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Hydrolyzed protein from *Lupinus albus* (INIA-Boroa variety) for the functional food industry: An example of partnership between farmers and academia

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ABSTRACT

This work presents investigations carried out to obtain by-products of bitter lupine (*Lupinus albus*), using a variety created specially in the Chilean Agricultural Research Institute (INIA). We present new information on obtaining protein isolates and hydrolysates as a way of adding value to bitter lupine, which promises to help return this crop to profitability in Chile. Lupine in this country is produced almost exclusively in the Araucanía Region, for the most part by small-scale farmers of the Mapuche ethnic group. An experimental design was drawn up based on one-factor experiments, for obtaining both protein isolate and protein hydrolysate. The best process conditions were achieved with stirring times of between 60 and 90 min; the concentrations (consistency of the mixture) were between 5 and 10%, with pH of the solution 12. The maximum yield achieved was 33% pure protein isolate (dry weight). After the protein isolates had been formulated, they were characterised by their functional properties: solubility, water retention capacity, lipid adsorption index, swelling capacity and foam stability. These properties are important for determining the quality of the protein hydrolysate, as they will affect its digestibility and thus its bioavailability in the organism. The protein hydrolysate was characterised by its complete proximal parameter, vitamins, total sugars, and amino acids. These results show that *Lupinus albus* (var. INIA-Boroa) is an excellent raw material for the existing functional foods industry.

1. Introduction

The most powerful trend in the market today is consumers' desire for foods and ingredients that are "naturally functional" (Mirosa & Mangan-Walker, 2018). This growing trend is indicative of the success of all the foodstuffs developed. It is the key driver of most innovation in healthier foods and drinks, from those of plant origin to the reappearance of whole milk products, the increase in green juices, blueberries, almonds, seaweed, and snack products. A natural, intrinsic health benefit gives the most convincing message (Puhakka et al., 2018).

Concentrating our attention within the wide range of food types, it has been suggested that food proteins are a source of high-quality proteins because they are a natural ingredient in functional foods. The typical food protein sources among plants and animals are meat, milk, eggs, fish, soya beans, wheat, etc. Proteins from serum contain the highest nutritional quality (protein digestibility-corrected amino acid score (PDCAAS) = 1) of the protein sources in our diet, with an even higher biological value than the milk protein casein. Serum proteins are rich in human essential amino acids (AA), branched-chain amino acids (BCAA), and AA which contain sulphur and promote metabolic regulation and protein folding (Almeida et al., 2015).

Consumption of plant proteins offers many advantages over animal protein. It has been shown that western diets – rich in meat, refined sugars, and fats – are worse in terms of promoting human health than

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plant-based diets, as they present a higher risk of developing certain diseases. Due to increasing consumer demand – attributable to various factors like health problems, vegetarianism, and religious restrictions – there is growing interest in plant proteins as a substitute for animal proteins. Among these protein sources, legumes like soya beans, lentils, peas, chickpeas, and lupins are still the biggest group (Ibrahim et al., 2019).

1.1. Lupins in Chile

Three species of lupine are cultivated in Chile: white lupine, *Lupinus albus* L.; narrow-leafed or "Australian" lupine, *Lupinus angustifolius* L.; and most recently yellow lupine, *Lupinus luteus* L. Andean lupine or tarwi (*Lupinus mutabilis Sweet*), has only been grown experimentally (Mera, 2016). White lupine, also known as bitter lupine, is grown for the most part by small-scale farmers of the Mapuche ethnic group. The original material received by the farmers in the 1960s was sweet – the German variety Multolupa – but after several years without renewal of the seed stock and exposed to repeated pollination with plants which carried the gene responsible to produce alkaloids, it has become bitter. Crossing with later varieties than Multolupa have made the type quite heterogeneous. In the 1990s, other bitter types of lupines were introduced from Italy (originating in Morocco), known among the farmers as high-calibre or giant (Mera, 2007).

Bitter lupine is not consumed in Chile; it has a niche export market in Mediterranean countries in Europe, mainly Spain, and the Middle East, mainly Egypt. The European market is more demanding and offers higher prices according to size, requiring beans with minimum calibre 13 mm. Calibres between 8 and 12 mm are exported to the Arab market; the larger the calibre, the higher the price paid. Chile's main competitor is Australia, which offers a lupine catalogued by importers as being of higher quality than the Chilean product (DIG, 2001).

In their original form, all these species of lupine contain alkaloids, toxic principles that impart bitterness to the green parts and beans. Alkaloids are concentrated in the beans, accounting for between 1.7 and 2.4% of the dry matter, although the variation may be greater. Alkaloids prevent lupine from being used in animal or human food without previous treatment, and it was therefore little used in antiquity. The search for naturally alkaloid-free forms of lupine was undertaken by Reinhold von Sengbusch in Germany, after he had developed a chemical method for determining its presence (Mera, 2016).

Lupine protein concentrate is a promising ingredient obtainable by a process of grinding with air, called dry fractionation. Some authors indicate that this is a more sustainable method than conventional moist extraction, and that it yields a protein concentrate with native functional properties. Extracting the oils from the lupine before dry fractionation would be a simple way of reducing the adherence forces and obtaining lupine protein concentrates with a higher protein content (Pelgrom et al., 2014).

Depending on the production conditions, protein isolates are characterised by poor techno-functional properties, particularly low foaming and emulsifying capacities, and unpleasant sensorial properties. Various approaches are described in the literature for altering protein structures to improve their techno-functional and sensorial properties; enzymatic hydrolysis has proved to be one of the most promising methods for modifying protein preparations to meet requirements (Angioloni & Collar, 2013). Enzymatic hydrolysis of proteins is currently used to obtain bioactive peptides with antioxidant, anti-hypertensive, or anti-diabetic activity. Many plant proteins have been used to obtain peptides with different bioactivities (Lammi et al., 2018).

Due to the nutritional potential of *Lupinus albus* (var. INIA-Boroa), and the selectivity of the enzymatic treatment for obtaining food products, this study proposes the use of commercial enzymes as an alternative treatment for obtaining a protein hydrolysate. Lupine protein can be purified and hydrolyzed using an experimental design with different conditions; it can then be converted into a functional food and used as an additive to create various extraordinary products for human consumption.

