PROTEASE ENZYME FROM CUCUMBER AS ANTIMICROBIAL AGENT

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ABSTRACT

Introduction: The following study was conducted to characterize the protease enzyme present in peel and flesh of cucumber; *Cucumis sativus*, which can be found easily in Malaysia. Method: The extracts from peel and flesh have been partial purified by undergoing ammonium sulphate precipitation and dialysis in order to obtain protease enzyme. The protease enzyme was tested for its concentration and enzyme activity. **Results:** The specific activity of the enzyme was found to be almost the same between peels and flesh which was 3.08 units/μg of protein in peels and 2.90 units/μg of protein flesh. The crude extracts and protease enzyme were then further tested for their antimicrobial activities against *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli*, *Bacillus cereus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Bacillus subitlis*, *Staphylococcus epidermidis* and *Candida albicans.* The crude extracts showed positive inhibition against *S.typhi* with zone of inhibition of 7.33±0.58 mm and 7.17±0.29 mm for flesh and peels respectively, while the zone of inhibition against *P.aeruginosa* was 7.33±0.29 mm and 7.33±0.58 mm for flesh and peels respectively. However, no inhibition zone was observed for partial purified protease tested on selected pathogenic microbes. **Conclusion:** the findings show that the ability of protease enzyme as antimicrobial agents varies based on the stage in partial purification from different plant parts.

KEYWORDS: *Curcumis sativus*, protease, enzyme activity, antimicrobial

INTRODUCTION

Microbes have the ability to evolve and transform themselves, making them resistant against antimicrobial agents thus rendering the drugs to be ineffective. In general, the bacteria has the genetic ability to transmit and acquire resistance to drugs in at least four different ways: (i) the mutagenic nature of bacterial DNA; (ii) the rapid multiplication of bacterial cells; (iii) the constant transformation of bacterial cells due to plasmid exchange and (iv) the uptake of plasmid by the pathogenic bacteria to develop antimicrobial resistance (Kaushik and Gopal, 2008). Some of the microbes can form biofilm which act as a protective shield against antimicrobial and thus greatly enhance their ability to survive. Thus, it is now an utmost importance to search for new antimicrobial agents from natural resources.

Among the best antimicrobial agents existed to date is the enzyme-based antimicrobials agents. Enzymes are protein molecules that act as biological catalyst and help in various reactions including synthesis and degradation of lipids, amino acid, carbohydrates, proteins, nucleic acids and all their metabolic intermediates. The lock-and-key properties of enzyme is said to be effective against microbes since it could only attack the target microbes and will not disrupt the friendly and necessary bacteria. This type of antimicrobials is now pursued by scientist to be studied and researched for its promising effectiveness against microbes.

There are many researches that have proved the effectiveness of enzyme against pathogenic microorganisms. A type of enzyme called lysozyme, is one of the best characterized hydrolases and is considered to be a self-defense enzyme, produced in serum, mucus and many organs of vertebrates. The supernatant of the lysozymes extracted from soft shelled turtle displayed antimicrobial activity against *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* and *Streptococcus faecalis* (Booncharoen and Thammasirirak, 2008). Since enzymes are also present in plants, it is interesting to know on whether the enzyme isolated from plant would also display the same antimicrobial properties. Among the plants that contain a large amount of protease are papaya and pineapple (Rao *et al.*, 1998), both plants which grow well in tropical area, same as cucumber.

Another type of plants which also has protease is cucumber. Cucumber is a type of tropical vegetables that is consumed either freshly or being cooked as a dish. Since cucumber can be found easily in Asia, they have been used as food source and also as traditional medicine especially in cosmetic, without apparent toxic effect. Having a high content of water, cucumber is effective as cooling agents and is distinctly diuretic (Uzodike and Onuoha, 2009). The seeds of the cucumber

are also found to be effective to treat ulcer (Saboo *et al*., 2013).Previous researchers have found that cucumber could offer many potential health benefits. However, most of the researches conducted on the medicinal and therapeutic values of cucumber are more focus on the bioactive compound that present in the plants, not the enzyme contents. Since there is now a hope on enzyme to be used as antimicrobial agents, thus it is a necessity to study the properties of the enzyme's extracted from this fruit and test it on whether this particular protease enzyme from the cucumber possess antimicrobial properties or not. This study aims to extract and characterize enzyme protease from peels and flesh of *Cucumis sativus* and investigate the antimicrobial properties of enzyme extracted.

