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PROTEIN RECOVERY FROM BARBADOS GOOSEBERRY (*Pereskia Aculeata* MILLER) LEAVES BY SALTING OUT AND ISOELECTRIC PRECIPITATION

RECUPERACIÓN DE PROTEÍNAS DE LA HOJAS DE ENREDADERA LIMÓN (*Pereskia Aculeata* MILLER) POR REMOCIÓN DE SALES Y PRECIPITACIÓN ISOELÉCTRICA

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Abstract

This study evaluated the protein recovery from extract of Barbados gooseberry leaves by the synergistic effect of salting out and isoelectric precipitation using a factorial design, varying salt type ($(NH_4)_2SO_4$, NaCl and KCl), salt concentration, and temperature. Temperature elevation increased salting out effect. The optimum salt concentration was 0.5 mol/L for $(NH_4)_2SO_4$ (%PP 69%), 1.5 mol/L for KCl (%PP 56%), and 2.5 mol/L for NaCl (%PP 61%). Research on isoelectric precipitation were performed. The most effective condition was found at 85 °C, using $(NH_4)_2SO_4$ at 0.8 mol/L to 1.0 mol/L and pH = 1.0. Results showed that temperature, salt type and salt concentration as well as the pH had effect on proteins precipitation from the crude BGB leaves extract by the synergistic effect of salting out and isoelectric precipitation. The medium conditions used in the experiments were capable of promoting high protein precipitation, what can be used as an industrial alternative to manufacturing protein concentrates.

Keywords: Barbados gooseberry, mucilage, ora-pro-nobis, protein concentrate, salting out extraction.

Resumen

Este estudio evaluó la recuperación de proteínas del extracto de hojas de enredadera limón por el efecto sinérgico de la precipitación de proteínas por sales y la precipitación isoeléctrica con el uso de un diseño factorial que varió el tipo de sal ($(NH_4)_2SO_4$, NaCl y KCl), la concentración de sal y la temperatura. La elevación de la temperatura aumentó el efecto de la precipitación de proteínas por sales. La concentración óptima de sal fue de 0,5 mol/L para $(NH_4)_2SO_4$ (%PP 69%), 1,5 mol/L para KCl (%PP 56%) y 2,5 mol/L para NaCl (%PP 61%). Se realizaron investigaciones sobre la precipitación isoeléctrica. La condición más efectiva se encontró a 85 °C por el uso de $(NH_4)_2SO_4$ de 0,8 mol/L a 1,0 mol/L y pH = 1,0. Los resultados mostraron que la temperatura, el tipo de sal y la concentración de sal, así como el pH, tuvieron efecto en la precipitación de proteínas del crudo extracto de hojas de BGB por el efecto sinérgico de la precipitación salina y isoeléctrica. Las condiciones de medio utilizadas en los experimentos fueron capaces de promover alta precipitación de proteína, lo que puede ser utilizado como una alternativa industrial para la fabricación de concentrados de proteínas.

Palabras clave: Enredadera limón, ora-pro-nobis, mucílago, concentrado de proteínas, extracción por remoción de sales.

1 Introduction

Several unexplored species from Brazilian vegetation are an excellent food source because of their high nutritional value and their economically viable cultivation (Takeiti *et al*., 2009). Among the species classified as non-conventional vegetables, the *Pereskia aculeata* Miller, popularly known as Barbados gooseberry (or ora-pro-nobis in Brazil) stands out due to the high protein content, is edible, and can be used in several food preparations (Takeiti *et al*., 2009; Lima Júnior *et al*., 2013). The leaves are also an emollient and have healing and anti-inflammatory properties (Martin *et al*., 2017).

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This perennial plant belongs to the family Cactacea and is characterized by the presence of mucilage and high digestibility proteins content, about 28.4% (Takeiti *et al*., 2009). The non-toxic properties of Barbados gooseberry (BGB) as well its balanced composition of essential amino acids, fiber, carbohydrate, calcium, phosphorous, magnesium and iron demonstrates the nutritional potential of the plant for food and pharmacological applications (Tan *et al*., 2005; Takeiti *et al*., 2009; Martin *et al*., 2017). The mucilage presents a potential use as hydrocolloids source as a thickener, gelling agent, emulsifying and stabilizing agent in food applications (Lima Júnior *et al*., 2013; Conceição *et al*., 2015).

The chemical compostion of the mucilage extracted from BGB leaves is characterized by containing 19% (w/w) of protein and 48% (w/w) of carbohydrate. Arabinogalactan type I is the major component, along with galactose, arabinose, rhamnose, fucose and partially esterified galacturonic acid (Martin *et al*., 2017).

The application of plant proteins in food and biotechnology industries ranges from its use as raw materials to high-added value products. However, these biomolecules are not obtained with the required degree of purity and an extensive sequence of extraction, separation and purification steps is required (Karaca *et al*., 2011; Lima Júnior *et al*., 2013; Chen *et al*., 2015; Silva *et al*., 2017).

Among the steps commonly employed in biomolecules purification such as proteins, the precipitation at their isoelectric point or salting out extraction are widely used due to their high recovery capacity and low costs, making this step present in more than half of the purification processes (Arogundade *et al*., 2006; Karaca *et al*., 2011; Sha *et al*., 2014; Chen *et al*., 2015). Furthermore, the excess of salt can be easily removed by dialysis (Sha *et al*., 2014).

