Malaysian Applied Biology (2024) 53(6): 87-96
https://doi.org/10.55230/mabjournal.v53i6.9

Research

The Investigation of Bio-Preservative Properties in *Plukenetia volubilis* L. (Sacha Inchi) Seeds Protein Extract For Food Spoilage Prevention

Noor Hasniza Md Zin[1]*, Widya Abdul Wahab[1], Mohammad Norazmi Ahmad[2], Zaiton Sapak[3], Noor Suhana Azahar[4]

1. Department Biotechnology, Kulliyyah of Science, International Islamic University Malaysia, Pahang, Malaysia
2. Department Chemistry, Kulliyyah of Science, International Islamic University Malaysia, Pahang, Malaysia
3. Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA (UiTM) Jasin, Melaka, Malaysia
4. Fakulti Sains dan Teknologi Industri, Universiti Malaysia Pahang Sultan Abdullah, Pahang, Malaysia
*Corresponding author: hasnizamz@iium.edu.my

ABSTRACT

The importance of food quality and safety has long been recognized by the food industry to cater to food spoilage. Food spoilage is caused by the loss of food quality from its original organoleptic qualities due to chemical and biological spoilage processes that may impact customer acceptance. This research aimed to evaluate the potential of Sacha inchi seeds protein as a multifunctional food preservative in controlling chemical (oxidation) and biological (microbial and protease activity) food spoilage. The antioxidant capacity revealed that the Sacha inchi seed protein has 73.72 ± 0.70% inhibition towards oxidation with the total phenolic content detected at the concentration of 463.13 ± 0.51 µg/mL in the protein extract. The tested food spoilage bacteria (*Escherichia coli* and *Micrococcus luteus*) and fungus (*Colletotrichum gloeosporioides*) were susceptible to Sacha inchi seed protein showing its potential antimicrobial properties. The Sacha inchi protein extract (46.65 µg/mL & 23.28 µg/mL) shows a significant inhibition for *E. coli* (30.5 mm) and *M. luteus* (33 mm), respectively, which indicated its effectiveness. Sacha inchi seed protein also displayed potential antifungal activities by suppressing the growth of *C. gloeosporioides* at inhibitory concentration percentages (IC%) of 1.5±0.12%, 5.85±3.89% and 5.90±1.98% after 2, 3 and 4-days of incubation, respectively. The caseinolytic plate assay revealed that Sacha inchi seed protein showed inhibition of trypsin digestion on casein with reduced inhibition diameter from 1.9±0.00 cm (negative control) to 1.05±0.00 cm. Following the effective protein separation by SDS-PAGE, zymography analysis revealed that a prominent protein band at 25 kDa showed protease inhibitory activity. This research contributes insights into the potential application of Sacha inchi seed extract as a bio-preservative in the food industry to combat food spoilage and it is aligned with SDG 2 for zero hunger.

Key words: Antioxidant, antimicrobial activity, bio-preservative, food spoilage protease inhibitory

Article History
Accepted: 5 November 2024
First version online: 25 December 2024

Cite This Article:
Md Zin, N.H., Wahab, W.A., Ahmad, M.N., Sapak, Z. & Azahar, N.S. 2024. The investigation of bio-preservative properties in *Plukenetia volubilis* L. (Sacha Inchi) seeds protein extract for food spoilage prevention. Malaysian Applied Biology, 53(6): 87-96. https://doi.org/10.55230/mabjournal.v53i6.9

INTRODUCTION

Food spoilage refers to the loss of food quality from its original organoleptic qualities observed during processing and storage which involves the process of undesirable changes in the physical, chemical, and organoleptic properties of the food (Onyeaka & Nwabor, 2016). The changes can be any undesired change in the natural color, taste, or texture of the food items, rendering them unfit for consumption due to the loss of quality and nutritional value (Karanth *et al*., 2023).

The rate of food spoilage is influenced by intrinsic and extrinsic factors. The extrinsic factors that can cause food spoilage include temperature, pH, water availability, the presence of spoilage microorganisms such as bacteria, fungi, and yeast, and the processing methods (Odeyemi *et al*., 2020). The intrinsic factors that contribute to food deterioration are the endogenous enzymes; lipases and proteases and chemical reactions; browning and oxidation that occur naturally within the food item (Pellissery *et al*., 2020). These factors affect the food shelf life by reducing the quality, safety, and visual appeal. Food spoilage can be a

microbial enzymatic activity, which will ultimately dominate food deterioration based on environmental conditions needed for microbial growth. Oxidation causes the breakdown of food components, resulting in changes in color, and flavor, and the formation of off-odors such as rancidity, which eventually leads to food spoiling. Endogenous proteases contribute significantly to the post-mortem breakdown of fish muscles, resulting in muscular disorganization and degeneration of myofibers (Singh *et al*., 2020).

Hence, a variety of techniques are available to ensure the safety and quality of the food such as refrigeration and freezing along with thermal preservation, chemical preservation, irradiation, and bio-preservation techniques. The bio-preservation technique can be described as the utilization of natural or controlled microbiota or antimicrobials to extend the shelf life of food products (Singh, 2018). The plant is one of the sources of bio-preservative which contains the components that can exhibit antimicrobial and antioxidant properties and inhibit protease activity. Previous studies suggested that legume seeds contain high-quality proteins, fats, flavonoids, saponins, vitamins, and minerals which can be applied in food systems for nutrition improvement and functionality (Chen *et al*., 2017). Sacha inchi seeds also have a high content of oil (54%) and protein (27%) as stated by Quinteros *et al*. (2016) and the protein may exhibit bio-preservative properties. Protease inhibitors can extend the shelf life of certain foods by preventing the activity of external and internal proteases during food preservation and processing (Bacha *et al*., 2017). Based on the active amino acid in the reaction sites, protease inhibitors can be categorized structurally into severalclasses, including cysteine, serine, aspartate, and metalloproteases.