MATERIAL AND METHOD

Protease Extraction

Three cucumbers which each weight around 160 g – 180 g were bought in Pasar Besar Kuantan and washed cleanly with distilled water and the outer skin was peeled off using a peeler. For every 100 g of cucumber samples, 100 ml phosphate buffer was added and the flesh part and the peel were blend separately. The blended cucumber was filtered through 4 layers of cheese clothes before being filtered using Whatman filter paper Number 1 to obtain the filtrates. The cucumber filtrates then were centrifuged at 15000 g for 10 minutes at 4^{o}C and the supernatant was collected while the cucumber pellets were discarded. The supernatants were then kept in 1.5 ml microcentrifuge tube and stored in -20 ^{o}C.

Ammonium Sulphate Precipitation and Dialysis

In this study, 80% ammonium sulphate precipitation is carried out to purify the protease. For every 40 ml of peels and flesh extract of cucumbers respectively, 21.31 g of solid ammonium sulphate is added slowly. The mixture is being mixed slowly to allow the ammonium sulphate to dissolve in the extract solution and the mixtures were allowed to precipitate overnight at 4^{o}C. The extracts were then centrifuged at 10 000 g for 15 minutes at 4^{o}C and the cucumber pellet were then recovered by dissolved it in 30 ml of phosphate buffer solution. Each mixture was then dialyzed using the Thermo Scientific SnakeSkin Dialysis Tubing 10K MWCO tube against the same buffer (1: 50 volumes) for overnight at 4^{o}C. The buffer was changed for every 4 hours. The final solution after the dialysis process completed was then collected and stored at -20^{o}C.

Protein Concentration Determination

Bradford method (Bradford, 1976) was carried out to determine the concentration of the protein in the extract. 5 µl of sample extract was mixed with 95 µl of 0.15 M sodium chloride, NaCl. Then, 1000 µl of Bradford reagent was added to the mixture. Five minutes after the Bradford reagent was added to the mixture, the absorbance of the mixture were read at 595 nm. A change in colour of Bradford reagent from reddish brown to blue indicates the presence of the protein. The absorbances of the samples were compared with the absorbance of bovine serum albumin at 595 nm.

Sodium Dodecyl Sulfate polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular size of the protein in the extract was detected by using one-dimensional discontinuous SDS-PAGE. The electrophoresis was carried out on 4% stacking and 12% resolving gel according to Mini-Protean® 3 Cell Instruction Manual from Bio-Rad, Hercules, USA. A total of 40 µg of protein for each extract was mixed with sample buffer and denatured at 95°C for 5minutes before being loaded into the wells on the polyacrylamide gel. A protein marker (Trail Mix™ Protein Markers) was mixed with sample buffer and 10 µl of the mixture was loaded to the designated well on the gel. The electrophoresis was carried out using using Mini-PROTEAN® Tetra System (Bio-Rad) in 1x electrode buffer containing 25 mM Tris-HCL (pH 8.3), 192 mM glycine and 0.1% (w/v) SDS at voltage 200V for 60 minutes. After 60 minutes, the gel was stained by adding the mixture of 0.1% (w/v) Coomasie blue (Thermo Scientific Pierce) and 10% (v/v) acetic acid and 40% (v/v) methanol just enough to cover the surface of the gel. After 1 hour of staining at 100 rpm, the gel was destanined for 10 minutes in a destain solution containing 40% (v/v) methanol and 30% (v/v) acetic acid for 10 minutes. It was then being destained with second destaining solution containing containing 5% (v/v) methanol and 7% (v/v) acetic acid for overnight. The image of the protein on the gel was observed for analysis.