Several authors report that the type and concentration of the salt have significant effect on protein precipitation (Machado *et al*., 2007; Sousa *et al*., 2007; Nishizawa *et al*., 2016; Fu *et al*., 2017; Kinugasa *et al*., 2017). Other common method is the pH adjustment, which has the advantage of high recovery and compatibility with the industrial environment (Elizondo-Garza *et al*., 2017). Arogundade *et al*. (2009) studied the effect of divalent cation (Ca^{2+} or Mg^{2+}) on the aggregation of African yam bean (*Sphenostylis stenocarpa*) protein in the various aqueous extraction media.

Sha *et al*. (2014) used different ammonium sulphate concentrations (20% to 40% w/w) to precipitate gelatin from bighead carp (*Hypophthalmichthys nobilis*). The effect of ionic strength and pH on the extraction of proteins from the broad bean (*Vicia faba* L.) was reported by Arogundade *et al*. (2006).

A three liquid-phase salting-out approach was used by Chen *et al*. (2015) to extract proteins from waste liquor obtained from sea cucumber and a two phase system was used by Vázquez-Villegas *et al*. (2015) to concentrate protein from alfalfa (*Medicago sativa*). Elizondo-Garza *et al*. (2017) tested the combination of pH and temperature to precipitate protein from skipjack tuna wash water. The extraction of hydrocolloids from *Pereskia aculeata* Miller was performed by Lima Júnior *et al*. (2013) using ethyl alcohol (95%) at a 3:1 proportion of alcohol to each L of filtrate solution. In this process, the authors were able to extract 10.47 g / 100 g of proteins from BGB. However, a long time (more than 270 min) was taken to obtain the mucilage containing proteins and hydrocolloids.

It is interesting to outstand the number of studies reported in the literature using salts as precipitant agents for purifying proteins from crude media by a unique step or as a fractionating method followed by a polishing stage (Machado *et al*., 2007; Sousa *et al*., 2007; Ninawe *et al*., 2008; Farinas *et al*., 2011; Sha *et al*., 2014; Chen *et al*., 2015; Dong *et al*., 2016; Purwanto, 2016). Nonetheless, to the best of our knowledge, there are no previous reports regarding protein extraction from a crude BGB extract leaves using salting out and isoelectric precipitation as a purification method.

Consequently, an increasing concern by the food industry towards using plant proteins as substitutes for animal source in new product formulations, combined to the feasibility of salts precipitation in protein purification processes justify obtaining protein concentrated extract from BGB leaves. In this study, it was evaluated the effect of temperature, salt type and salt concentration on the precipitation of proteins from crude BGB extract leaves. Research on isoelectric precipitation of BGB proteins were performed at 85 °C, varying the pH and the ammonium sulphate contents in order to evaluate the recovery of protein from BGB by the synergistic effect of salting out and isoelectric precipitation.

2 Materials and methods

2.1 Sample preparation

The BGB branches were harvested at Federal University of Lavras (Lavras, MG, Brazil) and its leaves were collected, sanitized and stored in polyethylene bags under freezing (-20 °C) conditions until the execution of the experiments. The BGB leaves were ground and homogenized in boiling water for 10 min using an industrial blender (Metvisa LG10, Brazil), at the rate of 1 kg of raw material to 2.5 L of distilled water. The samples were maintained in a thermostatic bath (Quimis, q 215-2) at 75 °C for 6 h. The extract was manually filtered with organza filter and subjected to a vacuum filtration by a buchner funnel coupled to a vacuum pump (Primar MC 1284, Brazil), using three layers of organza as filter médium (Lima Júnior *et al*., 2013; Conceiçao *et al*., 2014). Finally, the homogenate was centrifuged (SP Labor SP-701, Brazil) at 4680 × g for 7 min and originated the crude extract of BGB leaves used in the precipitation assays.

2.2 Effect of temperature, salt type and concentration on protein precipitation

Protein precipitation assays were performed according to Arogundade *et al*. (2006) with slight modification. Five milliliter aliquots of the BGB crude extract were added in 15 mL centrifuge tubes. A pre-defined mass of the salts ($(NH_4)_2SO_4$, KCl or NaCl) were weighed on analytical balance (ATY224, Marte Científica) and added directly to the tubes according to each desired molar concentration (0, 0.5, 1.5 and 2.5 mol/L). The tubes were kept at the desired temperature (25, 55 or 85 °C) for 1 h. The precipitated protein was separated by centrifugation at 2800 × g for 30 min. The initial protein content and remaining soluble protein was determined spectrophotometrically at 595 nm according to the Bradford method (Bradford, 1976). The analytical curve was constructed using standard bovine serum albumin solutions at concentrations ranging from 0.1 mg/mL to 1.0 mg/mL.

The percentage of precipitated protein (PP) was calculated according to Eq. (1).

$$\%PP = \left(\frac{C_I - C_S}{C_I}\right) \times 100 \tag{1}$$

where PP is the percentage (%) of precipitated protein, C_I is the initial protein concentration (mg/mL) and C_S is the supernatant protein concentration (mg/mL).

2.3 Combined effect of salting out and isoeletric precipitation

Salting out and isoelectric precipitation assays were performed as described in the section 2.3, using 5 mL of BGB extract solution and ammonium sulfate salt at different molar concentrations (0 to 2.5 mol/L). The pH of the samples were adjusted to values ranging from 1.0 to 12.0 by the addition of pure phosphoric acid or NaOH (5 mol/L). Final pH values of the samples were verified on a pH meter (mPA-210, MS Tecnopon). The samples were maintained at 85 °C for 1 h. The precipitated protein was separated by centrifugation and the remaining protein content was determined according to the Bradford method (Bradford, 1976). The percentage of precipitated protein was calculated according to Eq. (1).

