**	**−2.47 ± 0.02**	**5.54 ± 0.08**	**26.22 ± 0.82**	**6.30 ± 0.25**
6	Q8GT31	Phytocalpain (EC 3.4.22.17) (Fragment)	RVTGRDAL	3.62 ± 0.13	10.72 ± 0.35	6.33 ± 0.11	0.22 ± 0.00	10.63 ± 0.02	29.08 ± 0.12	−4.03 ± 0.06
7	A0A059Q1W5	Cation/H (+) antiporter	SIAGVTSYL	−2.13 ± 0.02	−0.59 ± 0.01	0.59 ± 0.01	−4.25 ± 0.05	9.42 ± 0.20	33.13 ± 0.76	−7.00 ± 0.08
8	A0A6B9MSZ0_9POAL	Tetraspanin-18	VMAAGL	−2.96 ± 0.13	0.27 ± 0.01	0.27 ± 0.01	−4.15 ± 0.05	5.46 ± 0.02	29.73 ± 0.57	−5.10 ± 0.03

The bolding signifies the chosen samples for further steps.

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3.4. Determination of Peptide–Microbe Interaction Mechanisms

The peptide–microbe pairs for which interaction mechanisms were characterized are listed in Table 6. In these assays, untreated microbes were used as the negative control and antibiotic-treated microbes as the positive control. Many differentially expressed proteins were identified between control and treated samples, and patterns of protein expression under different treatments were straightforwardly visualized by a Venn diagram [26] (Figure 2).

Table 6. Selected peptides and bacterial plant pathogens used for the determination of antibacterial mechanisms by shotgun proteomics.

Bacterial Plant Pathogen	Peptide No.	Peptide Sequence
X. oryzae pv. *oryzae*	1	PQLAVF
X. oryzae pv. *oryzae*	5	MDRFL
P. carotovorum	2	VQLMNSL
A. rhizogenes	2	VQLMNSL

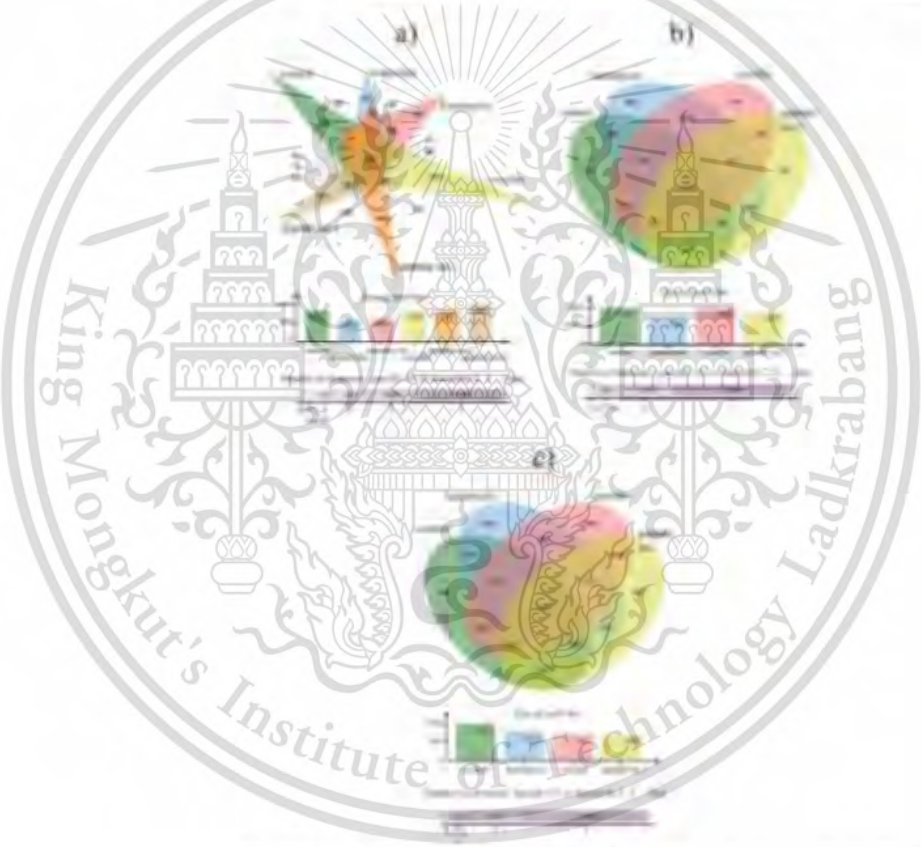

Figure 2. Venn diagram summary of protein expression in bacterial plant pathogens treated with selected peptides and antibiotics: (a) *X. oryzae* pv. *oryzae*, (b) *Pectobacterium carotovorum*, and (c) *A. rhizogenes*. The number of elements means the number of proteins detected in each treatment.

