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ORIGINAL ARTICLE

Extraction and characterization of proteins from banana (*Musa Sapientum L*) flower and evaluation of antimicrobial activities

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Abstract Ultrasonic assisted alkaline extraction of protein from banana flower was optimized using response surface methodology. The extracted proteins were characterized by Fourier transform infrared spectroscopy and molecular weight distribution was determined by gel electrophoresis. The maximum protein yield of 252.25 mg/g was obtained under optimized extraction conditions: temperature 50 °C, 30 min extraction time and 1 M NaOH concentration. The alkaline extraction produced a significantly high protein yield compared to enzymatic extraction of banana flower. Chemical finger printing of proteins showed the presence of tyrosine, tryptophan and amide bonds in extracted protein. Alkaline and pepsin assisted extracted banana flower proteins showed characteristic bands at 40 and 10 kDA, respectively. The extracted proteins showed antibacterial effects against both gram positive and gram negative bacteria. The high protein content and antimicrobial activity indicate the potential applications of banana flower in the food and feed industry.

Keywords Banana flower protein · Alkaline extraction · SDS-PAGE · FPLC

Introduction

The banana (*Musa sapientum* L) is a typical climacteric fruit and considered as one of the most important staples in tropical regions. In 2014, 126 million tons of banana were produced in more than 150 countries, making bananas and plantains the tenth most important global food crop and export commodity (FAOSTAT 2014). All banana plant parts have been reported to have medicinal values (Singh et al. 2016; Soorianathasundaram et al. 2016). Banana flower is byproduct of banana plantain and being normally consumed as vegetables in many parts of the world. In commercial harvesting, one bunch of banana produces one bell which contains several flowers. Despite having high nutritional values, these flowers have only been used as organic materials and bio-fertilizer in plantation (Mohapatra et al. 2010).

The demand of the products containing functional components has been increased immensely in recent years. Food waste and agro-food industrial by products contain various functional and bioactive compounds including phenolics, antioxidants, dietary fibers, flavonoids, anthocyanins, proteins, peptides, enzymes etc. (Jayathilakan et al. 2012; Waldron 2009) The recovery of bioactive compounds and reutilization is possible in food chain as additives, supplements, fortification or as minor ingredients (Baiano 2014; Roselló-Soto et al. 2015; Smithers 2008).

Plant proteins are of higher interest in food, feed, pharmaceuticals and cosmetic industries (Kitts and Weiler 2003). Banana blossoms have commercially been produced as canned banana flower in brine, dehydrated vegetable, pickle and pharmaceutical products (Wickramarachchi and Ranamukhaarachchi 2005). In addition, banana flower is rich source of dietary fiber, magnesium, iron, copper, antioxidant compounds, flavonoids and dopamine (Bhaskar

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et al. 2011; Jamuna and Nandini 2014; Kang et al. 2014; Ramu et al. 2014). Plant proteins lack some of the essential amino acids unlike animal protein, so it is advisable to consume a mixture of plant proteins to meet the nutritional requirement (Young and Pellett 1994). The banana flower contains high quality proteins because of its well-balanced composition, including essential amino acids leucine, lysine and threonine in amounts greater than 30 mg/g of protein (Sheng et al. 2010). Valorization of banana flowers provides the additional benefits in reducing the banana waste and also act as source of valuable compounds in preparation of functional foods.

Plants have always been a good source of antimicrobial compounds and considered to be safe for human consumption (Sadiq et al. 2017; Tam et al. 2015). Some peptides of plant origin can selectively inhibit the growth of gram positive or negative bacteria, while others can suppress the growth of fungi and viruses (Reddy et al. 2004; Shai 2002). The plant derived peptides possess therapeutic properties including, anticancer, anti-oxidative, chitinase and proteinase inhibitory activities (Hoskin and Ramamoorthy 2008; Sinha et al. 2014). Antimicrobial peptides tend to have direct effects on microorganisms such as, damaging or destabilizing the bacterial, viral or fungal cell membrane (Shai 2002). The objective of this study was to optimize the extraction of proteins from banana flower and characterization of extracted proteins for molecular weight distribution and antimicrobial potential.