Sacha inchi, scientifically known as *Plukenetia volubilis* L., is native to the Amazon forest and has been cultivated in Malaysia. It has been utilized as a dietary supplement or an additional food due to its high nutritional value, containing omega-3, omega-6, and omega-9 fatty acids (Wang *et al*., 2018). It has increasingly been acknowledged for its potential health advantages for humans. In recent years, there has been an increasing interest in leveraging the therapeutic potential of the Sacha inchi plant due to its high content of unsaturated fatty acids. Currently, many existing products use Sacha inchi as an ingredient, such as cosmetics, food and beverages as well as dietary supplements. In addition, Sacha inchi has been officially approved by the European Union (EU) and Food Safety Authority of Ireland (FSAI) to be a food ingredient and there is much research on its chemical properties justifying that it is good for human health. The Sacha inchi seed has been reported to have high antioxidant activity in selected varieties as reported by Lemus-Conejo *et al*. (2024). Besides, the plant seed possesses antimicrobial activity against pathogenic microbes (Jang *et al*., 2020). Previous studies have mentioned that there was an improvement in the textural properties of pork sausages and fish fingers after the addition of Sacha inchi protein (Rawdkuen & Ketnawa, 2019). The results showed that the shelf life of the fish finger added with protein hydrolysates had been prolonged, which suggested the potential of the Sacha inchi protein hydrolysates as a food preservative.

With the available research, Malaysia's agricultural sector has started to support the cultivation of Sacha inchi due to the potential benefits that will be acquired from it especially in terms of economy (Siaw, 2022). This study aimed at elucidating the bio-preservative properties of protein isolated from Sacha inchi seeds including antimicrobial potential, and antioxidant capacity in addition to their protease inhibitory properties to reduce the food deterioration process.

MATERIALS AND METHODS

Protein isolation of Sacha inchi seeds

Protein isolation from Sacha inchi seeds was conducted according to Puntambekar and Dake (2017) with some modifications. Briefly, 1 g of seeds were homogenized in 5 mL of 0.1 M phosphate buffer, pH 6.8. The homogenate was vigorously mixed and centrifuged at 10,000 rpm for 20 min at 4°C to collect the clear supernatant. The collected clear supernatant was subjected to ammonium sulfate precipitation at 0-60% saturation. The precipitate obtained was further dialyzed using a pre-treated dialysis tube (10,000 MWCO) (Pierce Biotechnology, USA). The dialyzed protein was subjected to Bradford assay for protein content determination using bovine serum (BSA) as the standard.

Antioxidant properties in Sacha inchi protein

The antioxidant properties of Sacha inchi protein extracts were estimated by measuring the total phenolic content and the 2,2-diphenyl-1-pycrylhydrazyl (DPPH) (Sigma-Aldrich, USA) scavenging activities according to Jaafar *et al*. (2020) with some modifications. The total phenolic content was estimated using gallic acid (R&M Chemicals, UK) as the standard, and the content was expressed in µg/mL. Meanwhile, the DPPH scavenging activity of the protein was determined by using the method by Shirazi *et al*. (2014) with slight modifications. Protein isolates were mixed with DPPH solution (0.1 mm

in methanol) and incubated in the dark for 45 min. The absorbance was measured at 517 nm by using a Genesys 10s UV-VIS spectrophotometer (Thermo Scientific, USA). The DPPH radical-scavenging activity was measured using Equation 1:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance control-Absorbance sample}}{\text{Absorbance sample}} \times 100 \quad \text{Equation 1}$$

Antimicrobial activity

Agar well diffusion method

The antibacterial assay was conducted according to Manandhar *et al*. (2019) with modifications. A sterilized cotton swab was used to evenly spread 100 µL of bacterial inoculum (*Escherichia coli* & *Micrococcus luteus*) onto the surface of Mueller-Hinton Agar (MHA) (Oxoid, UK) plates. After punching the agar to create wells with a diameter of approximately 6 mm, 50 µL of protein isolates were added to each well. The positive and negative controls were streptomycin sulfate (Calbiochem, Germany) and sterile distilled water, respectively. The plates were incubated at 37°C for 16 hr. Following incubation, measurements were made of the clear zone diameter that formed around the wells, signifying the zone of inhibition.

Meanwhile, the antifungal assay was carried out according to the work of Jose *et al*. (2009) with modifications. Approximately, 2 mm × 2 mm of a potato dextrose agar (PDA) (Merck, Darmstadt, Germany) cube of fungal inoculum (*Colletotrichum gloeosporioides* and *Aspergillus niger*), was prepared. The PDA plate was punched to form a well 6 mm in diameter at the center of the agar. An amount of 50 µL of isolated protein and 25 mg/mL nystatin (positive control) (Duchefa, Haarlem, Netherland) were respectively added to each well of each plate. Then, the cube of inoculum was carefully added into each well containing the respective protein isolate and control. A PDA plate containing only the inoculum cube was used as a negative control. The plates were then incubated for 4 days at 37°C and 28°C for *A. niger* and *C. gloeosporioides,* respectively. The diameter of fungal mycelium was measured and recorded.