2.4 Determination of zeta potential

Zeta potential measurements were performed by a particle electrophoresis instrument (Zetasizer Nano-ZS, Malvern Instruments, UK) using capillary cells equipped with gold electrodes. The waiting time prior to registration of the measures was 2 min. An electrophoresis assay was performed to calculate the zeta potential (ζ) of the samples by measuring the electrophoretic mobility (μ) or of the particle velocity, using Laser-Doppler velocimetry technique and light scattering analysis (Ly *et al*., 2008).

Zeta potential was calculated using the electrophoretic mobility data by Henry relation, represented at Eq. (2):

$$\zeta = \frac{3\eta\mu}{2\epsilon f(\kappa R_H)} \tag{2}$$

where η is the solvent viscosity (Pa s^{-1}), μ is the electrophoretic mobility (V Pa^{-1} s^{-1}), ϵ is the medium dielectric constant (dimensionless), $f(\kappa R_H)$ is the Henry function (dimensionless), κ^{-1} is the Debye length (measured thickness of the double electric layer around the molecule) (nm), and R_H is the particle radius (nm). Two values are usually used as an approximation for determining the Henry function: 1.5 or 1.0. Since ζ determination is commonly performed in aqueous medium and moderate electrolyte concentration, 1.5 is adopted for

$f(\kappa R_H)$, which is referred to as the Smoluchowski-Kramers approximation. The electrophoretic mobility conversion in zeta potential was made automatically by the Malvern software data analysis. Samples were appropriately diluted using ultra pure water. Zeta potential values were determined in triplicate and the average values and standard deviation are reported (Carrera *et al*., 2014).

2.5 *Absorption capacities of oil and water of the precipitated protein*

Absorption capacities of water and oil were determined using a method adapted from Beuchat (1977). Samples of approximately 0.25 g of precipitated protein were weighed and put in graduated centrifuge tubes, in which was added a volume of 2.5 mL of distilled water or soybean oil. Tubes were stirred for 1 minute until forming a suspension. After 30 minutes, with occasional stirring, tubes were centrifuged at 1170xg for 10 min. The resulting supernatant volume was measured, and the water and oil absorption capacities were expressed as volume (mL) of water or oil retained per mass (g) of protein.

2.6 *Antibacterial activity of the protein precipitated of Barbados gooseberry extract*

Antibacterial analysis was conducted at the Food Microbiology Laboratory of the Department of Food Sciences (Federal University of Lavras/MG). The bacteria selected for the experiment were *Staphylococcus aureus* GL 4384 (EMBRAPA Gado de leite - Juiz de Fora/MG), enteropathogenic *Escherichia coli* (EPEC) INCQS 00181 (CDC O55), *Salmonella enterica* subspecies enterica serovar Enteritidis S64, and *Pseudomonas aeruginosa* INCQS 0025 (ATCC 15442) (Oswaldo Cruz Foundation (Fiocruz) - Rio de Janeiro/RJ). Stock cultures were stored under freezing conditions (glycerol: 15 mL, bacterial peptone: 0.5 g, yeast extract: 0.3 g, NaCl: 0.5 g, distilled water: 100 mL) until their use.

Cultures were thawed at room temperature and reactivated by inoculating 100 μL of bacteria in tubes containing 10 mL of Tryptone Soy Broth (TSB) followed by incubation at 37 °C for 24 h, excepting for the *Pseudomonas aeruginosa*, which was incubated at 30 °C/24 h. The standardization of the activated inoculums was performed by spectrophotometer growth curve (O.D. 600 nm) and by plating in Tryptone Soy Agar (TSA) until cultures count reached 108 CFU/mL.

The antimicrobial activity of the precipitated proteins from the *Pereskia aculeata* Miller extract was performed using agar diffusion according to the Clinical and Laboratory Standards Institute (CLSI, 2005). The previously activated microorganisms were inoculated in TSA by spread plate and discs (6 mm) were embedded with the precipitate protein (10 μL) and added to medium. Chlorofenicol 0.1% (m/v) was used a negative control. Plates were incubated at the optimum growth temperature for each bacterium during 24 h and the inhibition halos were measured around the discs. The procedure was made in triplicate.

2.7 *Experimental design and statistical analysis*

The influence of the factors type of salt (potassium chloride, sodium chloride and ammonium sulfate), molar concentration of salt (0, 0.5, 1.5 and 2.5 mol/L) and temperature (25, 55 and 85 °C), on the precipitation of proteins from the BGB crude extract was evaluated using a $4 \times 3 \times 3$ factorial design, with three replicates in the completely randomized block design.

The isoelectric precipitation was performed in 12 x 4 factorial design, with three replicates in the completely randomized block design. The effect of pH (1.0 to 12.0) and the concentration of ammonium sulfate (0.5, 1.5 and 2.5 mol/L) on the protein precipitation of BGB extract samples were studied. The temperature was set at 85 °C. The temperature value (85 °C) and salt type (ammonium sulfate) were chosen based on the results from the temperature, salt type and salt concentration evaluation tests on protein precipitation (section 2.2).

The results were submitted to regression analysis and the model's accuracy (Eq. 3) was evaluated by the Fisher's statistical test (F-test), lack of fit and coefficient of determination (R2). Student's t-test was performed for each coefficient of the equation. All statistical analyses were performed using SAS v.9.0 software (SAS Institute, Cary, NC, USA).

$$Y = \beta_0 + \sum \beta_i \chi_i + \sum \beta_{ii} \chi_i^2 + \sum \beta_{ij} \chi_i \chi_j + e \qquad (3)$$

where Y stands for percentage of precipitated protein (%), β_0 is the model intercept, χ_i and χ_j are the levels of the independent variables, e is the error, and β_i, β_{ii} and βij are the linear, quadratic and interaction coefficients.