2. Materials and methods

2.1. Separating the skin from the bean and alkaloids removal

Beans of *Lupinus albus* (INIA-Boroa) were supplied by Cooperativa Campesina Boroa Limitada, a cooperative located in the district of Nueva Imperial, Araucanía Region, Chile (38°44′00″S 72°57′00″W). They were received, stored, and conditioned in the installations of the bioprocess laboratories of the Engineering Faculty of Universidad Católica de Temuco.

The conditions for separating the skin from the bean required a prior study which tested three different treatments (Oven-drying; Soaking in distilled water; Boiling in water). However, due to cross-sectional knowledge that boiling is the most appropriate methodology for removing the skin of legume beans in general, then the Boiling in water treatment was chosen. To establish the best working concentration in the laboratory of lupine beans in solution, the weight/volume ratio was set at 10%. The total alkaloids (TA) present in the INIA-Boroa variety were determined experimentally using the methodology reported by Magalhães et al. (2017).

Treatment A – the first method tested is one already used by some lupine farmers in the area. We followed their method of removing the bitterness, which consists in leaving the lupine beans to soak for 96 h (4 days) at room temperature, changing the water every 24 h. This method was replicated with some modifications, and the lupine concentration was fixed in an aqueous solution of NaCl at 0.01% (lupine beans in solution, 10% weight/volume).

Treatment B – After establishing the conditions for separating the skin from the bean, we eliminated the alkaloids from the "skinned" bean using a methodology based on heat treatment at 90 °C. A 3^2 factorial design was used with three concentrations of NaCl: 0.04%, 0.03% and 0.02% w/v for 15, 30 and 45 min. Boiling times were counted from when the water started to bubble. This procedure was repeated as often as necessary until analysis showed that the level of alkaloids was below that required by European regulations (lupine beans in solution, 10% weight/volume).

2.2. Basic extraction

2.2.1. Control experiment

Starting with the best results in separating the skin from the bean and removing the alkaloids, the treated beans were then crushed and ground. The methodology reported by Lqari et al. (2002) was used to obtain the protein isolate. It consists in preparing a suspension of lupine flour (20 g) in 200 mL of NaOH 1 N solution (weigh 4.0 g NaOH and bring up to 200 mL), to obtain pH 12 in the mixture, and stir for 1 h. Centrifuge at 8000 G and retain the supernatant. Three further extractions are carried out with half the volume of the alkaline solution with the solid portion recovered. The supernatants are grouped and analysed to determine the nitrogen content: the sediment is oven-dried at 50 °C, weighed and analysed. The pH of the soluble proteins is adjusted to the isoelectric point (IEP) (pH 4.3) and the precipitate formed is recovered by centrifuging at 8000 G. The precipitate is washed with a solution of HCl at 0.5 N (4.9 mL of HCl in 100 mL of water) to adjust to pH 4.3 and finally lyophilised.

2.2.2. Postulate

The protein was extracted by the response surface method for the experimental design. Optimization of the treatments to obtain protein isolate was calculated with the Design-Expert software (Test version 12.0.0, Stat-Ease, Minneapolis, Minnesota, USA). Based on single-factor experiments, the three independent variables involved, and their levels were selected as follows: X_1 flour-basic solution ratio (w/v): 5%, 10%

and 15%; X_2 pH of the aqueous solution adjusted to 8, 9 and 10; X_3 mechanical stirring times: 20, 30 and 40 min. In the following stage, each insoluble residue of the previous stage is re-used at a concentration of 20% w/v with NaOH 1 N solutions at pH 8, 9 and 10. They are stirred for 10 min, then centrifuged and the supernatants are recovered.

2.3. Acid Precipitation

The protein is precipitated from the supernatants obtained by basic extraction, and the pH is regulated with HCl solution at 0.5 N (4.9 mL HCl in 100 mL of water). It is stirred for 15 min and centrifuged to obtain a protein paste which will be lyophilised. The total protein is determined.

2.4. Characterization of the functional properties of the formulations

Solubility: The solubility – or rather the dispersibility – of the protein material represents the capacity of a protein sample to form a colloidal solution. The solubility of proteins depends on the pH, temperature, ionic strength, and concentration of the solution. It is expressed as the nitrogen solubility index (%NSI) and is quantified by dispersing the protein material at I % w/v in water at a temperature of 20 °C; the pH is adjusted with HCl for the acid range and NaOH for the alkaline.

Water retention capacity: Bryant and Hamaker's (1997) method was used. This parameter was measured by placing 0.5 g of sample in a centrifuge tube and adding an excess of water (3 mL), and then stirring for 1 min. The tubes were left to rest at 24 °C for 30 min and then centrifuged at 3200 rpm. The volume of water retained was measured. The % of water retained (%WR) in the samples was calculated using equation (1).

$$\%WR = \frac{mL\ water\ retained}{grams\ of\ sample} x\ 100 \quad (1)$$

% WR is the expression of the volume of water retained by each 100 g of protein concentrate.

Lipids adsorption index: The method described by Granito et al. (2013) was used. This property was determined by adding an excess of oil (3 mL) to the sample (0.5 g) in graduated centrifuge tubes and stirring manually for 1 min. The tubes were left to rest at 24 °C for 30 min and then centrifuged at 3200 rpm. The volume of excess oil was measured. The % of lipids retained (%LR) in the samples was calculated using equation (2).

$$\%LR = \frac{mL\ oil\ retained}{grams\ of\ sample} x\ 100 \quad (2)$$

% LR represents the quantity of oil adsorbed by 100 g of protein concentrate.

Swelling capacity: The methodology proposed by Hernández-Medina et al. was used to determine the solubility and the swelling capacity (SC) of the starches. An aliquot of 10 mL of sample and 30 mL of water was placed in a graduated test-tube, shaken with inversion to ensure that all the material was wetted, and left to rest for 2 h, after which the increase in volume of the sample was recorded. The % swelling capacity (%SC) in the samples was calculated using Equation (3).

$$\%SC = \frac{mL\ final\ volume}{mL\ initial\ volume} x\ 100 \quad (3)$$

The % SC is the measurement of the degree of swelling suffered by the protein in the presence of an excess of water at ambient temperature (20 °C).