Materials and methods

Materials

Banana flowers were obtained from a local market in Pathumthani province, Thailand. All the chemicals used were of analytical grade and used without further purification. Sodium hydroxide pellets (NaOH), hydrochloric acid (HCl), ammonium sulphate, ethanol (99% v/v), were purchased from Merck chemicals co ltd. UK. Acrylamide/Bis Solution (40%), 10X Tris Buffer, Tris hydrochloride, ammonium persulphate, Tricine Sample Buffer, SDS-PAGE low molecular weight standards were supplied by BIO-RAD, USA. Coomassie Blue G-250 dye and sodium dodecyl Sulphate were purchased from Carlos Ebra chemicals, NSW, Australia.

Sample preparation

Banana flowers were cleaned, cut into small pieces and dried in oven at 40 °C for 2 days. The dried banana flowers were firstly grinded in a mechanical grinder (Philips Co. Ltd., China) and made powder using mortar and pestle. The powder sample was separated by sieve to particle size (50 μm) and stored at 4 °C until the use.

Ultrasonic assisted alkaline extraction

The alkaline extraction of protein from banana flower was performed by using the method described by Vanvi and Tsopmo (2016) with slight modifications. The powdered banana flower (1 g) was soaked into 10 ml of NaOH (0.5, 1.0 and 1.5 M) for 30 min. The sample solutions were then exposed to ultrasonic waves using a probe ultrasonic reactor (UP200S, 200W, Hielscher, Teltow, Germany) having a fixed frequency of 24 kHz. Ultrasonication temperature and time were controlled for optimum output of protein. The samples were then centrifuged (Centrikon T-324, Germany) at 10,000 rpm for 30 min to remove the suspended particles. Protein concentration in the supernatant was determined by Bradford method (Bradford 1976).

Experimental design for alkaline extraction

The extraction of proteins from banana flower was optimized using response surface methodology (RSM) with a three level Box-Behnken experimental design (BBD). For ultrasonic assisted extraction, the effect on protein yield (Y) was observed in relation to the three independent variables including X_1 (extraction temperature; 40, 50 and 60 °C), X_2 (Extraction time; 15, 30, 45 min) and X_3 (NaOH concentrations; 0.5, 1 and 1.5 M). The quadratic model of response surface is shown in Eq. 1.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_{ij} \quad (1)$$

where X_1 = extraction temperature (°C), X_2 = Extraction time (sec) and X_3 = NaOH concentration (M).

Enzymatic extraction of banana flower protein

The enzyme assisted extraction of banana flower was carried out by following the method of Ngoh and Gan (2016), with slight modifications. The powdered sample (1 g) was added to a flask containing 10 ml of enzymatic solution (pepsin, 0.1% w/v, prepared in 0.02 M HCl). The sample was then incubated in a shaking incubator (Gallenkamp, UK) at 200 rpm and 40 °C for 6, 12 and 24 h followed by centrifugation at 10,000 rpm for 30 min. The supernatant was collected for further analysis.

Purification of banana flower protein

The collected raw protein solution from alkaline extraction and enzyme extractions were precipitated by centrifugation at 6000 rpm for 20 min in the presence of ammonium sulphate at 50% saturated salt. The precipitated protein was washed with acetone and collected protein was then passed through dialysis membrane (1000 Dalton molecular weight cut off; Spectrum Laboratories, California, USA) against distilled water for 24 h (Zhang et al. 2016). Distilled water was changed for every 2 h until the conductivity of water phase remained constant. The dialyzed solution was freeze dried (Scanvac Cool Safe 55-4, Denmark) at − 55 °C for 24 h to obtain the lyophilized protein powder.