Enzyme Activity Assay

The enzyme activity assay was based on a method proposed by Keay *et al.,* (1970) with some modification. The assay was carried out following the protocol in the Universal Protease Activity Assay by Sigma Aldrich. Casein was used as the substrate. Firstly, 2 g casein was dissolved in 50 mM potassium phosphate buffer solution pH 7.5 in a temperature less than 80 °C for 10 minute. In a 15 ml falcon tube, 1 ml of the diluted casein were being mixed with 1 ml of the cucumber extract and then left to incubate for 10 minutes at 37 °C water bath. After 10 minutes of incubation, 2 ml of 0.4 M trichloroacetic acid was added to the mixture to arrest the reaction and the mixture were incubated for another 10 minutes in 37 °C water bath. The mixtures then were filtered through

Whatman filter paper Number 1 to remove any insoluble substances. Then, 1 ml of the clear filtrate was then mixed with 5 ml of 0.4 M sodium carbonate (Na_2CO_3), and 1 ml of Folin & Ciocalteu's Phenol reagent and the mixture were incubated in 37 ^oC waterbath for 30 minutes before the absorbance was measured at 660nm. The absorbance was compared with the standard curve prepared by using L-tyrosine as the standard. The amount of L-tyrosine will be calculated and being incorporated into the following formula:

Enzymes activity (unit/ml) =

$$\frac{(\mu\text{mole tyrosine equivalents released}) \times (11)}{(1) \times (10) \times (1.5)}$$

Where;

11= Total volume (in milliliters) of assay
10= Time of assay (in minutes) as per the Unit definition
1= Volume of Enzyme (in milliliters) of enzyme used
1.5 = Volume (in milliliters) used in colorimetric determination.

Antimicrobial Test by Disc Diffusion Assay

The disc diffusion assay or also known as Kirby-Bauer disk diffusion was carried out to determine the susceptibility of microbe to antimicrobial compound. Eight pathogenic bacteria and one fungus were used to test the potential antimicrobial properties of enzyme extract of cucumber. They were *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli*, *Bacillus cereus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus epidermidis* (bacteria) and *Candida albicans* (fungus). All the microbes were obtained from Microbiology Laboratory at the Kulliyyah of Science, International Islamic University Malaysia (IIUM). All microbes were cultured onto nutrient agar to obtain single colony and all plates of bacteria were left to incubate for 24 hours at 37 ^oC while *C. albicans* culture were incubated in room temperature for 48 hours. A single colony from each species on the nutrient agar were taken and inoculated in a universal bottle containing Mueller-Hintons broth. Then they were left to incubate for another 12 hours at 37 ^oC and 30 ^oC for bacteria and fungus respectively. After 12 hours of incubation, the optical density of incubated bacterial broth was checked by using spectrophotometer with wavelength of 625 nm. If the bacterial suspension was too turbid, it then was diluted by adding more sterile Mueller-Hinton (MH) broth. If the suspension was not turbid enough, more bacteria were added.

The final optical density of the bacteria was approximately 0.1 OD while the fungus was 0.6 OD. The bacterial and fungal inoculums at approximately 10 µl were spread evenly on the surface of MH agar by using sterile cotton bud. The enzyme extract of cucumber was first being filtered using sterile filter 0.45 µm to avoid any contamination coming from the extract itself. Blank discs (Oxoid) impregnated with 30µl of cucumber's enzyme extract and were let dried under sterile condition. The impregnated discs then were placed on MH agar surface that have been inoculated with the bacteria. Each cucumber extract was assayed in triplicate. 10 µg of streptomycin discs (Oxoid) and 15 µg of nystatin disc (Oxoid) were used as positive control. Blank disc impregnated with 30 µl of distilled water on the other hand was used as negative control. The inoculated plates were incubated for 24 hours at 37°C for bacteria and 48 hours at 37°C for fungi. The clear zones of inhibition were measured after incubation period.