Foaming capacity (FC): 50 mL of a protein suspension at 5% w/v was prepared and stirred with an electric whisk at maximum speed for 6 min. The % FC of the samples was calculated using Equation 4.

Table 1
Parameters and methods used for complete proximal analysis, vitamins, total sugars and amino acids (General methods of tests and analysis for food products).

Parameter	Method
Water content or humidity	ISO 18787:2017
Ash	AOAC (2005) Ash of Flour (Direct Method), Method 923.03
Total protein	ISO 1871:2009
Determination of fatty matter	AOAC INTERNATIONAL, Volume 80, Issue 2, March 1, 1997, Pages 359–372
Energy content	The chemical composition of American food materials/by W.O. Atwater and Chas. D. Woods. Wellcome Collection. Public Domain Mark
Carbohydrates	AOAC INTERNATIONAL, Volume 104, Issue 6, November–December 2021, Pages 1465–1478
Raw fibre	ISO 5498:1981
Total sugars	AOAC SMPR 2018.001
Amino acids	AOAC INTERNATIONAL, Volume 88, Issue 3, May 1, 2005, Pages 877–887
Vitamins	ISO 20633:2015

$$\%FC = \frac{weight\ of\ suspension\ before\ whisking - g\ 50\ mL\ of\ foam}{g\ 50\ mL\ of\ foam} x\ 100$$

Foam stability (FS): An aliquot of 50 mL of foam was transferred to a sintered glass funnel. The time required for the first drop to drain, and the weight of liquid that drained in 60 min, were taken as indicators of foam stability.

2.5. Obtaining protein hydrolysate

The reference methodology for obtaining protein hydrolysate was that reported by He et al. (2015) which is basically hydrolysis parameters, including temperature, time and the ratio of alkaline protease, were optimized by response surface methodology with a central composite design. For this study, the commercial enzymes used were alcalase and papain. Ensure that the humidity of the protein isolate is less than 2% then grind in a 200 μm sieve. To carry out the enzyme hydrolysis experiment, the procedures are detailed below: pour 300 mL of buffer solution into an Erlenmeyer flask with a small magnetic bar on a slightly heated stirring plate (between 40 and 50 °C). While stirring the buffer solution, dissolve the amount of protein isolate necessary for the experiment (6, 18 or 30 g). Stir this mixture for 10 min and, then weigh the amount of enzyme needed for the experiment (0.09; 0.27 or 0.45 g) in a watch glass; pour into the Erlenmeyer flask and seal immediately.

For the next part, place the flask on an orbital shaker to carry out hydrolysis, setting the temperature (40, 50 or 60 °C) and time required for the experiment (60, 90 or 120 min). At the end of the time, remove the flask from the shaker and place it in a cuvette at low temperature to stop hydrolysis. Leave the flask in the cuvette for 15 min. Pour the whole contents of the flask into Falcon tubes for centrifuging. Set the centrifuge parameters to 6 °C, 10 min and 3000 rpm. After centrifuging, pour the supernatant into weighed and labelled Falcon tubes, and freeze for lyophilization. After lyophilization, weigh the Falcon tubes and record the values in a spreadsheet.

2.6. Characterization of the protein hydrolysate

Finally, the samples with the best yields in the complete proximal parameters, vitamins, total sugars, and amino acids were characterised (Table 1).

Table 2
Experimental design of response surface design for protein isolate yield.

Consistency %	pH	Time	Protein isolate yield		
			Experiment	Standard Deviation	Predicted
		min	%	%	%
10	10	60	28.15	0.24	27.72
10	10	60	28.21	0.24	27.72
10	12	30	27.32	0.71	27.38
5	12	60	32.68	0.71	32.29
15	8	60	9.23	0.75	9.61
5	10	90	25.04	0.74	27.31
5	10	30	26.77	0.74	27.09
10	8	90	13.26	0.75	13.19
10	12	90	33.76	0.71	31.87
15	10	90	27.38	0.74	27.07
15	10	30	21.09	0.74	18.82
15	12	60	25.44	0.71	27.64
10	10	60	28.1	0.24	27.72
5	8	60	15.67	0.75	13.47
10	10	60	26.44	0.24	27.72
10	12	60	31.09	0.27	31.12
10	8	30	7.32	0.75	9.21

3. Results and discussion

3.1. Alkaloids removal

Wink (1994) explained that *Lupinus albus* develops quinolizidine alkaloids naturally as a chemical defence system, and these impart a bitter taste even when present in low concentrations. Thus, the traditional use of this variety of lupine in the Mediterranean diet requires an extensive detoxification process, which involves leaching in water and boiling in water and salt. Prepared in this way, the beans are kept in a light brine and used as an aperitif in Mediterranean countries and Portugal. Unfortunately, processing the beans in this way eliminates a large part of the soluble protein together with the bitter alkaloids (principally lupanine), the oligosaccharides and the phytate.

The best treatment for removing the alkaloids was treatment B, under the conditions: NaCl concentration 0.2%, boiling time 45 min. Initial analysis of the beans (before the removal process) indicated a total alkaloid content of 1.39 ± 0.03% in the INIA-Boroa bitter lupine bean. The literature establishes a maximum value of 12.73% alkaloids in *Lupinus albus* sp. (Kroc et al., 2016), depending on the variety of the bean, however there are no previous studies of alkaloid contents in the INIA-Boroa variety to provide a reference. The best sample selected showed removal of 99.15% of the alkaloids (leaving a remnant of 0.85 ± 0.05%).

3.2. Response surface method for the experimental design

The experimental yield of the protein isolates and its standard deviations according to the factorial design are listed in Table 2. The results showed that the yield varied from 7.32% to 33.76%. The standard deviations varied in an interval of 0.4–1.1%, and the maximum yield was achieved with the conditions $X_1 = 10\%$, $X_2 = 12$ and $X_3 = 90$ min. The independent yield variables are related by the following second order polynomial equation:

$$Y = 27.72 - 2.13X_1 + 921X_2 + 2.12X_3 - 0.20X_1X_2 + 2.0X_1X_3 + 0.12X_2X_3 - 1.15X_1^2 - 5.82X_2^2 - 1.49X_3^2$$

where *Y* is the protein isolate yield and X_1, X_2 and X_3 are the independent variables for the flour:basic solution ratio (w/v), pH of the aqueous solution, and mechanical stirring times respectively.