Characterization of extracted protein

Chemical finger printing of protein

A Fourier Transmission Infra-Red (FT-IR) spectrometer (Perkin Elmer, USA) with a middle range infrared light source (4000–500 cm^{-1} wavenumbers) was used to analyze the chemical finger-printing of banana flower protein. The protein sample was grounded with potassium bromide (KBr) to a fine powder, placed under high pressure with compression dye until formation of a pellet and then evaluated by FTIR (Sadiq et al. 2015). The FTIR spectra for both banana flower protein and standard bovine serum albumin (BSA, BIO-RAD, USA) were compared for chemical finger printing.

Size exclusion chromatography of banana flower protein

Size exclusion chromatography was performed using a fast protein liquid chromatography (FPLC) system with an UV detector at 280 nm (Wang and Ng 2006). The sample (1 ml) was injected into the column (Superdex 75 HR 10/30 column, Amersham Biosciences) and eluted with a mobile phase of 0.05 M Tris–HCl buffer (pH 9.0) at a flow rate of 0.25 ml/min. Each separated fraction was collected separately in 1.5 ml micro centrifuge tubes and analyzed on SDS-PAGE for molecular weight characterization.

Determination of the molecular weight of extracted protein using sds-page

The molecular mass of extracted protein was estimated by using SDS-PAGE system, using pre-cast gels (Mini PROTEAN, TGX, 12% BIORAD, California, USA) following the method of Zhang et al. (2007), with some modifications. Protein sample was mixed with laemmli sample buffer and β-mercaptoethanol in 1:1 ratio and heated up to boiling temperature for 5 min. Electrophoresis was performed in vertical gels in a Mini-Protean II cell (BIO-RAD Laboratories, Richmond, CA, USA) at 200 V for 45 min. After electrophoresis, the gel was cut in two vertical parts. One part was fixed and stained with Coomassie brilliant blue R-250 (1 g/L) in 50% v/v methanol and 10% v/v acetic acid for 20 min and then de-stained with 10% glacial acetic acid and 5-10% v/v methanol. Molecular mass was determined by comparison with broad range MW standard Markers (2–250 kDa) (BIO-RAD).

Antimicrobial effects of banana protein

The agar well-diffusion method was used to evaluate the antibacterial effects of banana flower proteins following the methods as described by (Sadiq et al. 2015), with some modifications. Each inoculum was adjusted to 0.5 McFarland standard and spread with the help of sterilized cotton swab on the surface of Muller Hilton Agar (MHA) (Himedia, India) plates. Wells of 8 mm in diameter were made on each plate with the help of sterilized cork-borer and 100 µl of different concentrations (5, 10 and 25 mg/ml) was introduced into the wells by using micropipette. Tetracycline (30 µg/ml) was used as a positive control whereas, polyethylene glycol was used as negative control. The plates were incubated for 24 h at 37 °C and results were interpreted by measuring the diameter of inhibition zone (mm) around the wells.

Data analysis

All experiments were carried out in triplicates and results are expressed as mean values with standard deviation (± SD) of three replicates. One-way analysis of variance (ANOVA) and Tukey's HSD tests were carried out to determine significant group differences ($p < 0.05$) between samples by using SPSS statistical software package (SPSS, version 22.0, USA).

Results and discussion

Alkaline extraction

The effect of extraction temperature, extraction time and alkali (NaOH) concentration on the protein yield of banana flowers are shown in Table 1. Protein yield ranged from 125.55 to 252.25 mg/g of dried crude banana flower. The variations in protein content by changing the extraction parameters indicated that extraction temperature, time and NaOH concentration greatly affected the protein yield (Hiranrangsee et al. 2016; Jain and Anal 2016). The maximum protein yield of 252.25 mg/g was obtained at a

Table 1 Box–Behnken experimental design with experimental yield of protein obtained by ultrasonic assisted extraction from banana flower (yield values are mean ± SD)