Inhibitory Concentration percentage (IC%)

The IC% determination on bacterial growth was done as described by Tehrani *et al*. (2012) with modifications. The serial dilutions of protein isolates were prepared using Mueller-Hinton Broth (MHB) (HiMedia, India) in 96-well microplates and tested against *Escherichia coli* and *Micrococcus luteus*. The plate was incubated at 37°C for 24 hr. The microplate reader was used to measure the absorbance value at 620 nm. Equation 2 was used to calculate the percentage of inhibitory concentration (IC%) for various protein isolates:

$$IC\% = \frac{OD_w - (OD_a - OD_c)}{OD_w} \times 100 \quad \text{Equation 2}$$

Where OD_w is the absorbance value of bacterial growth control, OD_c is the absorbance value of a sample containing only protein isolates as a control, and OD_a is the absorbance value of bacterial suspensions containing different concentrations of protein isolates. The lowest concentration of protein isolates that completely prevents bacterial growth is known as the minimum inhibitory concentration (MIC).

Meanwhile, the IC% for fungal growth was according to Jose *et al*. (2009) with some modifications. Approximately 2 mm x 2 mm of a PDA cube of inoculum, containing active mycelium from 3-5 days old fungal culture was prepared. The PDA plate was punched using a yellow micropipette tip to form a well (about 6 mm in diameter) at the center of the agar. An amount of 50 µL of protein isolates, as well as nystatin 25 mg/mL (positive control) were respectively added into each well of each plate. Then, the prepared inoculum was carefully added into each well containing the respective extract. A PDA plate containing only the inoculum cube was used as a negative control. The plates were then incubated for 4 days at 37°C and 28°C for *A. niger* and *C. gloeosporioides*, respectively. The diameter of fungal mycelium was measured and recorded on Days 2, 3, and 4. The percentage of inhibitory concentration of protein isolates was calculated and recorded, following Equation 3.

$$IC\% = \frac{(Dc - Ds)}{Dc} \times 100 \quad \text{Equation 3}$$

Where Dc is the diameter of mycelium that was subjected to no treatment and Ds is the diameter of mycelium that was treated with sample extracts. After four days of cultivation on PDA plates, the fungi reached their maximal growth, which was regarded as 0% growth inhibition. When the mycelium's radial growth revealed less than 2.5 mm in diameter, it was deemed to be 50% inhibited; 100% inhibition, on the other hand, indicated that there was no growth on the plate.

Caseinolytic plate assay

Casein agar (Solarbio, China) plates containing 1% (w/v) agar and 1% (w/v) casein (Sigma-Aldrich, USA) were utilized for the initial evaluation of protease inhibition activity. A metallic plunger was used to drill wells on the agar plate. The center well contains 0.1 mg/mL trypsin (Solarbio, China) whereas the surrounding wells contain Sacha inchi protein isolates. Plates were incubated in an incubator for 12 hr at a temperature of 37°C. After the incubation, 10 µL of trichloroacetic acid (TCA) (Sigma-Aldrich, USA) was added to allow the inhibition of the process for 15 min. The decrease in the diameter of the trypsin zone of inhibition was observed in the presence of the inhibitor compared to that of the negative control plate, which only contained 0.1 mg/mL trypsin surrounded by phosphate buffer, pH 6.8 (Mohan *et al*., 2018). The positive control plate consists of 0.1 mg/mL trypsin at the center of the well and 1 mg/mL pronase E (Solarbio, China) at the surrounding wells. The diameter of the trypsin inhibition was measured for each treatment.

SDS-PAGE and reverse zymography

The Sacha inchi protein extracts were separated on the 12% polyacrylamide gel (Bio-Rad, USA) to determine the molecular mass range of the proteins followed by reverse zymography to validate the existence of protease inhibitor activity according to Mohan *et al*. (2018). The SDS-PAGE (Bio-Rad, USA) was carried out at 100 V and 200 A until the bromophenol blue reached the bottom of the gel. The gel was stained in a staining solution (a mixture of 0.1% Coomassie blue R250 (Sigma-Aldrich, USA) in 20% methanol (R&M Chemicals, UK) and 100% glacial acetic acid (R&M Chemicals, UK)) and destained using a destaining solution (a mixture of 10% methanol & 7% glacial acetic acid). In reverse zymography analysis, 1% gelatin (Sigma-Aldrich, USA) was copolymerized into polyacrylamide matric before protein separation using SDS-PAGE. Following the electrophoretic run, the gel was subjected to incubation with a 2.5% (w/v) solution of Triton X 100 (Merck, Darmstadt, Germany) for 30 min to eliminate traces of sodium dodecyl sulfate (Merck, Darmstadt, Germany). Subsequently, the gel was rinsed with phosphate buffer; pH 6.8, 0.1 M. The gel was then exposed to 0.1 mg/mL trypsin for 12 hr at room temperature. Following gelatin hydrolysis, the gel underwent a thorough rinsing with distilled water and was subsequently stained. Suppression of protease activity was observed as colored bands on a transparent surface following staining with Coomassie brilliant blue.