The pH of the mixture is an important factor which can influence protein isolation. The yield affected by the reaction pH is shown in Fig. 1. To investigate the concurrent effect of the parameters designed in this study and find the best preparation conditions, the contour plots were created according to Equation (1). The interactions X_1 and X_2, and X_2 and X_3, have the greatest influence, from which it may be deduced that the consistency of the mixture must be calculated according to the pH of the solution, and the stirring time must be calculated according to the solution, but that the consistency has no influence on the stirring time or vice-versa.

Table 3 shows the analysis of variance for the response surface model. The F-value of 25.32 produced by the model indicates that the

Table 3
Analysis of variance for the quadratic surface model.

Source	Sum of squares	Degree of Liberty	Mean squared	F Value	p-value
Model	971.53	9.00	107.95	25.32	0.0002
X1-Consistency	36.21	1.00	36.21	8.49	0.0225
X2-pH	746.71	1.00	746.71	175.15	<0.0001
X3- stirring time	35.87	1.00	35.87	8.41	0.0230
X1X2	0.16	1.00	0.16	0.04	0.8519
X1X3	16.08	1.00	16.08	3.77	0.0932
X2X3	0.06	1.00	0.06	0.02	0.9070
$X1^2$	5.58	1.00	5.58	1.31	0.2901
$X2^2$	141.74	1.00	141.74	33.25	0.0007
$X3^2$	9.35	1.00	9.35	2.19	0.1821
Residual	29.84	7.00	4.26		
Lack of Fit	27.63	4.00	6.91	9.39	0.0481
Pure Error	2.21	3.00	0.74		

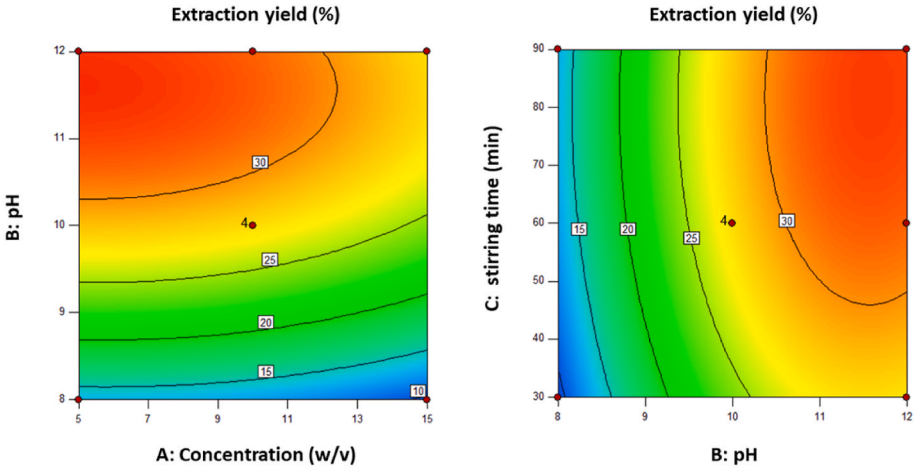

Fig. 1. Contour plot of yield of protein isolate by factors X_1 and X_2, X_2 and X_3.

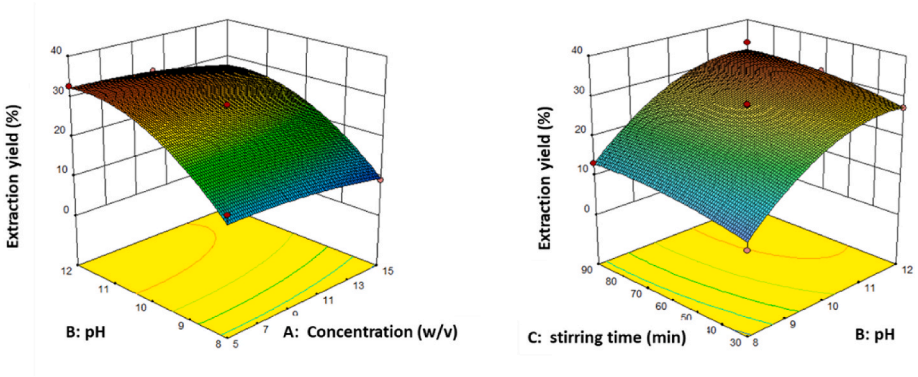

Fig. 2. Response surface for optimum experimental conditions.

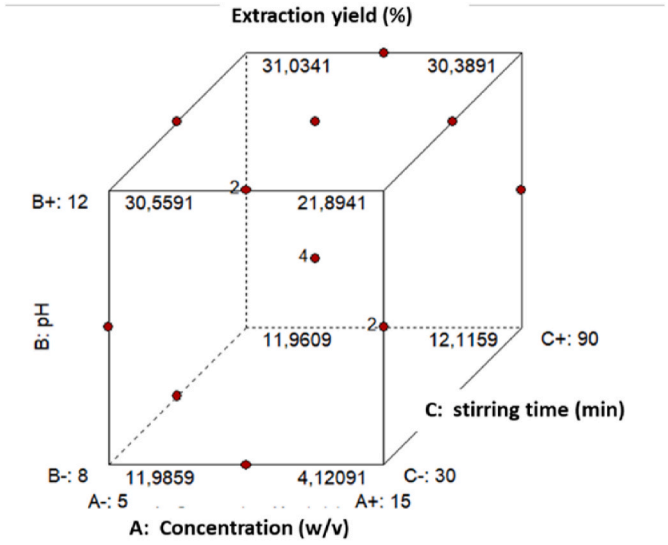

Fig. 3. Response surface for optimum experimental conditions.

model is highly significant, and the p-value of less than 0.0001 shows that the terms of the model are also significant. The coefficient of determination (R^2) of the model (0.9702) is close to 1, with no significant lack of fit ($p > 0.0481$).

The regression model represents the true relation between the responses and the independent variables within the range of experimental variables (Li et al., 2011). It can therefore be confirmed from the analysis that the response surface model is appropriate for optimising the preparation conditions in this investigation. The linear term X_2 (pH) and the quadratic term X_2^2 are significant ($p < 0.001$), indicating that the protein isolate yield was significantly affected by the preparation condition to the same degree.