Run	Temperature (°C)	Time (min)	NaOH (M)	Protein (mg/g dry matter)
1	60	30	1.5	163.19 ± 2.00
2	40	30	0.5	172.72 ± 0.77
3	40	15	1	125.55 ± 1.53
4	60	45	1	228.51 ± 0.81
5	50	15	0.5	191.59 ± 1.62
6	60	15	1	144.12 ± 1.27
7	40	30	1.5	163.19 ± 1.75
8	50	30	1	231.96 ± 2.58
9	50	15	1.5	175.36 ± 2.24
10	60	30	0.5	186.92 ± 1.73
11	50	30	1	252.25 ± 3.37
12	50	45	0.5	228.51 ± 4.94
13	50	45	1.5	200.01 ± 2.00
14	50	30	1	251.25 ± 2.21
15	40	45	1	172.93 ± 1.85

temperature of 50 °C, 30 min extraction time and 1 M of NaOH concentration.

The results obtained after running the trials of Box-Behnken experimental design were fitted to a factorial equation to explain the dependence of protein yield. The value of R^2 was close to 1 (0.9325) which indicated the accuracy and well fit of polynomial model. Multiple regression equation was obtained from the polynomial model that represents the relationship between response and independent variables as shown in Eq. 2.

$$\begin{aligned} Protein = & -869.79 + 37.47\,A + 5.10\,B + 152.77\,C \\ & + 0.16\,AB + 2.69\,AC - 1.09\,BC - 0.42\,A^2 \\ & - 0.16\,B^2 - 126.14\,C^2. \end{aligned} \quad (2)$$

The contour or three-dimensional response surface plots were generated by the regression model and interactive effects of independent variable on response were determined as shown in Fig. 1.

Both the extraction time and NaOH concentration displayed a significantly quadratic effect on the protein yield in the response surface and contour plots. The extraction time demonstrated a linear increase of the response when the time of extraction was between 25 and 45 min. The prediction obtained from statistical analysis showed that the extraction temperature, extraction time and NaOH concentration had positive linear effect and negative quadratic effect on the protein yield.

At industrial scale, UAE gains worthwhile economic benefits due to improved extraction efficiency and extraction rate. The equipment used for UAE is commercially viable and scalable at industrial level (Vilkhu et al. 2008). UAE leads to reproducible food processes that can be completed in seconds or minutes with high reproducibility, reduced processing cost, improved purity of final product and consuming only a fraction of energy compared to conventional extraction methods (Chemat et al. 2017). In various previous studies, e.g. rice bran protein extraction (Chittapalo and Noomhorm 2009), tea leaves aroma extraction (Xia et al. 2006), antioxidants from mung beans (Lai et al. 2010) it was reported that ultrasonic treatment improved the yield significantly. Two main types of physical phenomena involved in ultrasonic assisted extraction are cavitation produced in the solvent by the passage of an ultrasonic wave and diffusion through the cell walls (Li et al. 2007). The temperature showed variable effects on protein yield from banana flower. Initially protein content was increased with increase in the temperature (40–50 °C), followed by decrease at 60 °C for all extraction treatments. The maximum protein yield (252.25 mg/g) was obtained when extraction was carried out at a temperature of 50 °C. The high yield of protein from banana flower indicated it as a good source of proteins, which are mainly located in the florets of banana flower. Sheng et al. (2010) reported that banana flower contained high concentration of proteins (1.62–2.07 g/100 g). They reported that the essential amino acid content in banana flower was about 39–45%, mainly glycine, leucine, alanine, and aspartic acid. The high alkaline concentration helps to break down the hydrogen bonds which leads to cell wall swelling and release of more pectinase. This weakens the cellulose and polysaccharides in the cell wall thus making extraction more efficient (Vilkhu et al. 2008).