Preservation test on raw fish flesh using Sacha inchi protein extract

The preservation test followed the methods described by Chakchouk-Mtibaa (2017) with some modifications. The fresh raw fish (*Decapterus maruadsi*) were purchased from a local market in Kuantan, Pahang, and brought back to the laboratory at IIUM Kuantan by preserving the fish at 4°C on slurry ice. Five grams of fish flesh was used for each treatment with Sacha inchi seed protein and negative control respectively. The mixture for each treatment was packed in zip zip-locked plastic bag and stored under refrigeration at 4°C. The samples were withdrawn at 0, 4, 8, and 12 days of incubation for sensory observation and pH measurement. The sensory attributes were evaluated by observing the odor and texture changes of the fish flesh. The odor of the fish flesh was observed by the presence of strongly fishy, rancid, and putrid odors for each incubation period. Meanwhile, the texture of the soft and spongy fish flesh was recorded for each incubation period. For the pH measurement analysis, 0.5 g of fish flesh was homogenized in 10 mL distilled water and the pH was determined by using a pH meter (Toshiba, Japan).

Data analysis

The analysis was done in duplicate. The data represented the mean of two replicates ± standard deviation (SD) and it was generated using Microsoft Excel 2016. Subsequently, the data were analyzed using SPSS version 27, which involved the application of Analysis of Variance (ANOVA) to identify significant differences for all analyses. A significance level of $P<0.05$ was considered as a statistically significant difference in all data sets.

RESULTS AND DISCUSSION

Antioxidant property

The total phenolic content of the Sacha inchi protein isolates was 463.13 ± 0.51 µg/mL with 73.72% of DPPH scavenging activities. This showed that the protein of Sacha inchi can prevent the oxidation process in food thus reducing the food spoilage process. The antioxidant activities are associated with the ability of the amino acids to donate protons to free radicals in which the protein from Sacha inchi can prevent the oxidative damage to biomolecules in the food products and act as free radical reducing agents, metal ion chelators as well as a free radical scavenger (Petchiammal & Hopper, 2014).

Antimicrobial properties

Sacha inchi protein isolates possessed significant antibacterial activities against *E. coli* and *M. luteus* through the observation of zone of inhibition on the agar plates. The diameter of the zone of inhibition was 30.5 ± 0.75 mm and 33.0 ± 0.00 mm concerning *E. coli* and *M. luteus*. The lower inhibitory concentrations of 46.56 µg/mL and 23.28 µg/mL of Sacha inchi protein were enough to inhibit the growth of *E. coli* and *M. luteus,* respectively. Sacha inchi protein also displayed potential antifungal activities against *C. gloeosporioides*, given by the abilities of the extract to suppress the fungal growth by 1.50 ± 2.12%, 5.85 ± 3.98% and 5.90 ± 1.98% at day 2, 3 and 4 of incubations, respectively which was statistically significant (p<0.05). Sacha inchi protein extracts also had potential antifungal activities against *A. niger* although the activities decreased rapidly over time. Protein extracts suppressed the *A. niger* growth at day-2 and day 3 of incubation by 25.85 ± 7.28% and 8.6 ± 1.22%, respectively, and were statistically different at p<0.05. The formation of a zone of inhibition and inhibitory concentration values of protein highlighted the potential of Sacha inchi as a natural preservative in food products. The presence of some protein populations in the seeds that are positively charged may induce the antibacterial effect (Sitohy & Osman, 2010). For instance, an oligomeric glycinin (11S globulin) contains six subunits that are acidic and positively charged. The presence of a positive charge on protein induces the interaction between protein and negatively charged bacterial membranes; for example, the phospholipids, which then will be disrupted and consequently disrupt the bacterial growth.

Protease inhibitor activities of Sacha inchi seeds protein

Casein agar plates are used in this experiment to visually identify protease inhibitor activities of Sacha inchi protein. The technique entails evaluating the capacity of Sacha inchi seed protein isolates to hinder protease activity by quantifying the inhibition of casein digestion by the trypsin. Figure 1 shows the inhibition of trypsin (0.1 mg/mL) against casein by Sacha inchi seeds' protein isolates. The presence of protease inhibitor resulted in a observable circular area with a reduced diameter of trypsin inhibition zone detected at 1.05 ± 0.07 cm when compared to the positive (1.7 ± 0.00 cm) and negative control (1.9 ± 0.00 cm).

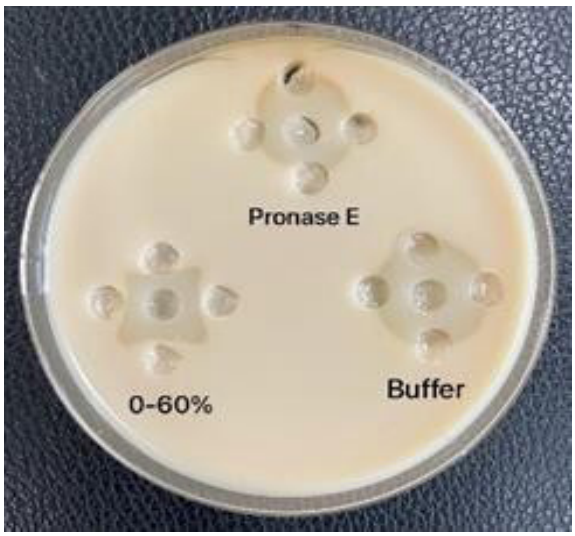

Fig. 1. Protease inhibitory activity of Sacha inchi seed protein on casein agar showing a reduction in zone of inhibition.