3 Results and discussion

The composition of the BGB extract leaves was 22.423% of crude protein, 1.230% of crude fiber, 52.803% of carbohydrate fraction, 23.543% of ash and 0% of fat in dry basis and 98.51% of moisture. Percentages of the precipitated protein from BGB and the initial pH of samples as a function of the concentration of salt and temperature are presented in Table 1. The minimum and maximum values were 41.09% and 71.20% for ammonium sulfate, 12.10% and 61.49% for sodium chloride, and 5.22% and 56.06% for potassium chloride. Standard deviations were lower than 5%.

Ninawe *et al*. (2008) separated xylanase from *Streptomyces cyaneus* by salt precipitation using 60% (w/v) of saturated ammonium sulfate (about 4.5 mol/L) overnight at 4.0 °C, and obtained 58.92% of precipitated protein. The yield was lesser than the one found in this work (68%) using lower concentrations of ammonium sulfate (0.5 and 1.0 mol/L) at 85 °C. Farinas *et al*. (2011) aiming at separating endoglucanase and xylanase produced by *Aspergillus niger* evaluated the precipitating agent, time and temperature. The highest precipitation efficiency for both enzymes was achieved with 80% (v/v) of saturated ammonium sulfate (about 10 mol/L) at 10.0 °C for 180 min, resulting in activity recoveries of 56% and 27%.

The present study found 56.24% of precipitation using 2.5 mol/L of ammonium sulfate at 25.0 °C for 60 min. Therefore, under notably milder conditions, it was possible to precipitate the same percentage of protein. Moreover, it facilitates the following purification stages, since the amount of salt to be removed is smaller. However, the efficiency of salting out depends on the experimental conditions and on the biomolecule to be extracted. Thereby BGB protein presented ease of extraction.

Studies with sodium chloride as a precipitant agent were developed by Fontanari *et al*. (2007) to obtain guava seed protein isolate. At pH 12.0, temperature of 25.0 °C and using 0.5 mol/L of the salt, it was obtained about 30% of precipitated protein. Machado *et al*. (2007) obtained precipitation of 27.67% of egg white proteins at pH 6.0, 25 °C and using 0.5 mol/L of the sodium chloride. At the same conditions of concentration and temperature, the present work found 27.95% of protein precipitation from BGB extract.

Experimental results were subject to regression analysis (Table 2). According to Table 2, the salt concentration and temperature interaction was significant ($p < 0.05$) to protein precipitation using ammonium sulfate and sodium chloride. For all tested salts, both linear and quadratic effects of the variables salt concentration significantly affected ($p < 0.05$) the precipitation.

Equations 4, 5 and 6 represents the mathematical models that describe the percentage of precipitation as a function of temperature and salt concentration for ammonium sulfate, sodium chloride and potassium chloride, respectively.

Table 1. Average results of percentages of precipitated protein (%) from BGB and initial pH of samples as a function of the salt concentration (C_{salt}) and temperature (T), for the different salt type (ammonium sulfate, sodium chloride and potassium chloride).

Assay	C_{salt}(mol/L)	T (°C)	PP (%)			Initial pH of samples		
			Ammonium sulfate	Sodium chloride	Potassium chloride	Ammonium sulfate	Sodium chloride	Potassium chloride
1	0	25	3.98 ± 0.62	3.68 ± 0.33	5.22 ± 0.56	4.89 ± 0.00	4.91 ± 0.02	4.98 ± 0.04
2	0.5	25	47.6 ± 2.03	27.95 ± 0.93	10.86 ± 1.91	4.81 ± 0.06	4.65 ± 0.07	4.79 ± 0.01
3	1.5	25	50.46 ± 0.56	31.95 ± 0.97	30.75 ± 4.24	4.63 ± 0.00	4.40 ± 0.01	4.76 ± 0.00
4	2.5	25	56.24 ± 2.00	46.95 ± 0.39	10.01 ± 1.99	4.57 ± 0.01	4.36 ± 0.02	4.74 ± 0.01
5	0	55	20.19 ± 1.22	19.24 ± 2.10	20.94 ± 2.86	4.94 ± 0.04	4.94 ± 0.02	4.97 ± 0.02
6	0.5	55	58.98 ± 1.53	45.38 ± 1.29	52.98 ± 1.42	4.80 ± 0.02	4.73 ± 0.03	4.78 ± 0.05
7	1.5	55	57.70 ± 2.89	48.98 ± 0.74	45.89 ± 1.99	4.66 ± 0.02	4.39 ± 0.03	4.72 ± 0.00
8	2.5	55	56.85 ± 1.22	51.66 ± 0.52	32.51 ± 0.76	4.57 ± 0.05	4.37 ± 0.05	4.69 ± 0.01
9	0	85	59.16 ± 3.05	37.91 ± 1.23	44.22 ± 2.17	4.94 ± 0.01	4.94 ± 0.01	4.93 ± 0.02
10	0.5	85	69.07 ± 3.39	44.92 ± 0.45	53.03 ± 1.12	4.83 ± 0.03	4.78 ± 0.00	4.89 ± 0.04
11	1.5	85	68.16 ± 0.38	56.58 ± 1.70	56.06 ± 1.79	4.74 ± 0.04	4.64 ± 0.03	4.76 ± 0.04
12	2.5	85	58.18 ± 0.32	61.49 ± 1.71	45.06 ± 2.63	4.64 ± 0.04	4.45 ± 0.02	4.74 ± 0.02