Enzyme assisted protein extraction

Figure 2 illustrates the influence of enzymes on yield of extracted protein. The maximum protein yield (102.98 mg/g, dry matter) was obtained after 6 h of incubation with pepsin. However, the protein yield was slightly decreased after 12 and 24 h of enzymatic extraction that was due to excess enzymatic hydrolysis resulting in decrease of protein content (Gani et al. 2015). Palm kernel expeller protein extraction by enzyme pepsin resulted in higher yield up to 6 h, followed by the decrease with increasing time (Ng and Mohd Khan 2012). The enzyme assisted extraction of proteins from banana flower yield was significantly higher than the protein content when compared to extractions assisted by 0.02 M HCl and distilled water separately.

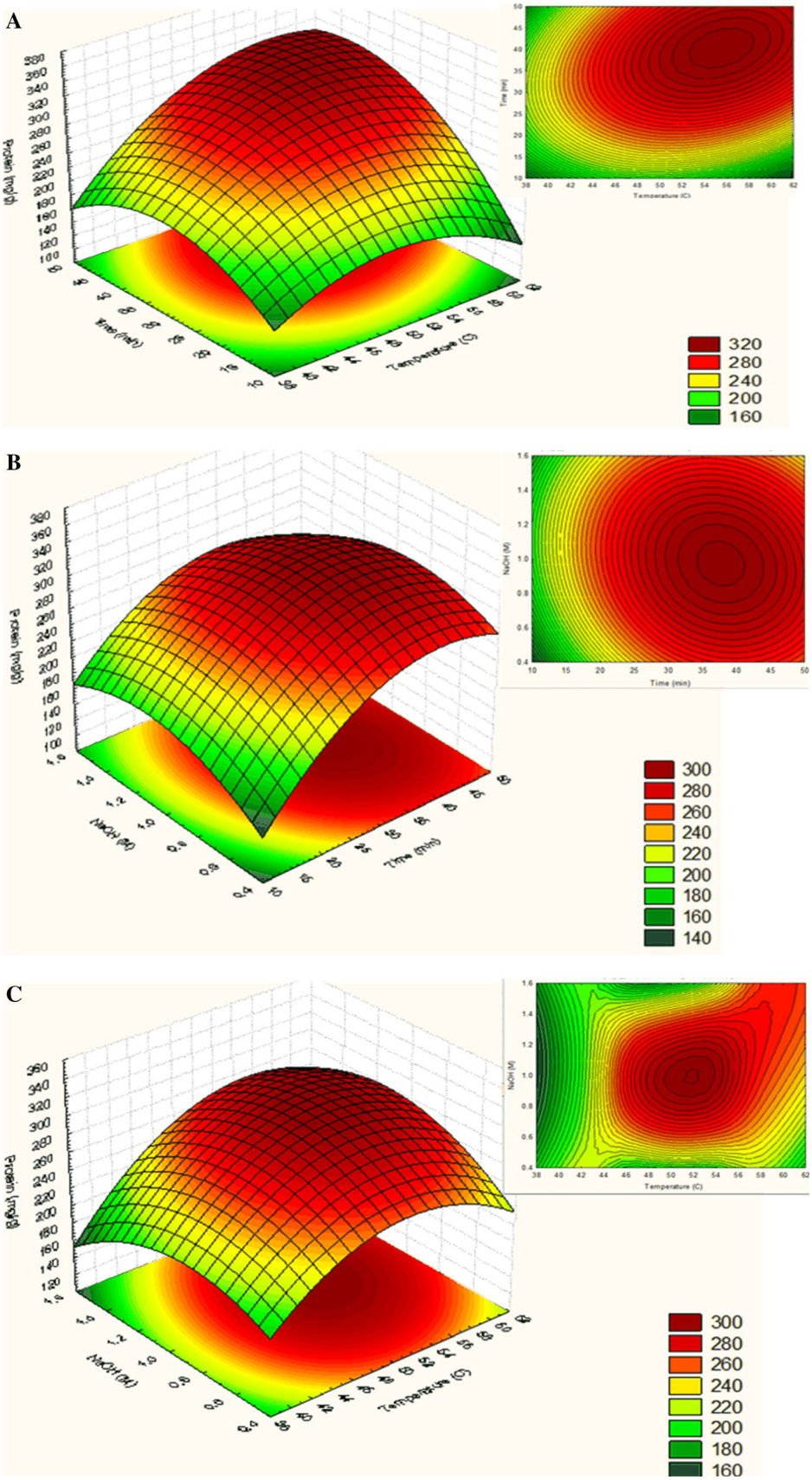

Fig. 1 Response surface plots of protein yield (mg/g); **a** as a function of temperature (°C) and time (min), **b** temperature (°C) and alkali concentration (M) and **c** extraction time (min) and alkali concentration (m)

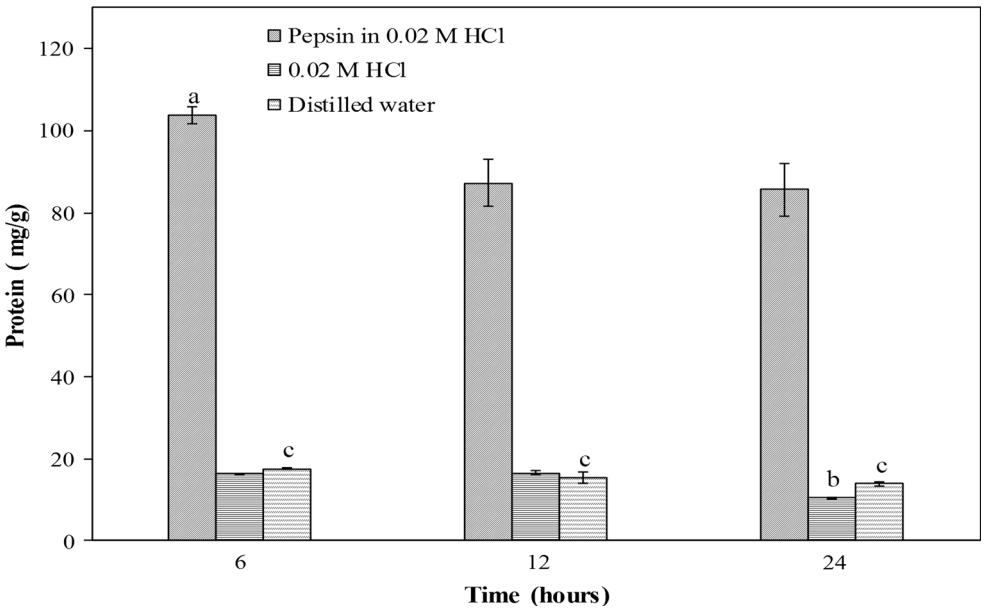

Fig. 2 Effect of extraction time and treatment condition on the banana flower protein extraction using pepsin in 0.02 M HCl, 0.02 M HCl and distilled water as extraction solvents. The letters (a and b) indicate means that are significantly ($p \leq 0.05$) different compared to all other means at same treatment condition), the letter "c" indicates the means that are not significantly different from each other at same treatment condition

Characterization of protein

The FTIR spectrum was used to identify the functional groups present in banana flower protein based on the peak values in the region of infrared radiation. The FTIR spectra of protein and assignment of bands to functional groups are shown in Fig. 3. The functional groups were assigned to its corresponding peaks in accordance with the results shown by Barth (2000). The functional groups corresponding to amino acids Tyr-OH, COOH (1175–1250 cm^{-1}), Gln, COO- (1475–1565 cm^{-1}), Tyr (1616 cm^{-1}), Trp (1340–1360 cm^{-1}) and Gln, NH_2 (1565–1655 cm^{-1}) were found in the banana flower protein. The FTIR spectrum of banana flower protein was compared with the standard BSA protein (dotted line for BSA). The characteristic absorption bands for tyrosine and tryptophan amino acid were found by at 1616 and 1340–1360 cm^{-1}, respectively. This result was in agreement with (Sheng et al. 2010) who reported the significant presence of tyrosine (51.8 mg/g) and tryptophan (31.2 mg/g) in banana flower. Tyrosine has been most intensely studied due to its interesting properties as it may take part in proton and electron transfer reaction (Dollinger et al. 1986).