A caseinolytic plate assay is a technique employed to screen for protease inhibitor activity and has been utilized in diverse research, including the evaluation of trypsin inhibitory action in plants (Zhang *et al*., 2021). The reduction area of trypsin activity known as the inhibition zone indicates the inhibitory

effect of the potential extracts used on protease action against casein (Mohan *et al*., 2018). The inhibitory activity of Sacha inchi protein shows its potential application as a natural food preservative as the inhibitor can retard several deterioration processes such as protein degradation due to endogenous and exogenous proteases during food processing and preservation. The use of an adequate amount of this natural protease inhibitor will extend the shelf-life of various foods and is known to improve the nutritional quality of food.

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of Sacha inchi seeds' protein at a concentration of 0.2 mg/mL is depicted in Figure 2. The gel separation yielded polypeptides with molecular weights ranging from 10 kDa to 60 kDa. The protein samples exhibited the maximum expression of polypeptides with a mass ranging from 25-35 kDa.

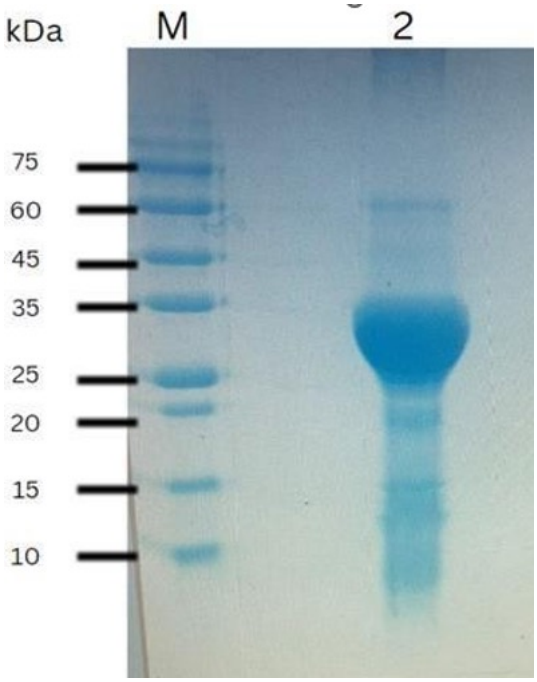

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of Sacha inchi seeds protein (0.2 mg/mL). M, Marker (kDa); Lane 2, Sacha inchi seed protein.

The proteins with molecular weights of 25 kDa exhibited significant band intensity when subjected to decreasing conditions. They would function as subunits of the primary proteins present in Sacha inchi. The findings were consistent with prior research that had analyzed the protein composition of Sacha inchi seeds. Sacha inchi proteins possess the disulfide bond within their structure. Furthermore, it could potentially include significant quantities of amino acids that contain sulfur (Rawdkuen *et al*., 2016). The proteins found in Sacha inchi were at the molecular weights of 63, 35, 17, and 11 kDa (Figure 2). Based on the SDS-PAGE image, this suggests that the protease inhibitor was shown by a protein band of molecular weight size between 25- 35 kDa. This finding aligns with the discovery made by Rawdkuen *et al*. (2018) that Sacha inchi protein hydrolysates, namely those within the size range of 25-35 kDa, demonstrate protease inhibition. The hydrolysates generated by crude papain and calotropis proteases exhibit antioxidant properties such as DPPH inhibition, suggesting their potential as protease inhibitors.

Reverse zymography was conducted by utilizing gel from SDS-PAGE analysis along with gelatin substrate and Sacha inchi protein isolates to investigate the presence of protease inhibitor activity. Figure 3 displayed the reverse zymography pattern of the protease inhibitor in the protein isolates of Sacha inchi seeds. Reverse zymography analysis of Sacha inchi protein treated with 0.1 mg/mL trypsin, revealed the presence of a distinct and prominent band (R1) at the molecular size of 25 kDa, whereas the other bands that were previously shown on SDS-AGE (Figure 2) disappeared after trypsin treatment. Trypsin is an enzyme that breaks down gelatin, except in the regions where inhibitory activity is seen.

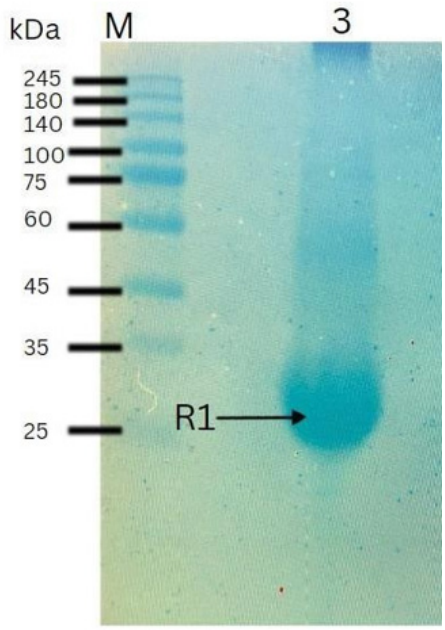

Fig. 3. Reverse zymography of the Sacha inchi seed protein.