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In total, 1445 proteins with altered expression were identified in *X. oryzae* pv. *oryzae* treated with peptide no. 1, and 1688 in the same pathogen treated with peptide no. 5 (Figure 2a). Among these, 304 and 452 proteins were uniquely expressed in association with peptides no. 1 and 5, respectively, and only a small number overlapped with expression profiles obtained under kanamycin, ampicillin, and oxycline treatments. *Pectobacterium carotovorum* was treated with peptide no. 2, in which condition 1837 proteins were found to be expressed (Figure 2b). Among those proteins, 733 were uniquely present under treatment with peptide no. 2, 214 were also expressed under oxycline treatment, and 145 also under kanamycin treatment. *A. rhizogenes* was likewise treated with peptide no. 2, which yielded an expression profile consisting of 983 proteins. A total of 447 proteins were unique to treatment with peptide no. 2, while 110 were also expressed under oxycline and 79 under kanamycin.

The proteins expressed in peptide-treated pathogen cells were further analyzed by consulting the UniProt database to identify Gene Ontology or functional annotations.

4. Discussion

4.1. Preparation of Protein Hydrolysates and Screening of Antibacterial Activity

In the initial protein extraction, sample proteins were extracted with 0.05 M sodium acetate, pH 4 for 1 h in keeping with previous reports that mild-acid extraction is appropriate for isolating plant proteins due to minimizing oxidation, polymerization of phenolic compounds, and irreversible protein binding [27]. Lay et al. [28] similarly extracted proteins from ornamental tobacco and petunia using 50 mM sulfuric acid; Pickardt et al. [27] found that increasing the concentration of sodium chloride enhanced the relative protein yield; Taniguchi et al. [29] adjusted the pH to 2.0 with 1 M HCl before hydrolyzing with pepsin. Following extraction, peptides in this study were heated in an autoclave (121 °C for 15 min) to remove heat-intolerant and heat-labile proteins, conformant to the method of Lay et al. [30].

Plant AMPs are normally smaller than 10 kDa [31]; accordingly, we used a semipermeable membrane to filter the diluted hydrolysates before purification. Notably, the shorter peptides are more cost-effective to synthesize; Gordon et al. [20] highlighted that peptides of less than ten amino acids could be a good option because the cost of synthesizing them is much lower than for long peptides. Therefore, we used a Vivaspin 20 with 3 kDa MWCO to retain only peptides of less than 3 kDa (approximately 27–28 amino acids). In 2017, Choi et al. [12] tried to identify an ssAMP effective against *X. citri* subsp. *Citri* using the PS-SPCL (positioning scanning of a synthetic peptide combinatorial library) technique; among fourteen investigated ssAMPs, they found three hexapeptides that showed bactericidal activity against *X. citri* subsp. *Citri* strains. Taniguchi et al. [29] also selected on peptide size using dialysis tubing.

In terms of inhibitory percentage against plant pathogenic bacteria as shown in Table 3, the protein hydrolysate from bagasse (AW6) ranked first for every tested pathogen. These results agree with a report by Velazquez-Martinez et al. [32] that found sugarcane bagasse with 2.2% crude protein to have a high antimicrobial activity against *Escherichia coli*, *Bacillus cereus*, and *Staphylococcus aureus*. That coconut residue hydrolysate ranked second in this work is consistent with a report that coconut AMPs (Cn-AMPs) have an extremely efficient antimicrobial activity against both Gram-positive and Gram-negative pathogenic bacteria including *E. coli*, *Bacillus subtilis*, *S. aureus*, and *Pseudomonas aeruginosa* [33]. Moreover, the antimicrobial activity of rice straw hydrolysate observed here aligns with the work of Park et al. [34], which reported rice straw extract to inhibit growth of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Finally, these results are consistent with our last study, in which we found coconut residue, peanut seed coat, and rice straw protein hydrolysates to have strong antimicrobial activity against the plant pathogens *Ralstonia solanacearum* and *Burkholderia cepacia*, achieving over 74% inhibition [35].

4.2. Peptide Purification

Antibacterial screening identified four effective hydrolysates (AW1, AW6, IW3, and IW4), after which the key next step was peptide purification. Normally, purification methods rely on intrinsic physio-biochemical properties such as size, overall net charge, solvent tolerance, and thermostability. Scott et al. [36] highlighted that some protein mixtures from waste samples should be separated according to the presence of polar and nonpolar side groups, while others may be separated on the basis of basic and acidic amino acids. A complex mixture of amino acids can also be separated using chromatographic purification techniques, with ion exchange chromatography being particularly important to develop in the separation of peptides from complex mixtures. This study accordingly employed multiple purification steps, namely reverse-phase chromatography to separate hydrophobic and hydrophilic fractions followed by cation exchange chromatography and pI-based purification (off-gel fractionation). Each purification step functioned to narrow the scope of bioactive peptides. After the initial reverse-phase chromatography, one fraction for each of the four samples exhibited antibacterial activity (the UBR fractions). However, after cation exchange chromatography, only one fraction from one sample, AW6 UBR-BC, demonstrated antibacterial activity. Following the final pI-based purification, five fractions exhibited antibacterial activity (wells 5, 6, 7, 13, and 18) and were selected for further analysis using LC–MS/MS and the Mascot software. Of the multitude of peptides identified as having high peptide scores and sizes smaller than ten amino acids, only eight were selected for synthesis and characterization of the mechanisms of their antimicrobial activity against plant pathogen cells.