Figure 3 also shows the observed amide I and amide II bands which was comparable to bovine serum albumin spectrum. Both trends demonstrated that the amide I and amide II are present in FTIR spectrum at 1600–1690 and 1480–1575 cm^{-1} respectively. Parmar et al. (2017) presented the secondary structure of kidney bean proteins by infrared analysis of amide 1-region (1600–1700 cm^{-1}) that corresponded to α- helix, β-sheet, β-turn and anti-parallel β-sheet. FTIR spectrum of amide I component of banana

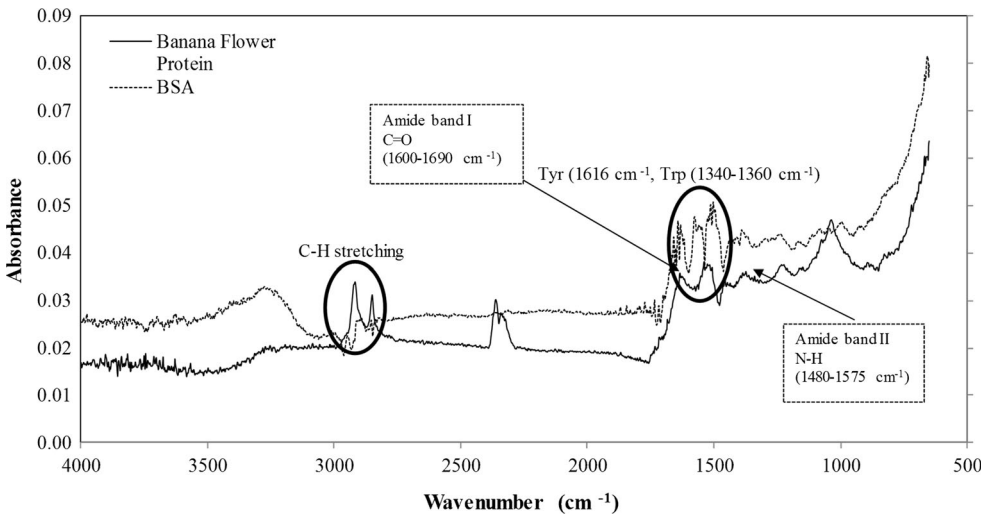

Fig. 3 FTIR finger printing of standard BSA (Bovine serum albumin) and banana flower protein after FPLC (Fast protein liquid chromatography) purification

flower protein revealed four major Gaussian bands centered at ~ 1635, ~ 1653, ~ 1664 and ~ 1683 cm^{-1} corresponding to β-sheet, α-helix, β-turn and anti-parallel β-sheets (β-A) conformations, respectively (Shevkani et al. 2015). The amide I bands arise due to stretching vibrations of the C=O bond of the peptide group (Ghumman et al. 2016). Moreover, the absorption associated with the amide II band primarily leads to bending vibrations of the N–H bonds (Kong and Yu 2007).

Protein purification and molecular weight characterization by SDS-PAGE

Protein fractions were separated based on their molecular weight (Fig. 4). All the fractions collected by FPLC purification were analyzed by SDS-PAGE. All the fractions (B2, B3, B5, B6, B7, B8 and B9) collected by FPLC showed characteristic bands at 40 kDa. Faint protein bands were observed for protein fraction obtained by pepsin assisted extraction at 10 kDa as shown in lane P1 and P2. The bands at low molecular weight from pepsin assisted extraction were due to excessive hydrolysis of proteins by enzyme (Berg et al. 2002).