Protease inhibitors at the 25 kDa molecular weight exhibited the greatest intensity of bands even after being treated with 0.1 mg/mL of trypsin. The distinct band at this size range was observed on the transparent surface following the Coomassie brilliant blue staining after trypsin digestion showing its protease inhibitory action against trypsin. The protease inhibitor interferes with trypsin activity by forming a 1:1 complex in these bands, preventing the digestion of gelatin (Patricia & Von, 2005). The outcome is the appearance of blue protease inhibitor bands contrastingwith a colorless background. In this study, Sacha inchi seed protein appears as an inhibitors of protease like trypsin. Previous findings indicate that Kunitz-type inhibitors have a molecular mass ranging from 18 to 26 kDa (Bijina *et al*., 2011). These inhibitors are predominantly monomeric or dimeric, with subunits connected by a disulfide bridge. Therefore, this is in agreement with the previous finding that the protease inhibitor obtained from Sacha inchi is a compact protein with molecular weight ranging from 25 to 35 kDa and falls into the Kunitz category of serine protease inhibitors (Rawdkuen *et al*., 2022). The suppression of trypsin digestion on the gelatin substrate by the protein extracts revealed the protease inhibitor presence in Sacha inchi seeds.

Preservation test on raw fish flesh

The pH of the fish flesh with or without protein treatment increased as the storage period increased reflecting the natural process of fish spoilage as presented in Table 1. However, an increment of pH was observed in the fish flesh treated with Sacha inchi seed protein throughout the incubation period of 12 days.

Table 1. pH values of fish flesh during storage at 4°C upon treatment with Sacha inchi protein isolates

Sample	Incubation time (day)			
	0	4	8	12
Control	6.41 ± 0.15	7.56 ± 0.14	8.00 ± 0.2	8.11 ±0.11
Sacha inchi protein	5.96 ± 0.15	7.16 ± 0.15	7.52 ± 0.03	7.85 ± 0.12

The pH of the fish flesh ranged from 6.41 ± 0.15 to 8.11 ± 0.11 post-incubation period in the control treatment. A slower pH incline was observed for the fish flesh treated with Sacha inchi seeds' protein with the pH values ranging from 5.96 ± 0.15 to 7.85 ± 0.12 throughout the incubation duration. This process indicated the preservation of the condition of the fish flesh. This fundamental positive result gives a good indicator of Sacha inchi seeds' protein to be used in the food industry as a bio-preservative. The increasing trend of pH values with the increase in storage period was corroborated with previous studies done by Sarika *et al*. (2019) and Chakchouk-Mtibaa *et al*. (2017) who have studied the bio-

preservative for fish and meat samples, respectively. The increase in pH value corresponded to the protein breakdown which induced the liberation of free amino acids (Chakchouk-Mtibaa *et al*., 2017). These amino acids undergo transamination, deamination, as well as decarboxylation in the cytosome, which then results in the formation of α-keto acids, ammonia, and amines (Zhuang *et al*., 2020). These nitrogenous compounds from the alkaline reaction reduced the quality of the fish samples and altered their physicochemical properties such as texture, moisture distribution, color, and water-holding capacity. According to Chan *et al*. (2022), the myofibrillar proteins at an isoelectric point (pI) value of ~5.5 have a strong protein-protein interaction, which would limit water penetration. The increase in pH during spoilage would lead to the loosening of the protein matrix allowing a greater interaction between water and proteins (Kraft, 1992). Moreover, there were also changes in their sensory attributes including odor and texture. Fish spoilage was typically associated with strongly fishy, rancid, and putrid odors (Tahiluddin *et al*., 2022). This foul odor was the result of the breakdown of components as well as the formation of new compounds in the fish samples (Ghaly *et al*., 2010). According to Zhuang *et al*. (2020), the foul odor comes from the volatile organic compounds that are produced through the decomposition of sulfur-containing amino acids, branched-chain amino acids, and aromatic amino acids. Additionally, Abbas *et al*. (2009) have stated that the fishy off odors might be associated with the degradation of trimethylamine oxide (TMA) that was caused by some spoilage bacteria. The fish sample supplemented with antimicrobial peptide had less fishy and rancid odor compared with the control and fish sample supplemented with Sacha inchi protein isolates after 4 4-day storage period at 4°C. In addition, the tissue of fish flesh became soft and spongy as the storage day increased due to the changes in protein structure and composition which agrees with the findings by Ghaly *et al*. (2010). Based on the observation, fish samples supplemented with Sacha inchi protein isolates remained firm in texture as compared to the control after 4 days of storage period. Other than the biological factors such as the fat and collagen proportion in the fish sample, the microbiological and autolytic processes also contributed to the myofibrillar protein degradation, which consequently led to the reduction of muscle firmness (Tahiludin *et al*., 2022). The color changes might be due to the lipid oxidation that was linked to the color oxidation in fish samples (Chakchouk-Mtibaa *et al*., 2017). Moreover, it was reported that the formation of metmyoglobin resulting from myoglobin oxidation was the major cause of the decrease in the redness of the fish flesh.