4.3. Peptide–Microbe Interaction Mechanisms

Protein expression Venn diagrams (Figure 2) revealed that the majority of proteins expressed by bacterial plant pathogens treated with the selected peptides were unique (that is, they were not also expressed in cultures treated with antibiotics). Thus, it could be concluded that peptides no. 1, 2, and 5 have mechanisms of action distinct from those of the tested antibiotics. Data from UniProt provided further insight into these mechanisms.

First, we examined the Gene Ontology annotations (biological process, molecular function, and cellular compartment) of all proteins expressed under peptide treatment (but not found in antibiotic treatment or control) and organized them into groups (Figure 3). For *X. oryzae* pv. *oryzae* treated with peptide no. 1 (Figure 3a), most expressed proteins (53.33%) related to the cell membrane and cell wall, and a smaller proportion related to DNA-related biological processes. These results suggest that the antimicrobial mechanism of peptide no. 1 in *X. oryzae* pv. *oryzae* is primarily related to the cell membrane and cell wall. It is possible that the peptide may have caused cell leakage and that some part of it also affected DNA-related processes.

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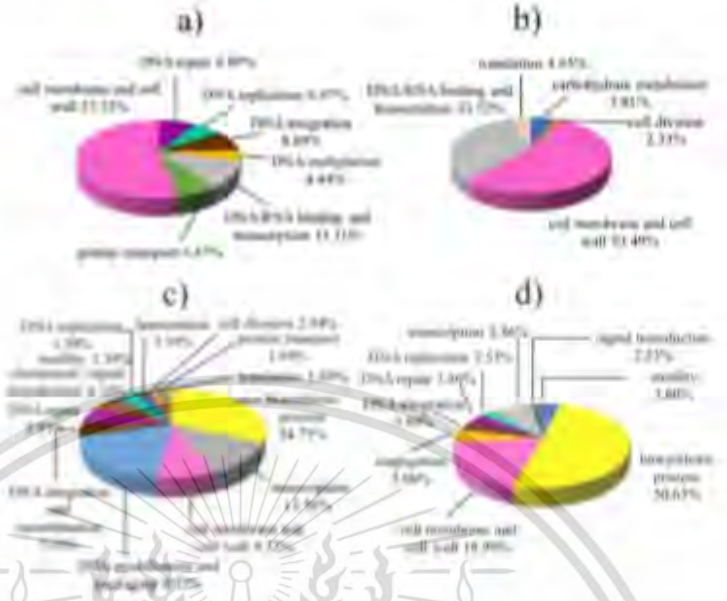

Figure 3. Functional profiles of identified proteins expressed in peptide-treated plant pathogens (but not found in antibiotic treated or control samples): (a) *X. oryzae pv. oryzae* with peptide no. 1, (b) *X. oryzae pv. oryzae* with peptide no. 5, (c) *Pectobacterium carotovorum* with peptide no. 2, and (d) *A. rhizogenes* with peptide no. 2.

Similarly, in *X. oryzae* pv. *oryzae* treated with peptide no. 5 (Figure 3b), the majority of expressed proteins (53.49%) related to the cell membrane and cell wall; this group included proteins involved in cell wall organization and integral components of membranes. Other expressed proteins had functions relating to DNA/RNA binding, transcription and translation, carbohydrate metabolism, and cell division. Overall, the functional profiles of proteins expressed in *X. oryzae* pv. *oryzae* treated with peptides no. 1 and 5 were similar, and those functions suggest these peptides primarily interact with the cell membrane directly, and secondarily may have some feature that interacts with intracellular targets such as DNA, RNA, and proteins. Thus, peptides no. 1 and 5 can be considered membrane-active AMPs.

In *Pectobacterium carotovorum* treated with peptide no. 2, expressed proteins were most commonly related to biosynthetic processes (34.75%); annotations in this group included the general biosynthetic process, peptidoglycan catabolic process, phosphopyruvate-dependent sugar phosphotransferase system, and polysaccharide biosynthetic process. Other prominent function groups included regulation of transcription, the cell membrane, and the cell wall, and terms relating to DNA. These results imply that peptide no. 2 mostly interacts with proteins involved in intracellular processes including biosynthesis, transcription and translation, DNA repair, and signal transduction. However, some part of the peptide may interact with the cell membrane and cell wall of *Pectobacterium carotovorum*.