Antimicrobial activity of different extracted protein from banana flower

Antibacterial activities of banana flower protein were evaluated by well-diffusion method using Mueller–Hinton agar medium. The results were interpreted in terms of diameter of inhibition zone. Alkaline extract of banana flower showed significant antimicrobial activity against gram positive and negative bacterial. *Staphylococcus aureus* was more sensitive to extracted protein than *Escherichia coli* at lower tested concentrations (5 and 10 mg/ml). The extract was effective against *Escherichia coli* at a concentration of 25 mg/ml with an inhibition zone of 14.30 ± 1.83 mm whereas, at 5 and 10 mg/ml there was no inhibition for *E. coli.* The banana flower protein extract showed inhibition diameter of 12.70 ± 0.56, 11.64 ± 1.06 and 8.84 ± 0.21 mm against *Staphylococcus aureus* at concentrations; 25, 10 and 5 mg/ml respectively.

The antimicrobial activity of proteins is related to bacterial lysis of the pathogen. The way antimicrobial peptides interact with the prokaryotic cell membrane has been extensively investigated (Phoenix et al. 2013). Since most antimicrobial peptides/proteins are cationic at physiological pH, they are prone to interact quite un-specifically through electrostatic interactions, with negatively charged molecules in the bacterial cell membrane. In Gram-negative bacteria those negatively charged molecules are lipopolysaccharide while in Gram positive bacteria these molecules are lipoteichoic acid or teichoic acid. Gram-positive bacteria have also been shown more susceptible than Gram negative bacteria, probably due to the lack of an outer membrane (Phoenix et al. 2013).

Conclusion

Bana flower is considered to be a byproduct of banana fruit harvesting. Valorization of banana flower yield high protein content. Fast protein liquid chromatography and chemical finger printing of banana flower extract indicated the presence of various peptides and amino acids. The isolated proteins were found to have significant

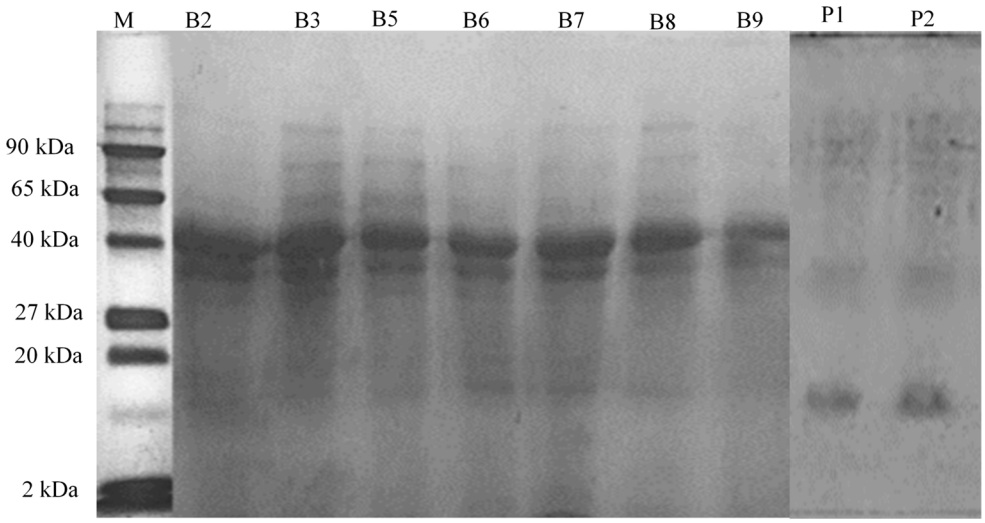

Fig. 4 SDS page profile of different fractions of banana flower protein after purification by FPLC (Fast protein liquid chromatography) and enzyme assisted extraction (M = standard protein marker, B2, B3, B5…B9 = different fractions obtained from FPLC, P1, P2 = proteins obtained by enzymatic extraction)

antibacterial potential against gram positive and gram negative bacteria. Thus, valorization of banana flower is an alternate approach to produce functional proteins and peptides for food and feed industries.

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