$$PP = -7.9480 + 56.4967 \times C_{salt} - 12.3929 \times C_{salt}^2 + 0.7373 \times T - 0.2989 \times C_{salt} \times T \quad (4)$$

$$PP = -3.3598 + 30.9454 \times C_{salt} - 5.6856 \times C_{salt}^2 + 0.4776 \times T - 0.0899 \times C_{salt} \times T \quad (5)$$

$$PP = -24.2182 + 32.3333 \times C_{salt} - 12.2197 \times C_{salt}^2 + 1.3284 \times T - 0.0067 \times T^2 \quad (6)$$

where PP is the percentage of precipitated protein (%), C_{salt} is the salt concentration (mol/L) and T is the temperature (°C). The models presented coefficient of determination greater than 82%, which means that much of the variation of the dependent variable is explained by the model. From contour plots graphs (Figures 1 A, B and C), it is possible to evaluate the maximum regions for percentage of precipitation of BGB proteins.

The percentage of precipitated protein using ammonium sulfate reached higher values (68%), in concentrations around 1.0 mol/L at 85 °C (Fig. 1A). Highest percentage values of precipitation using sodium chloride were obtained at experimental conditions of 2.5 mol/L at 85 °C (Fig. 1 B). For potassium chloride, the maximum regions were found with concentration near to 0.75 mol/L at 60 °C (Fig.1C).

Type and salt concentration significant affect the protein stability in aqueous solution, being able to precipitate protein or increasing its solubility. Two types of salts are known. The first group of salts is called lyotropic or cosmotropic and is efficient in protein precipitation (salting out effect) and the second group is classified as chaotropic and is efficient in solubilizing protein (salting in effect) (Baldwin, 1996; Tadeo *et al*., 2007). Cosmotropic ions have the ability to structure water molecules and stabilize proteins, establishing strong interactions with water molecules and promoting hydrophobic interactions, which reduces protein solubility. In contrast, chaotropic ions disrupt water molecules and hydrophobic interactions, increasing protein solubility (Zhang *et al*., 2006). Nucci and Vanderkooi (2008) stated later that the positions of some ions in the series might alternate depending on the nature of the system. Regardless of the salt type, increasing temperature resulted in higher protein precipitation. This behavior is related to the increase in thermal kinetic energy in the medium that causes protein unfolding, exposure of hydrophobic groups, aggregation, and precipitation, i.e., decreased solubility (Parkin, 2017).

As expected, salt concentration directly affected protein precipitation. It is observed that the higher the amount of precipitated protein, the higher the sodium chloride concentration at the same temperature (Fig. 1 B). In general, high salt concentrations increases ionic strength, increasing the attractive interactions between protein molecules and causing precipitation (Parkin, 2017).

Table 2. Regression analysis performed for percentage of protein precipitate of Barbados gooseberry extract.

Source	Ammonium sulfate			Sodium chloride			Potassium chloride		
	GL	SS	F value	DF	SS	F value	GL	SS	F value
C_{salt}	1	5324.86	78.52*	1	1597.54	60.71*	1	2081.17	63.99*
$C_{salt} \times C_{salt}$	1	2331.09	34.37*	1	490.65	18.64*	1	2266.39	69.68*
T	1	61.09	0.9	1	299.41	11.38*	1	976.54	30.02*
$T \times T$	1	81.33	1.2	1	45.61	1.73	1	295	9.07*
$C_{salt} \times T$	1	1779.56	26.24*	1	160.76	6.11*	1	58.73	1.81
Model	5	10270.94	30.29*	5	8296.43	63.05*	5	10125.74	62.27*
Error	30	2034.56		30	789.48		30	975.73	
LOF	6	1962.82	109.45*	6	756.51	91.78*	6	858.56	29.31*
Pure error	24	71.73		24	32.97		24	117.17	
Total	35	12305.49		35	9085.9		35	11101.47	
R^2		0,828			0,908			0,907	

DF: degrees of freedom, SS: sum of squares, LOF: lack of fit, T: temperature (°C), C_{salt}: salt concentration (mol/L). *($p < 0.05$).

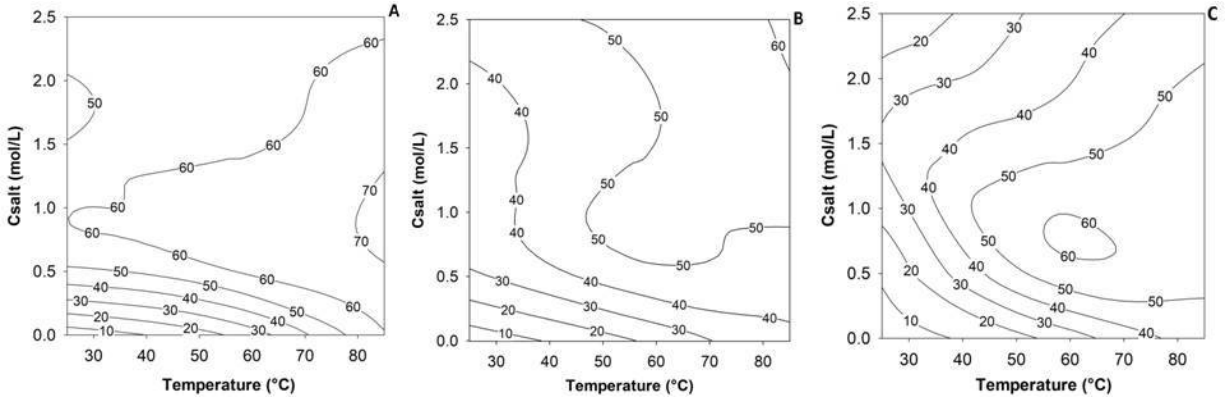

Fig. 1. Percentage of precipitated protein (%) from Barbados gooseberry crude extract as a function of temperature (°C) and the molar salt concentration (mol/L) for ammonium sulfate (A), sodium chloride (B) and potassium chloride (C).