The response surface plots (Fig. 2) and the cube plot (Fig. 3) are the best methods for investigating the concurrent effect of the pH on the other parameters proposed in this study, principally because they optimise the resources available.

Finally, to obtain the best process conditions, stirring times of between 60 and 90 min are recommended with concentrations (consistency of the mixture) between 5 and 10%, and pH of the solution 12.

The results of the analysis of the functional properties of the protein isolate obtained are given below in Table 3. In general, the solubility increased proportionally with the pH, so the isolate could be a promising option for use in drinks prepared from alkaline plants. The differences found in the lipids adsorption and water retention values may be due to their conformation, surface hydrophobicity, presence of lipophile groups and non-polar amino acids in the samples which presented the greatest oil absorption capacity and the lowest water retention; or possibly to the partial denaturalisation of the proteins during extraction, leading to the exposure of hydrophobic groups of amino acids (Sathe et al., 1982).

3.3. Functional properties of the formulations

All the samples presented low values for swelling capacity, with high hydrophobicity; as a result, the addition of buffer would exercise a

Table 4
Functional properties of the formulations of protein isolate.

(%)/pH/min	%						
	Yield	Solubility in pH 7–9	Water retention capacity	Lipids adsorption index	Swelling capacity	Foaming capacity	Foam stability
10/12/90	65	77	45	61	22	83	85
10/12/30	64	77	47	58	24	88	87
15/10/30	62	76	42	59	21	80	80
15/12/60	62	77	44	60	18	82	82
5/10/90	59	75	45	63	22	87	85
5/12/60	58	78	52	60	20	83	84
10/8/30	56	75	45	59	22	78	80
10/8/90	55	77	40	57	23	78	81
15/8/60	54	76	38	55	22	80	80
10/10/60	52	78	42	59	19	84	83
10/10/60	51	75	48	60	15	85	85
15/10/90	50	77	44	60	17	84	85
10/10/60	50	76	45	59	20	83	84
5/8/60	50	78	38	59	22	83	85
10/10/60	49	78	45	60	19	83	84
5/10/30	48	75	37	55	20	79	81
10/10/60	27	77	45	60	20	78	81

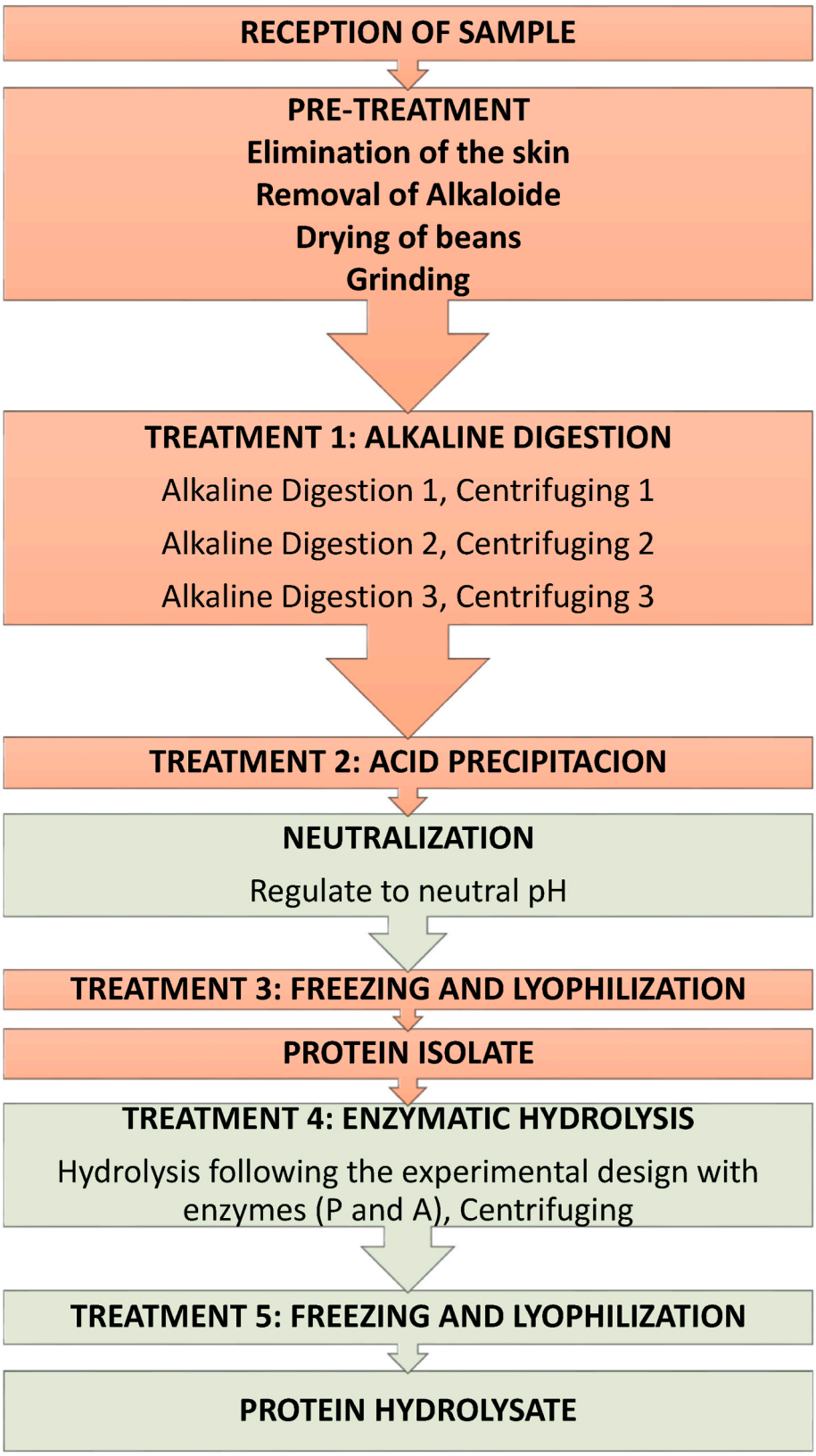

Fig. 4. Process for obtaining protein hydrolysate from *Lupinus albus* (var. INIA-Boroa).

certain pressure on the samples, leading to compaction of the powder and finally to reduced swelling capacity. The last two columns of Table 4 show the foaming capacity and the foam stability. The pH 10 and 12 treatments produced a greater foaming capacity in the protein with basic pH, whereas the greatest stability was obtained with pH 7. The treatments at pH 8 presented lower foaming capacity; this difference may be because a more acid pH is needed in the precipitation stage, so the impact of the pH on the functional properties is greater.