CONCLUSION

In conclusion, a protein isolated from Scha inchi seeds has shown its capacity as a bio-preservative agent in reducing fish flesh spoilage process when tested through the antioxidant capacity test, antibacterial activity test, proteolysis inhibitory test as well, and preservative test on raw fish flesh. From this study, it is demonstrated that the seeds' protein was recorded with total phenolic compounds that give positive scavenging activities in DPPH inhibition. Meanwhile, antimicrobial tests revealed that the seeds' protein has antibacterial and antifungal properties when tested on the selected food spoilage pathogens namely *E. coli*, *M. luteus*, and *C. gloeosporioides.* Meanwhile, the antibacterial and antifungal inhibitory concentration percentage (IC%) suggested that Sacha inchi protein extract demonstrated concentration-dependent antibacterial activity. In the proteolysis test by caseinolytic assay, the proteins have potent inhibitory activity against the trypsin enzyme, one of the destructive endogenous enzymes involved in the food spoilage process. The SDS-PAGE unveiled the seed protein exhibiting molecular weights ranging from 10 kDa to 60 kDa. Following reverse zymography, it is revealed that a prominent protein band at the molecular weight size of 25 kDa was observed on the transparent surface following the Coomassie brilliant blue staining after trypsin digestion. The suppression of trypsin digestion on the gelatin substrate in the acrylamide gel by the protein extracts revealed the presence of protease inhibitors in Sacha inchi seeds. This study contributes valuable insights into the potential application of Sacha inchi seed protein isolates as a natural and sustainable alternative to synthetic preservative agents in the food industry. The findings hold promise for developing novel, eco-friendly strategies to combat food spoilage and enhance the shelf life of various food products.

ACKNOWLEDGEMENTS

We would like to thank the Kulliyyah of Science, IIUM for providing the facilities. This study was funded by the IIUM-UMP-UiTM Research Collaboration Grant (SRCG20-048-0048).

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abbas, K.A., Saleh, A.M., Mohamed, A. & Lasekan, O. 2009. The relationship between water activity and fish spoilage during cold storage: A review. Journal of Food, Agriculture and Environment, 7(3/4): 86-90.

Bacha, A.B., Jemel, I., Moubayed, N.M.S. & Abdelmalek, I.B. 2017. Purification and characterization of a newly serine protease inhibitor from Rhamnus frangula with potential for use as therapeutic drug. 3 Biotech, 7(2):148. https://doi.org/10.1007/s13205-017-0764-z

Bijina, S.C., Basheer, S.M., Elyas, K.K., Bahkali, A.H. & Chandrasekaran, M. 2011. Protease inhibitor from Moringa oleifera leaves: Isolation, purification, and characterization. Process Biochemistry, 46(12): 2291- 2300. https://doi.org/10.1016/j.procbio.2011.09.008

Chakchouk-Mtibaa, A., Smaoui, S., Ktari, N., Sellem, I., Najah, S., Karray-Rebai, I. & Mellouli, L. 2017. Biopreservative efficacy of bacteriocin BacFL31 in raw ground Turkey meat in terms of microbiological, physicochemical, and sensory qualities. Biocontrol Science, 22(2): 67-77. https://doi.org/10.4265/bio.22.67

Chan, S.S., Roth, B., Jessen, F., Jakobsen, A.N. & Lerfall, J. 2022. Water holding properties of Atlantic salmon. Comprehensive Reviews in Food Science and Food Safety, 21(1): 477-498. https://doi.org/10.1111/1541-4337.12871

Chen, Z., Wang, J., Liu, W. & Chen, H. 2017. Physicochemical characterization, antioxidant and anticancer activities of proteins from four legume species. Journal of Food Science and Technology, 54(4): 964-972. https://doi.org/10.1007/s13197-016-2390-x

Ghaly, A.E., Dave, D., Budge, S. & Brooks, M.S. 2010. Fish spoilage mechanisms and preservation techniques. American Journal of Applied Sciences, 7(7): 859-877. https://doi.org/10.3844/ajassp.2010.859.877

Jaafar, N.F., Ramli, M.E. & Mohd Salleh, R. 2020. Optimum extraction condition of *Clitorea ternatea* flower on antioxidant activities, total phenolic, total flavonoid and total anthocyanin contents. Tropical Life Sciences Research, 31(2): 1-17. https://doi.org/10.21315/tlsr2020.31.2.1

Jang, M., You, S. & Kim, G-H. 2020. Evaluation of antibacterial and antioxidant activities of Sacha inchi (*Plukenetia volubilis* L.) leaf from Myanmar. Korean Journal of Food Preservation, 27: 781-790. https://doi.org/10.11002/kjfp.2020.27.6.781

José, D.L.S., Ma., G.J., Rafael, S. & Joel, E.L. 2009. Root extracts from Mexican avocado (*Persea americana* var. *drymifolia*) inhibit the mycelial growth of the oomycete *Phytophthora cinnamomi*. European Journal of Plant Pathology, 124: 595-601. https://doi.org/10.1007/s10658-009-9446-y

Karanth, S., Feng, S., Patra, D. & Pradhan A.K. Linking microbial contamination to food spoilage and food waste: the role of smart packaging, spoilage risk assessments, and date labeling. Frontiers in Microbiology, 14: 1198124. https://doi.org/10.3389/fmicb.2023.1198124

Kraft, A. A. 1992. Psychotropic Bacteria in Foods: Disease and Spoilage. CRC Press, Boca Raton, Florida. 288 pp. https://doi.org/10.1201/9781466593503

Lemus-Conejo, Ana, Alvaro Villanueva-Lazo, Maria E. Martin, Francisco Millan, and Maria C. Millan-Linares. 2024. Sacha inchi (*Plukenetia volubilis* L.) protein hydrolysate as a new ingredient of functional foods. Foods,13: 2045. https://doi.org/10.3390/foods13132045

Manandhar, S., Luitel, S. & Dahal, R.K 2019. In vitro antimicrobial activity of some medicinal plants against human pathogenic bacteria. Journal of Tropical Medicine. https://doi.org/10.1155/2019/1895340

Mohan, M., Kozhithodi, S., Nayarisseri, A. & Elyas, K.K. 2018. Screening, purification and characterization of protease inhibitor from *Capsicum frutescens*. Bioinformation,14(6): 285-293. https://doi.org/10.6026/97320630014285