As shown in Figure 1A and C, for the same temperature level, a decay in the variable PP can be observed after ammonium sulfate and potassium chloride concentrations reach 1.3 mol/L and 0.9 mol/L, respectively. Salts dissociate into ions that interact with opposing charge groups on the protein, diminishing electrostatic interaction among the molecules and increasing their solubility. As much as the salt concentration is increased, the ions begin to compete with the protein for the water molecules. If the ions-water interaction is strong, protein molecules interact with each other, resulting in precipitation. However, in a saturated salt solution there is an excess of ions and electric charge on the protein surfaces, which causes an electrostatic repulsion between these molecules and hence prevents their precipitation (Parkin, 2017).

These results demonstrate that the effectiveness of the salts in protein precipitation by salting out strongly depends on the nature of the salt. This fact can be elucidated based on the Hofmeister series (Baldwin, 1996; Tadeo *et al*., 2007). Two different anions are present in the evaluated salts (sulfate and chloride). The salt with sulfate anions was more effective for induction of the precipitation by salting out, which can be justified by the position of these two anions in the Hofmeister series. Sulfate anion is in a more favorable position, and since it is more cosmotropic, the precipitated protein content is higher in the presence of this salt (Bradford, 1976; Ly *et al*., 2008).

Potassium chloride and sodium chloride present the same anion, however they differ by the cations. According to Hofmeister series, the sodium cation appears to be less or equally cosmotropic than potassium and their position may alternate depending on the nature of the system being evaluated (Bradford, 1976; Ly *et al*., 2008; Nucci and Vanderkooi, 2008). In the present study, sodium was more efficient in inducing salting out. The addition of sodium chloride led the proteins to lose the solubility and to aggregate more intensely. Sousa *et al*. (2007) evaluated the effect of pH and salt concentration on the solubility and density of egg yolk proteins. The authors found that at pH 3.0 and salt concentration of 0.05 mol/L, a protein solubility of 55.75% and 19.06% was obtained using sodium chloride and ammonium sulfate, respectively. The amount of precipitated protein is inversely proportional to the solubility of these biomolecules, thus sodium chloride was less effective in inducing the salting out effect, reaffirming that dissociated ions in salt solutions are in agreement with Hofmeister series.

From the predicted models in Eq. (4) to (6), it was generated the curves depicted in Figs. 2 A and B, in which each independent variable was varied at time while another independent variable was fixed, in order to determine conditions that maximize the dependent variable PP. According to the Figs. 2 A and B, it may be observed that the optimal precipitation conditions for BGB proteins was achieved using $(NH_4)_2SO_4$ at 85 ºC, in which %PP was higher than 70% between 1.0 mol/L to 1.5 mol/L of this salt. Based on the experimental and predicted results of the salt precipitation, isoelectric precipitation tests were performed combining the salting out effect using the ammonium sulphate salt and setting the temperature at 85 °C (Table 3), since the experimental condition of higher temperature and ammonium sulfate resulted in higher efficiency in the precipitation process of BGB proteins.

Table 3. Average results of percentages of precipitated protein (%) from BGB and final protein precipited concentration as a function of the concentration of the salt ammonium sulfate (C_{salt}) and pH, at 85 °C. The initial protein concentration of crude extract leaves of BGB was 0.399 mg/ml.

pH	PP (%)				Final protein precipitated concentration (mg/mL)			
	0 mol/L	0.5 mol/L	1.5 mol/L	2.5 mol/L	0 mol/L	0.5 mol/L	1.5 mol/L	2.5 mol/L
1	22.77 ± 2.00	86.95 ± 0.85	81.70 ± 2.18	79.35 ± 0.67	0.09 ± 0.01	0.35 ± 0.00	0.33 ± 0.01	0.32 ± 0.00
2	52.32 ± 0.53	84.85 ± 0.79	76.99 ± 0.76	73.90 ± 1.53	0.21 ± 0.00	0.34 ± 0.00	0.31 ± 0.00	0.30 ± 0.00
3	70.84 ± 1.15	77.45 ± 1.74	71.10 ± 1.11	68.31 ± 0.25	0.28 ± 0.00	0.31 ± 0.01	0.28 ± 0.00	0.27 ± 0.00
4	68.93 ± 2.12	67.68 ± 2.15	70.44 ± 1.47	57.56 ± 0.76	0.28 ± 0.01	0.27 ± 0.01	0.28 ± 0.00	0.23 ± 0.00
5	65.59 ± 1.20	67.40 ± 0.64	64.85 ± 1.01	58.74 ± 2.27	0.26 ± 0.00	0.27 ± 0.00	0.26 ± 0.00	0.23 ± 0.01
6	59.70 ± 2.61	63.77 ± 1.45	65.58 ± 1.35	47.62 ± 0.80	0.24 ± 0.01	0.25 ± 0.01	0.26 ± 0.00	0.19 ± 0.00
7	47.30 ± 0.85	51.28 ± 0.74	63.08 ± 1.53	37.69 ± 1.77	0.19 ± 0.00	0.20 ± 0.00	0.35 ± 0.01	0.15 ± 0.01
8	36.10 ± 0.70	37.67 ± 0.24	42.62 ± 2.00	40.78 ± 0.66	0.14 ± 0.00	0.15 ± 0.00	0.17 ± 0.01	0.16 ± 0.00
9	24.16 ± 1.22	93.28 ± 7.25	-	-	0.10 ± 0.00	0.37 ± 0.03	-	-