RESULTS AND DISCUSSIONS

Protein Quantification

The protein concentration for each samples were shown in Table 1 below. The protein concentration of crude extract of each peel and flesh of cucumber extracts were higher than the partial purified extracts.

Table 1 Protein concentration of cucumber extracts

Sample of cucumber extracts	Protein concentration (µg/µl)
Flesh A	4.86
Peel A	5.26
Flesh B	4.05
Peel B	4.77

Note: A, crude extract; B, partial purify protease; Flesh, protease from cucumber flesh; Peel, protease from cucumber peel.

Enzyme Activity

The protease activity and specific activity of the cucumbers' samples were as in Table 3.2 followed.

Table 2 Enzyme activity (units/ml) and enzyme specific activity (units/µg) of different cucumber extracts

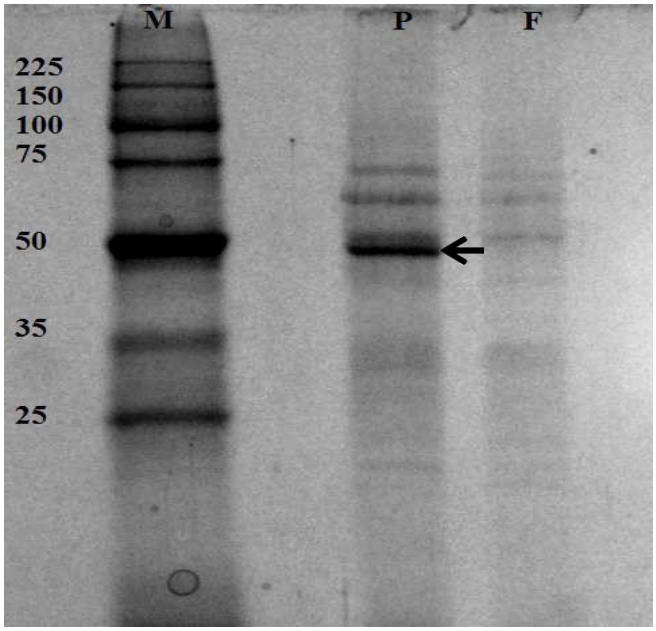
Sample of cucumber extracts	Enzyme activity (units/ml)	Enzyme specific activity (units/µg of protein)
Flesh A	6.93	1.42
Peel A	9.35	1.78
Flesh B	11.74	2.90
Peel B	14.70	3.08

Note: A, crude extract; B, partial purify protease; Flesh, protease from cucumber flesh; Peel, protease from cucumber peel.

From the Table 2 above, the enzyme activity of partial purified protease was higher than the crude extracts. The peels extracts also showed higher enzyme activity compared to flesh's extracts. This result is compatible with the research carried out by Artthaisong *et al*. (2012), which recorded the enzyme activity of cucumber's flesh to be 12.22 unit/mL at 37 ^{o}C. The enzyme specific activities were also higher for both peel extracts of crude extracts and partial purified protease which are 1.778 units/µg and 3.081 units/µg respectively compared to crude extract of flesh (1.424 units/µg) and flesh extract after partial purification (2.896 units/µg). Both peel and flesh extract after partial purification showed higher enzyme activities compared to crude extracts.

Protein separation by SDS-PAGE

The crude cucumber extracts were separated on the polyacrylamide gel and the result was presented in Figure 1. The SDS-Page was carried out to determine the size of the protein present in the peels and flesh of cucumber extract.



Figures 1: SDS-PAGE of cucumber peel and flesh crude extracts. P, peel extract of cucumber; F, flesh extract of cucumber; M, protein marker (in KDa size). The arrow shows the high protein expression in peel sample.