Table 5
Experimental results of the degree of hydrolysis using the enzyme alcalase.

Group	Run	Factor 1	Factor 2	Factor 3	Response 1
		Enzyme Alcalase (%w/w)	pH	Reaction time (min)	Degree of hydrolysis (%)
3	5	1.5	7	120	25
2	4	1.0	7	92	21
4	10	1.0	7	84	20
1	1	1.0	6	92	17
4	9	1.0	6	120	17
1	2	1.0	7	60	15
3	6	1.5	6	60	15
3	7	1.5	8	83	15
5	12	0.5	7	120	14
4	8	1.0	8	120	12
5	13	0.5	6	60	11
5	11	0.5	8	83	9
2	3	1.0	8	60	8

Table 6
Experimental results of the degree of hydrolysis using the enzyme papain.

Group	Run	Factor 1	Factor 2	Factor 3	Response 1
		Enzyme Papain (%w/w)	pH	Reaction time (min)	Degree of hydrolysis (%)
2	5	1.5	8	120	21
1	4	1.0	7	91	18
3	7	1.0	8	60	18
5	12	1.0	7	120	16
5	13	1.0	8	87	16
4	10	0.5	8	120	14
2	3	1.5	7	60	13
3	8	1.0	7	92	13
4	9	0.5	7	60	10
1	1	1.0	6	120	8
2	6	1.5	6	95	7
1	2	1.0	6	60	5
4	11	0.5	6	96	4

Starting from the process of obtaining protein hydrolysate, the following process diagram shows all the stages of the transformation of bitter lupine (Fig. 4).

The procedure for determining the degree of hydrolysis starts with the preparation of the enzymes in buffer solution, followed by production of hydrolysate from the protein isolate; the reaction is then stopped by cold, and centrifuging is performed. The supernatant is subjected to the reagent OPA in different dilutions. After 3 h, the absorbance is read at wavelength 341 nm. Finally, the values are noted, and the reading curve is estimated and substituted in the formula (Equation (6)).

$$GH: Vb \times Nb / Mp \times Ht \times \alpha \times 100 . \quad (6)$$

Where.

GH: Degree of hydrolysis
Vb: Volume of base consumed during the process (ml)
Nb: Normality of the base used (meq/ml)
Mp: Mass of proteins: (g)
α: Degree of dissociation, which will depend on the pH and the temperature used during the process (Source: Adler and Niseen, 1978)
Ht: Total number of peptide bonds contained in the protein molecule (8 meq/g).

3.4. Obtaining and characterization of hydrolyzed protein

The results obtained for the degrees of hydrolysis in both enzymes proposed are given below (Table 5, Table 6).

The above tables show that for treatments with the enzyme alcalase, the best results for GH were runs 5 and 4 of groups 3 and 2 respectively. The best way of using this enzyme was at concentrations of 1.0 and 1.5% w/w (the weight of protein isolates to be hydrolyzed was set at 50 g) at pH 7, with the longest reaction times (120 and 92 min). Acceptable ranges of GH in proteins in general vary between 15 and 30%. The behaviour of the proteolytic enzymes in plant proteins presents a lower hydrolysation range, with a maximum of 27%; this treatment may therefore be considered to meet expectations. The treatments with the enzyme papain present lower GH values, with maximum 21%, while the pH that produced the best action is higher than with alcalase (pH 8).

In analysis of the split-plot response surface statistical model in the hydrolysate treatments, p-values lower than 0.0500 indicate that the terms of the model are significant, indicating that the variables represent differences, and in this case, X_1, X_2, X_3, X_2^2 are significant terms of the model.

Applying the square root equation to predict the behaviour of the variables in the treatment with the enzyme alcalase, we have the following:

$$\sqrt{(\text{degree of hydrolysis})} = -2.804.644 - 0.085139\ (\text{enzyme}) + 860.681\ (\text{pH}) + 0.061775\ (\text{time}) + 0.035779\ (\text{enzyme}) * (\text{pH}) + 0.011208\ (\text{enzyme}) * (\text{time}) + 0.001196\ (\text{pH}) * (\text{time}) - 0.123823\ (\text{enzyme})^2 - 0.648341\ (\text{pH})^2 - 0.000405\ (\text{time})^2.$$

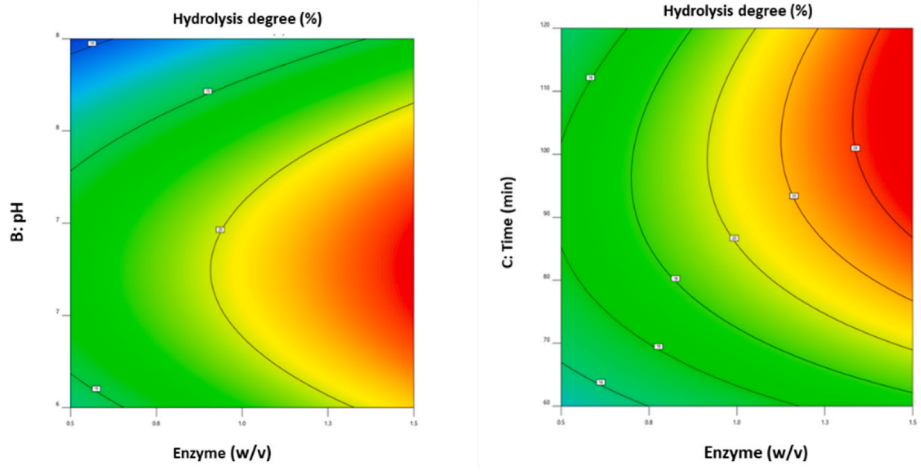

Fig. 5. Contour plot of GH by the factors pH (X_2) and reaction time (X_3) in function of the quantity of enzyme alcalase (X_1).