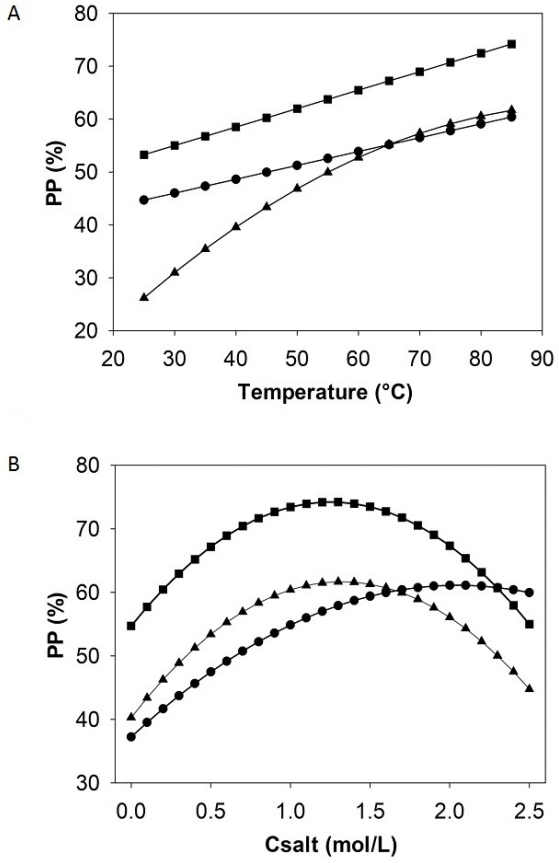

Fig. 2. Predicted results for percentage of precipitated BGB protein (%) as a function of (A): temperature using 1.3 mol/L of ammonium sulfate (▪), 2.4 mol/L of sodium chloride (●) and 1.3 mol/L of potassium chloride (▲); (B) molar salt concentration using ammonium sulfate (▪), sodium chloride (●) and potassium chloride (▲) at 85 ºC.

The precipitation results of BGB proteins by the synergistic effect of salting out and isoelectric precipitation are presented in Table 3. It is noticed that the final concentration of precipitated proteins is elevated, and most of the treatments presented %PP higher than 60% (Bacigalupe *et al.*, 2017), amount found in soy protein concentrate, a common protein concentrate used as a supplement for human and animal diet and obtained from vegetable source. Then, it highlights the potential of extracting BGB protein by salting out techniques and isoelectric precipitation and use these processes as industrial alternative to manufacturing protein concentrates. As presented in Table 3, it was verified that the percentage of precipitated protein (PP%) was not quantified at pH 9 for the concentrations of 1.5 mol/L and 2.5 mol/L of ammonium sulfate and for the pH's 10, 11 and 12 in all salt concentrations tested, since NaOH volumes used under these conditions were high, resulting in the dilution of salt concentrations of the samples. The experimental results (Table 3) were subjected to regression analysis (Table 4).

The relationship between the independent variables pH and salt molar concentration and the response variable PP% can be examined by the contour plots depicted in Figure 3, which were generated from the experimental results. From Fig. 3, it may be noted that the maximum PP% was obtained at pH 1.0 and for $(NH_4)_2SO_4$ concentrations between 0.8 mol/L and 1.0 mol/L. For salt concentrations higher than approximately 0.4 mol/L, it is observed that the increase of pH decreased the precipitation percentage, for the same salt concentration. This result is also be shown observing the negative regression coefficient for the pH presented in Eq. (7), indicating that higher pH resulted in lower PP%.

Table 4. Regression analysis performed for percentage of protein precipitate as a function of pH and concentration of ammonium sulfate.

Source	DF	SS	F value
pH	1	16888.78	8917.86*
$pH \times pH$	1	4681.91	2472.21*
C_{salt}	1	325.29	171.77*
$C_{salt} \times C_{salt}$	1	2592.97	1369.18
$C_{salt} \times pH$	1	1157.95	611.44*
Model	5	25646.9	53.55*
Error	96	9194.74	
LOF	28	9065.96	170.97*
Pure error	68	128.78	
Total	101	34841.64	
R^2	0.74		

DF: degrees of freedom, SS: sum of squares, LOF: lack of fit, C_{salt}: concentration of ammonium sulfate (mol/L). *($p < 0.05$).

The Eq. (7) represents the mathematical model that describes the percentage of precipitation as a function of ammonium sulfate concentration and the medium pH.

$$PP = 46.9519 + 28.1020 \times C_{salt} - 8.0284 \times C_{salt}^2 + 9.3107 \times pH - 1.3527 \times pH^2 - 1.4963 \times C_{salt} \times pH \quad (7)$$

where PP is the percentage of precipitated protein (%) at 85 °C, C_{salt} is the salt molar concentration (mol/L) and pH is the pH.

According to the zeta potential analysis, it was found that the isoelectric point of the proteins present in the crude BGB extract was around pH 1.7, indicating that in pH 1.0, the optimal precipitation pH, the proteins were positively charged, what combined with the excess of cations from the dissociation of ammonium sulfate, resulted in reduced protein solubility in the medium. Maurer *et al*. (2011) found that the chymosin solubility decreased with ionic strength increasing from 0.6 mol/L $(NH_4)_2SO_4$, reaching its minimum at pH 5.0 and ammonium sulphate 0.8 mol/L and 1 mol/L, the same salt concentration region found in this study, but in higher pH value.