Based on the SDS-PAGE result, the protein bands were distributed between the molecular weight of 75 kDa and 50 kDa. There are differences in band intensity which can be seen from the gels whereby that some of the proteins were expressed higher in peels compared to flesh. The protein in peels was expressed higher at the size of 50 kDa, while the protein in flesh at similar band size was expressed lower. A research conducted by Siritapetawee *et al*., (2012), found a 48-kDa protease (AMP48) from jackfruit, so it can be suggested that the protein band expressed at about 50 kDa is protease as shown by black arrow (Figure 1).

Antimicrobial Properties

Both crude extracts and partial purified extracts from peel and flesh were respectively tested against 8 selected bacteria and one type of fungus. The results were assayed by measuring the zone of inhibition after an overnight growth of pathogenic microbes. The values of the zone of inhibition of each experiment were calculated from the mean triplicate test. Antibiotic streptomycin and nystatin disc was used as positive control and phosphate buffer was used negative control for the test. The results showed that there was no zone of inhibition for partial purified extract for all microorganisms while there are positive antibacterial activities of crude extract of cucumber against *Pseudomonas aeruginosa, Salmonella typhi and Candida albicans* showed by the inhibition zone. The mean zones of inhibition are tabulated in Table 3.

Table 3: Average diameter of zone of inhibition (mm) for each cucumber extracts.

Microbial strains	Samples				Positive control		Negative control
	Flesh A (± 145.8 µg/disc)	Peel A (± 147.3 µg/disc)	Flesh B (± 145.8 µg/disc)	Peel B (± 143.1 µg/disc)	Streptomycin (10 µg/disc)	Nystatin (100 µg/disc)	Phosphate buffer (30 µl/disc)
S. aureus	-	-	-	-	15	NA	-
S.epidermidis	-	-	-	-	17	NA	-
B.cereus	-	-	-	-	18	NA	-
S.typhi	7.33±0.58	7.17±0.29	-	-	15	NA	-
P.eruginosa	7.33±0.29	7.33±0.58	-	-	14	NA	-
K.pneumoniae	-	-	-	-	15	NA	-
E.coli	-	-	-	-	17	NA	-
B.subtilis	-	--	-	-	18	NA	-
C.albicans	-	10.33±0.58	-	-	NA	27	-

Values are mean ± SD from triplicate tests. Diameter of inhibition zone (mm) include diameter of the disc which is 6 mm. Flesh A and Peel A are crude extracts from flesh and peel respectively. Flesh B and Peel B were partial purified protease from flesh and peel respectively. NA indicates not available while (-) indicates no inhibition.

In the antimicrobial test, disc diffusion assay is being used since it is one of the easiest qualitative methods to observe antimicrobial properties of a substance against microorganisms. From the results in Table 3, it can be observed that the crude extract of the peels and flesh of cucumber have low antimicrobial properties. The zone of inhibition against *Pseudomonas aeruginosa* and *Salmonella typhi* were between 7 – 9 mm which indicates low and weak antimicrobial properties as similar to diameter of zone of inhibition produced by Johnson and Case (1995). The zone of inhibition of crude extract of cucumber's peels against *Candida albicans* showed moderate antimicrobial activities since the zone of inhibition is 10.33±0.58 mm. Based on Table of Diameter of Zone of inhibition (Johnson and Case, 1995), diameter of zone of inhibition ranges from 11 to 15 mm is considered as having moderate antimicrobial properties. This result somehow correlates with past research conducted by Siritapetawee *et al*., (2012) which have found that a 48-kDa protease (AMP48) from jackfruit displayed antimicrobial properties against *Pseudomonas aeruginosa* and *Candida albicans.*

In another research carried out by Sotiroudis, *et al.,* 2010, stated that volatile oil of cucumber have antimicrobial activity against several microbes, one of them is *Pseudomonas aeruginosa* and fungi *Candida albicans*. The volatile oil of the cucumber also showed antimicrobial activities against *Klebsiella pneuomoniae* and *Escherichia coli* and in another research, phyto-constituents (E, Z)-2, 6-nonadienal (NDE) and (E)-2-nonenal (NE) present in cucumber have been found to have antimicrobial activities against *Escherichia coli* as well (Cho *et al*, 2004). However, phosphate buffer extraction of cucumber in this study shows no inhibition to both *Klebsiella pneuomoniae* and *Escherichia coli.* This might be due to the substances that are responsible to inhibit the growth of such microorganisms that have been omitted during the extraction process.