Finally, we examined the functional classifications of proteins expressed by *A. rhizogenes* after treatment with peptide no. 2 (Figure 3d). Again, more than half were related to biosynthetic processes (50.63%); specific terms included the carbohydrate metabolic process and transport, fatty acid and lipid biosynthesis, and amino acid metabolism and peptidoglycan biosynthesis. In addition, a small number of proteins had functions relating

to the cell membrane and cell wall. Overall, the functional classifications of proteins expressed in *P. carotovorum* and *A. rhizogenes* after treatment with peptide no. 2 are similar in that most related to metabolic processes. Thus, peptide no. 2 could be called an intracellular-active AMP.

Among the eight peptides investigated, three were found to effectively inhibit the growth of *X. oryzae* pv. *oryzae*, *P. carotovorum*, and *A. rhizogenes*. Peptides no. 1 and 5 were determined to be membrane-active AMPs, and peptide no. 2 an intracellularly active AMP. In general, membrane-active AMPs are usually unstructured when in aqueous solution, and form an α-helical structure in the presence of a lipid membrane. Under appropriate conditions, membrane-active AMPs create transmembrane pores or channels that allow leakage of intracellular molecules, eventually leading to cell death [37]. Various modes of action have been proposed on the basis of the arrangement of AMPs on a membrane: namely, the barrel-stave pore, toroidal pore, or carpet mechanism. Furthermore, the precise peptide–microbe mechanism is dependent on lipid membrane composition, peptide structure, peptide concentration, temperature, and pH [38].

In contrast, intracellularly active AMPs can inhibit or kill microbial cells without causing membrane disruption. This type of AMP interacts with targets inside the cells, namely DNA, RNA, or proteins [39].

Several AMPs are reported to have only one mode of action regardless of their concentration; for example, apidaecin exhibits a nonmembrane-lytic mode of action in every concentration and condition [40]. However, many AMPs are reported to have dual mechanisms contingent on peptide concentration. Typically, concentrations above the minimum inhibitory concentration (MIC) lead to membrane lysis with the AMP acting as a detergent, whereas concentrations lower than the MIC cause membrane penetration and targeting of macromolecules within cells [41]. This intracellular action causes inhibition of metabolic processes and also leads to the death of the bacteria. As illustrated in Figures 3c,d, the intracellular mechanism of peptide no. 2 is related to nucleic acids (DNA/RNA), lipids, and proteins. This is consistent with other findings regarding the mechanisms of intracellular-active AMPs in general. These results conform to several previous reports.

Interestingly, while peptides no. 1 and 5 have primarily membrane-active mechanisms of action, they were also associated with expression of some proteins related to metabolic processes. At the same time, peptide no. 2 is primarily an intracellular-active AMP, but is also implied to affect the cell wall and cell membrane. These results are similar to some previous findings. Shi et al. [42] reported that melittin, an AMP from Apis mellifera, disrupts the cell membrane, making holes that result in cytoplasm leakage, but it also may inhibit the biosynthesis of both DNA and proteins. In addition, the bactericidal peptide indolicidin has exhibited more than one mechanism of antimicrobial action. In aqueous solution, it takes on an amphipathic and globular conformation, distinctly unlike the structures adopted in lipids, and these different structures feature different functions. Gel retardation and fluorescence quenching experiments revealed indolicidin to have DNA-binding properties and to interact with lipid bilayers at different concentrations [43].

5. Conclusions

Protein hydrolysates of AW1 (rice straw), AW6 (bagasse), IW3 (peanut seed coat), and IW4 (coconut residue) exhibited antibacterial activity against bacterial plant pathogens including *X. oryzae* pv. *oryzae*, *X. citri*, *Pectobacterium carotovorum*, and *A. rhizogenes*. Peptides PQLAVF, VQLMNSL, and MDRFL were identified as novel peptides derived from AW6 (bagasse) that inhibited the growth of *X. oryzae* pv. *oryzae*, *Pectobacterium carotovorum*, and *A. rhizogenes*. Of those, VQLMNSL did not show any effect against the PGPRs *Bacillus subtilis* and *Pseudomonas fluorescens*. Regarding the mechanism of effect, PQLAVF and MDRFL might cause cell leakage and interfere with DNA-related processes in *X. oryzae* pv. *oryzae*, while VQLMNSL interferes with the biological processes of *Pectobacterium carotovorum* and *A. rhizogenes*.

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Academic publications

1. Ditsawanon, T., Parinthawong, N., Phaonakrob, N. and Roytrakul, S. 2022. "Protein hydrolysates from agricultural wastes for plant bacterial disease control." **International Journal of Agricultural Technology** 18(2):479-488.
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