The ability to absorb water or oil can be defined as the capacity of a protein to retain a certain amount of such liquid when added (Kaushik *et al*., 2016).

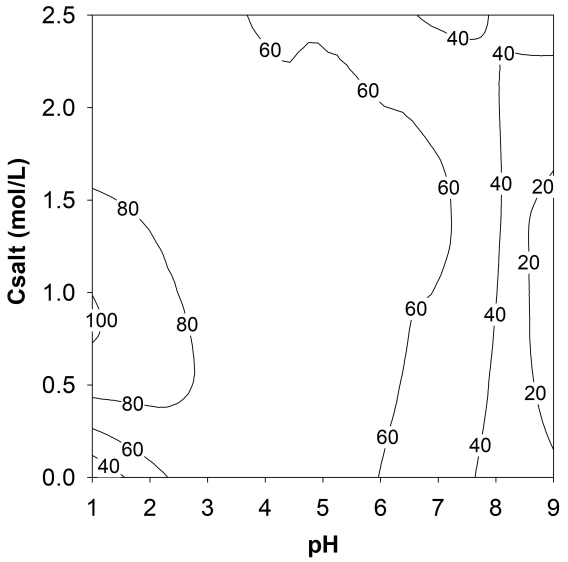

Fig. 3. Synergistic effect of salting out and isoelectric precipitation (%) of proteins from crude BGB extract leaves using ammonium sulfate at 85 ºC.

In the present study, the precipitated protein fraction of the BGB extract showed water and oil absorption capacities of 0.2 mL/g and 6.0 mL/g, respectively. Higher values for oil absorption over water absorption can be justified by the hydrophobic character of the proteins extracted from *Pereskia aculeata* Miller leaves. According to Kinsella (1976), proteins with larger proportion of nonpolar amino acids increase the lipophilic characteristics of the molecule, what may explain the result encountered.

It is observed that the four bacteria tested were sensible to the precipitated protein obtained by the BGB extract, presenting the following inhibition halos: *Salmonella enterica*: 8.99 ± 1.32 mm; *Escherichia coli*: 8.54 ± 1.16 mm; *Pseudomonas aeruginosa*: 8.50 ± 0.87 mm; *Staphylococcus aureus*: 7.01 ± 0.15 mm. The precipitated fraction of the OPN extract inhibited the growth of all strains and exhibited potent antibacterial activity, what can be verified by larger diameters of inhibition halos for these microorganisms (Fig. 4).

Similar results were found by Jimenez *et al*. (2011), for capulin (*Prunus serotina subsp capuli*) extracts and by Souza *et al*. (2016). in a study about the chemical composition and biological activities of *Pereskia aculeata* Miller leaves.

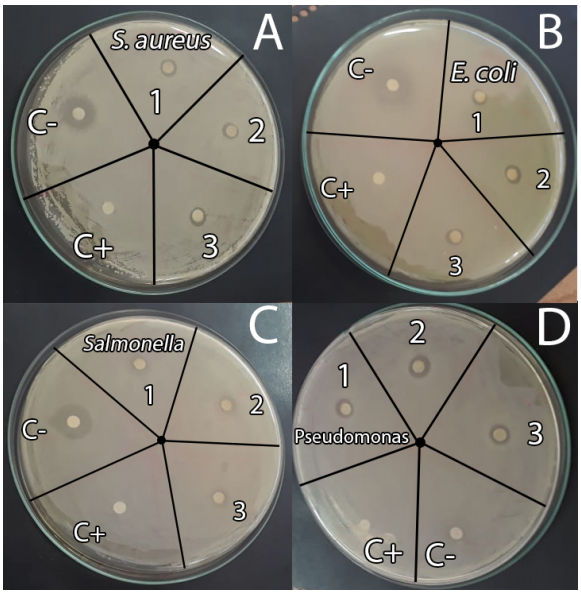

Fig. 4. Typical inhibition zones created by positive control (chloramphenicol) (C+), negative control (TSA) (C-) and protein precipitated from Barbados gooseberry extract (1, 2 e 3): (A) *Staphylococcus aureus*, (B) *Escherichia coli*, (C) *Salmonella enterica*, (D) *Pseudomonas aeruginosa*.

The authors performed tests with mucilages of *P. aculeata* precipitated from different solvents (petroleum ether, chloroform and methanol), in which the extract of lower polarity showed antimicrobial potential on the bacteria *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*.

Conclusions

pH in order to obtain a protein concentrate from Barbados gooseberry extract by processes of salting out and isoelectric precipitation. Increases in temperature resulted in increased precipitated protein content, and the temperature of 85 °C was the most effective for salting out. Salt type and concentration also influenced protein precipitation. The highest percentages of precipitated protein were obtained using ammonium sulfate at molar concentrations of 1.0 mol/L (%PP 69%), showing the effectiveness of salts in protein precipitation. Finally, by the isoelectric experiments, it was found that pH influenced the protein recovery from BGB extract. Using the optimum conditions of salt type and temperature, it was found that the highest protein precipitation was found at 85 °C, using ammonium sulfate at 0.8 mol/L to 1.0 mol/L and pH = 1.0. In this way, it is noticed that the BGB leaves extract might be used as source of protein concentrates for food application, since by salting out technique combined with isoelectric precipitation it was possible to obtain high percentage of precipitated proteins.

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Disclosure

The authors declare no conflict of interest.

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