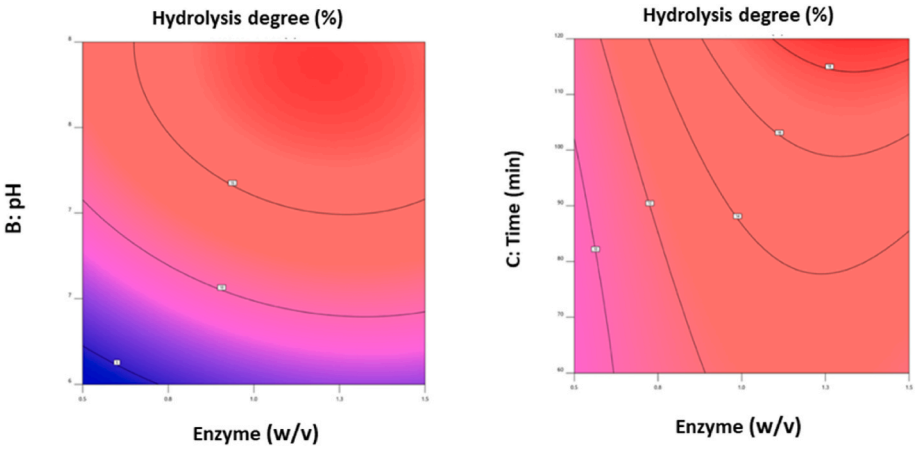

Fig. 6. Contour plot of GH by the factors pH (X2) and reaction time (X3) in function of the quantity of enzyme papain (X1).

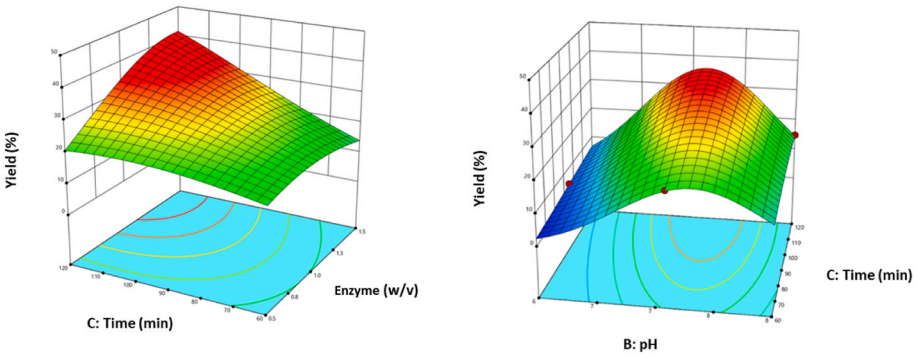

Fig. 7. Response surface plots of the yield by factors pH (X_2) and reaction time (X_3) in function of the quantity of the enzyme alcalase (X_1).

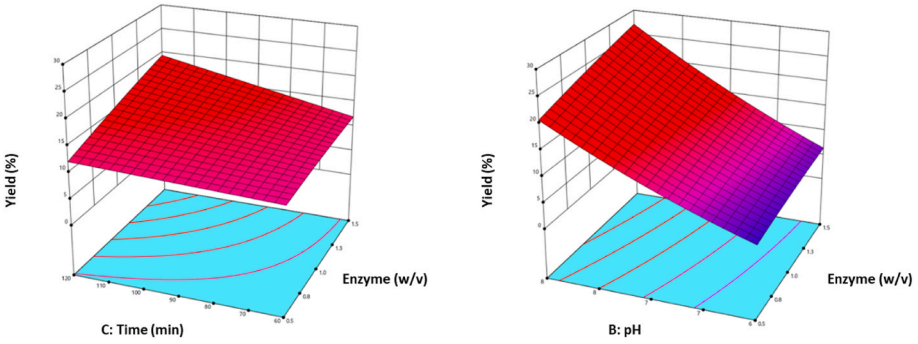

Fig. 8. Response surface plots of the yield by factors pH (X_2) and reaction time (X_3) in function of the quantity of the enzyme papain (X_1).

For the enzyme papain with the log transformation, we have the following format for predicting treatments:

Ln (degree of hydrolysis) = −1.906.216 + 253.165 (enzyme) + 502.316 (pH) +0.011781(time) - 0.136283(enzyme) * (pH) +0.004100 (enzyme) * (time) −0.002363 (pH)* (time) - 0.766311 $(\text{enzyme})^2$–0.295400 $(\text{pH})^2$ + 0.000033 $(\text{time})^2$.

In the protein hydrolysate treatments, similar behaviours can be observed to those of the protein isolate treatments in relation to the variables used. In general, it was the variables pH and reaction time that determined the responses in this project. Fig. 5 shows the critical spaces estimated in the statistical model selected; the enzyme alcalase shows the best performance at pH 7 with reaction times longer than 90 min.

Fig. 6 shows observation contours estimated in the statistical model chosen for the enzyme papain. On this basis we estimate that the best performance was obtained at pH 8, with reaction times greater than 90 min (as with alcalase).

To investigate the concurrent effects of the pH and the reaction time on the difficult variable (enzymes) in this study, and find the optimum preparation conditions, the best type of graph is a surface plot (Figs. 7 and 8), mainly because it optimises the available resources.

With the enzyme alcalase, the reaction time has a greater influence on the yield, since it produces a greater surface area and a maximum value of 35% of the protein isolate (using 50 g).

With the enzyme papain, the pH has a greater influence on the yield, with pH above neutral producing a maximum yield of 27% protein isolate (using 50 g). Finally, to obtain the best process conditions, we recommend reaction times between 90 and 120 min, with concentration between 1.0 and 1.5% w/w of enzymes. With the enzyme alcalase the best pH is 7, whereas with papain it is 8.

Below we detail the analysis results for five selected samples of

Table 7
Results of the Proximal analysis (A – Alcalase) (P – Papain).