Odeyemi, O. A., Alegbeleye, O. O., Strateva, M. & Stratev, D. 2020. Understanding spoilage microbial community and spoilage mechanisms in foods of animal origin. Comprehensive Reviews in Food Science and Food Safety, 19(2): 311- 331. https://doi.org/10.1111/1541-4337.12526

Onyeaka, H., & Nwabor, O. 2022. Food Spoilage - an overview. In: Food Preservation and Safety of Natural Products. H.N. Onyeaka and O.F. Nwabor (Eds.). Academic Press, United Kingdom. 272 pp. https://doi.org/10.1016/B978-0-323-85700-0.00005-8

Patricia A.M.S.B. & Johannes W.V.H. 2005. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. BioTechniques, 38:73-83. https://doi.org/10.2144/05381RV01

Pellissery, A. J., Vinayamohan, P. G., Amalaradjou, M. A. R. & Venkitanarayanan, K. 2020. Spoilage bacteria and meat quality. In: Meat Quality Analysis. A.K. Biswas and P.K. Mandal (Eds.). Academic

Press. pp. 307-334. https://doi.org/10.1016/B978-0-12-819233-7.00017-3

Quinteros, M., Vilcacundo, R., Carpio, C. & Carrillo, W. 2016. Digestibility and anti-inflammatory activity in vitro of sacha inchi (*Plukenetia volubilis* L.) proteins. Asian Journal of Pharmaceutical and Clinical Research, 9(3): 303-306.

Rawdkuen, S., D'Amico, S. & Schoenlechner, R. 2022. Physicochemical, functional, and in vitro digestibility of protein isolates from Thai and Peru sacha inchi (*Plukenetia volubilis* L.) oil press-cakes. Foods , 11:1869. https://doi.org/10.3390/foods11131869

Rawdkuen, S., Murdayanti, D., Ketnawa, S. & Phongthai, S. 2016. Chemical properties and nutritional factors of pressed-cake from tea and sacha inchi seeds. Food Bioscience15: 64-71. https://doi.org/10.1016/j.fbio.2016.05.004

Rawdkuen, S., Nurdalila, R. & Suttiporn P.S. 2018. Characterization of sacha inchi protein hydrolysates produced by crude papain and calotropis proteases. LWT (Food Science and Technology), 96: 18-24. https://doi.org/10.1016/j.lwt.2018.08.008

Sarika, A.R., Lipton, A.P. & Aishwarya, M.S. 2019. Biopreservative efficacy of bacteriocin GP1 of *Lactobacillus rhamnosus* GP1 on stored fish filets. Frontiers in Nutrition. 6: 29. https://doi.org/10.3389/fnut.2019.00029

Shirazi, O.U., Khattak, M.M.A.K., Shukri, N.A. M. & Nasyriq, M.N. 2014. Determination of total phenolic, flavonoid content and free radical scavenging activities of common herbs and spices. Journal of Pharmacognosy and Phytochemistry, 3(3): 104-108.

Siaw, F. 2022. Sacha inchi, a superfood that could help Malaysia's economy [WWW Document] Rise Malaysia.com. URL https://risemalaysia.com.my/sacha-inchi-nutribah/8964/ (accessed 9.6.23).

Singh, J., Singh, B. (2020) Inhibition of post-mortem fish muscle softening and degradation using legume seed proteinase inhibitors. Journal of Food Science and Technology, 57: 1-11. https://doi.org/10.1007/s13197-019-04044-6

Singh, V. P. 2018. Recent approaches in food bio-preservation-a review. Open Veterinary Journal, 8(1): 104-111. https://doi.org/10.4314/ovj.v8i1.16

Sitohy, M. & Osman, A. 2010. Antimicrobial activity of native and esterified legume proteins against Gram-negative and Gram-positive bacteria. Food Chemistry, 120(1): 66-73. https://doi.org/10.1016/j.foodchem.2009.09.071

Tahiluddin, A., Marıbao, I., Amlani, M. & Sarri, J. 2022. A review on spoilage microorganisms in fresh and processed aquatic food products. Food Bulletin. 1: 21-36. https://doi.org/10.29329/foodb.2022.495.05

Tehrani, M.H.H., Fakhrehoseini, E., Nejad, M.K., Mehregan, H. & Mojdeh, H-V. 2012. Search for proteins in the liquid extract of edible mushroom, Agaricusbisporus, and studying their antibacterial effects. Iranian Journal of Pharmaceutical Research,11: 145-50.

Wang, S., Fang, Y. & Kakuda, Y. 2018. Sacha inchi (*Plukenetia volubilis* L.): Nutritional composition, biological activity, and uses. Food Chemistry, 265: 316- 328. https://doi.org/10.1016/j.foodchem.2018.05.055

Zhang, X., Yao Shuai, Han, T., Li, C., & He, L. 2021. Novel method for the quantitative analysis of protease activity: the casein plate method and its applications. ACS Omega, 6(5): 3675-3680. https://doi.org/10.1021/acsomega.0c05192

Zhuang, S., Hong, H., Zhang, L. & Luo, Y. 2020. Spoilage-related microbiota in fish and crustaceans during storage: Research progress and future trends. Comprehensive Reviews in Food Science and Food Safety, 20(1): 252- 288. https://doi.org/10.1111/1541-4337.12659