The extract for both peels and flesh of cucumbers after partial purification on the other hand showed no inhibition zone against all microorganisms. From this result, it can be suggested that the partial purified protease enzyme of cucumber did not have any antimicrobial properties. It can also be deduced that the antimicrobial activities which the crude extracts displayed are coming from the bioactive compound or some other type of protein present in the plants. Besides that, even though the crude extract showed the presence of protein according to the result from protein quantification, there is another non-protein substance residue in the crude extract which is not being detected in this study. Many past researches have also proved the presence of bioactive compound and natural product in cucumber which can inhibit the growth of pathogenic microorganisms. After ammonium sulphate fractionation, most of the bioactive compound might be defected and only selected enzymes are left in the samples. This result correlate with the study by Jewell (2000), which showed that the supernatant of *Burkholderia* strain 2.2 N displayed antimicrobial activities against several microorganisms but the purified protease from the bacteria showed no antimicrobial activities.

There are many factors that can influence the antimicrobial activities. The characteristics and

susceptibility of each microorganism towards antimicrobial agents also can influence the result of this test. Generally, plant extracts are more active in inhibiting the growth of Gram-positive bacteria compared to Gram-negative bacteria (Basri and Fan, 2005). However, in this study, the extract showed antimicrobial activities against two types of Gram-negative bacteria which are *S.typhimurium* and *P.eruginosa.* Besides that, the concentration of the protein in the extracts might also affect the result of the test. The peels samples which have more protein in it displayed slightly larger zone of inhibition compared to the flesh samples. In addition of that, this test was first carried out with only 10 µl of samples per discs, which has approximately 48.6 µg of protein and it does not show any inhibition against any bacteria.

Since the usage of enzyme as antimicrobial agents is still a new subject, the real mechanism on how specific enzyme acts as antimicrobial agents is still a confusing subject to many. There are many theoretical mechanisms on how the enzyme could attack the pathogenic microorganism. The enzymes may attack the microbial cells by degrading major structural components of the cells. They could also be a catalyst and thus induce the production of antimicrobial substances. The enzymes could prevent biofilm formation or disrupt the existing biofilms by degrading the molecular compound that holds the biofilm together. By disrupting the biofilm formation, the microorganisms are now more susceptible to antimicrobes (Thallinger, *et al.,* 2013).

CONCLUSION

This study was designed to characterise the enzyme protease from each peels and flesh of cucumbers respectively. The amount of the protein is found to be higher in the peels sample compared to the flesh samples. The enzyme assays were carried out at 37^{o}C and the specific enzyme activity is found to be higher in peels samples. The ammonium sulphate fractionation were also carried out to concentrate the amount of the protein in the samples and it is found that the enzyme specific activity of flesh and peels extract after ammonium sulphate fractionation were higher than the crude extract of cucumber. From the SDS-PAGE the size of the protease were found to be distributed at 75 kDa and 50 kDa with a different in intensity of the band which indicates that some protein were expressed higher in peels compared to flesh. From the disc diffusion assay, it can be observed that the protease isolated after 80% ammonium sulphate fractionation does not possess any antimicrobial properties even though the crude extract of peels and flesh of cucumber showed positive antimicrobial activities against *S.typhi* and *P.aeruginosa.*

ACKNOWLEDGEMENT

The authors wish to acknowledge full gratitude to the International Islamic University Malaysia (IIUM) for funding this project through RIGS16-107-0271 and Final Year Project allocation in the Kulliyyah of Science.

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