Sample	ENERGY [Kcal/100 g]	%						
		HUMIDITY	ASH	PROTEIN	FATS	Carbohydrates	FIBRE*	TOTAL SUGARS*
A-1	393.6	10.2	4.37	43.32	2.35	39.76	1.19	0.66
A-10	384.90	10.0	4.52	32.69	0.90	51.89	1.14	0.86
A-9	393.10	18.2	3.82	52.07	1.93	23.98	1.85	0.78
A-5	408.90	27.0	3.43	60.58	4.04	4.95	2.14	1.05
A-4	403.60	24.4	4.23	56.16	3.93	11.28	1.12	0.91
P-13	398.30	9.1	4.33	35.14	3.16	48.27	1.00	0.47
P-12	389.50	8.9	4.84	26.59	1.96	57.71	1.08	0.45
P-7	390.10	8.8	4.43	32.93	1.73	52.11	1.19	0.75
P-5	393.40	9.0	4.05	35.83	2.04	49.08	0.56	1.20
P-4	395.40	9.2	4.53	42.53	2.79	40.95	0.85	0.80

Table 8
Vitamin contents in selected samples of lupine protein hydrolysate.

SAMPLE	Vit-C*	Vit-D +	Vit-A +	Vit-E +
A-1	4.247	21.343	0	0
A-10	3.364	17.823	0	0
A-9	4.923	12.712	0	0
A-5	3.504	20.212	0	0
A-4	9.312	36.625	0	0
P-13	8.663	7.456	0	0
P-12	4.223	17.744	0	0
P-7	5.568	20.202	0	0
P-5	4.895	25.107	0	0
P-4	4.676	29.483	0	0

* mg/100 g.
+ mg/mL.

protein hydrolysate with the enzyme alcalase and five samples with the enzyme papain (Table 7) (see Table 8).

In these results we particularly note the contents of protein and raw fibre. Since it is considered as a complementary food for balanced nutrition, the protein content offered (over 50% of total protein) is acceptable, and so is the raw fibre content of 2 g in a total of 100 g.

For a balanced diet, the World Health Organization recommends 90 mg/100 g of vitamin C per day (diet of 2000 Kcal) and at least 0.3 μg/mL of vitamin D per day (FAO, 2018). The results of the analysis indicate that protein hydrolysate from bitter lupine provides 10% of the indicated amount of vitamin C, and easily exceeds the recommended minimum daily intake of vitamin D (36 μg/mL).

Arginine is a conditionally essential amino acid (it is only needed in the diet under certain conditions) and can stimulate the immunological function by increasing the number of leukocytes. Arginine is involved in the synthesis of creatine and polyamines, and in DNA. It can reduce the cholesterol to improve the capacity of the circulatory apparatus and stimulate the release of growth hormone (somatropin), as well as reducing levels of body fat and facilitating recovery in athletes through its effects of removing ammonia (muscle residues resulting from anaerobic exercise) from the muscles and converting it into urea which is excreted in the urine. It is used in the biosynthesis of creatine. It is generally found in certain ergogenic products that contain nitric oxide (NO), as it strengthens vasodilatory effects (Benítez et al., 2012).

In Table 9, comparing the arginine content in protein hydrolysate produced from bitter lupine in this study with other widely reported foods which are known sources of this essential amino acid, we may conclude that this experimental product has great potential as a functional food due to the considerable nutritional contribution of its components, including amino acids.

4. Conclusions

The results report the distinctive characteristics of bitter lupine, variety INIA-Boroa, and its potential to produce new products. It shows that bitter lupine beans without the skin contain almost 35% proteins (dry base), with a very interesting amino acids profile and significantly higher arginine content than other high-protein foods such as milk, meat, and soya beans. Arginine is an essential amino acid involved in many of the activities of the endocrine glands. It can stimulate the immunological function by increasing the number of leukocytes, reduce cholesterol and stimulate the release of growth hormone. It reduces levels of body fat and is used in the synthesis of creatine. The proteins in lupine offer excellent functional properties, such as the ability to be incorporated into water as an emulsifying and foaming agent. Protein isolate was obtained that served as a raw material for protein hydrolysate. The method of hydrolysing the protein isolate with the enzyme alcalase is the most suitable for obtaining a high yield with an acceptable protein content, as well as providing a larger number of amino acids

Table 9
Amino acid contents in samples of lupine protein hydrolysate.

Amino acid	A-1	A-10	A-9	A-5	A-4	P-13	P-12	P-7	P-5	P-4
Asp	2.91	4.60	5.25	4.75	4.75	2.95	2.21	2.83	2.95	3.57
Glu	7.15	12.08	13.14	12.68	12.68	8.59	6.57	7.90	8.95	9.43
Ser	1.63	2.74	3.13	2.94	2.94	1.74	1.34	1.69	1.83	2.14
Gly	0.86	1.38	1.71	1.47	1.47	1.11	0.96	1.06	1.10	1.35
Hys	1.08	1.78	1.85	1.99	1.99	1.11	1.56	0.96	1.17	1.33
Arg	3.58	5.76	6.43	6.26	6.26	3.94	2.03	3.63	4.05	4.65
Thr	1.13	1.71	2.00	1.86	1.86	1.14	0.85	1.04	1.08	1.37
Ala	0.62	1.04	1.18	1.21	1.21	0.85	0.57	0.68	0.76	1.00
Pro	1.87	2.79	3.35	3.08	3.08	1.73	1.45	1.83	1.94	2.25
Tyr	1.88	2.75	3.32	2.93	2.93	1.76	1.31	1.69	1.75	2.19
Val	1.42	1.95	2.42	2.11	2.11	1.47	1.10	1.33	1.36	1.68
Ile	1.87	2.97	3.77	3.24	3.24	1.89	1.38	1.77	1.89	2.58
Leu	2.99	4.79	6.09	5.35	5.35	3.13	2.33	2.99	3.15	4.22
Phe	1.92	2.83	3.45	3.03	3.03	1.84	1.41	1.73	1.79	2.33
Lys	1.78	2.90	3.50	3.24	3.24	1.89	1.53	1.81	2.06	2.45

Results reported in g/100 g of sample.

available in the product (degree of hydrolysis).

Credit author statement

Patricia E. Oliveira: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Sergio F. Benavides: Data curation, Writing – review & editing. Daniel Huenufil: Investigation, Visualization. Pablina Zarate: Investigation, Visualization. Ximena Petit-Breuilh: Conceptualization, Data curation, Writing – review & editing, Supervision, Project administration.

Data availability

No data was used for the research described in the article.

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