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Protein hydrolysates from agricultural wastes for plant bacterial disease control

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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

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

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 °C, 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.

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^{i}	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*.

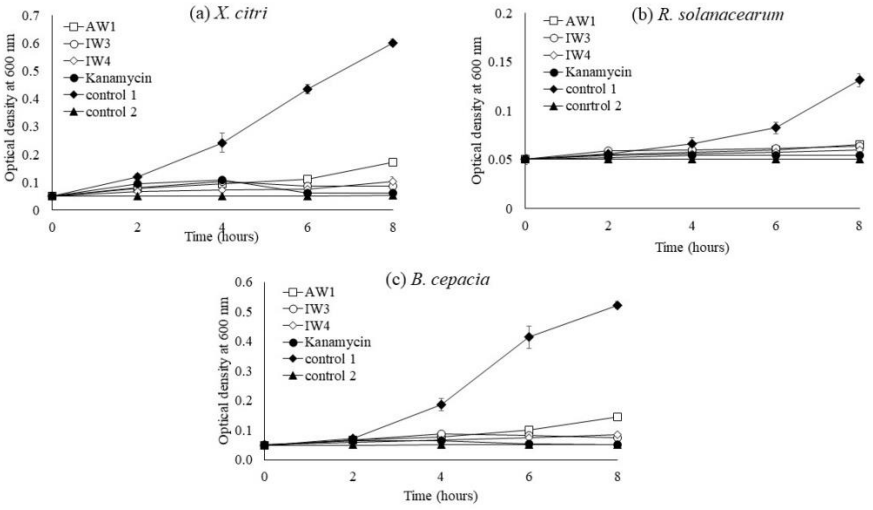

Figure 1. Antibacterial activity of 100 µg/ml protein hydrolysates from AW1, IW3 and IW4 against the pathogens (a) *X. citri*, (b) *R. solanacearum* and (c) *B. cepacia*. Control 1 was bacterial growth, without antibiotic, and control 2 was PBS, without microorganisms

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.

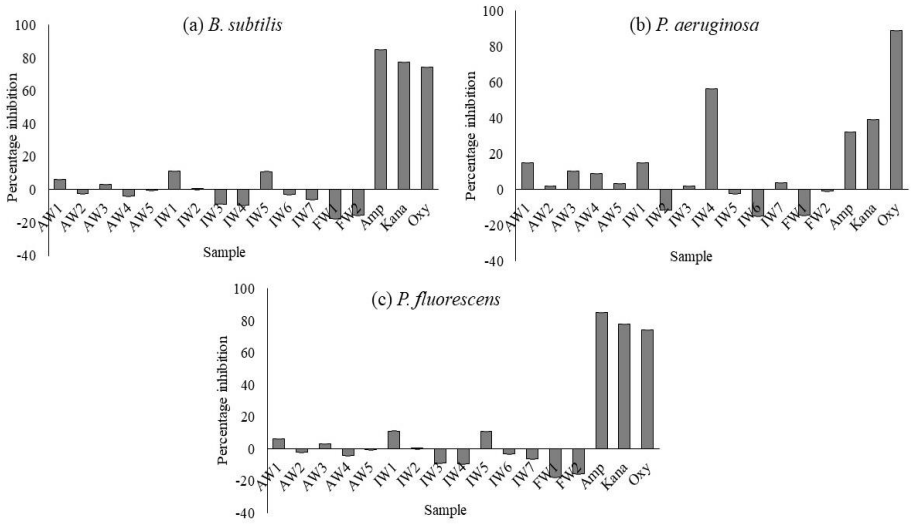

Figure 2. PGPR growth inhibition (a) *B. subtilis*, (b) *P. aeruginosa* and (c) *P. fluorescens*

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,

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.

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