

Production of lupinus angustifolius protein hydrolysates with improved functional properties

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RESUMEN

Producción de hidrolizados proteicos de *Lupinus angustifolius* con mejores propiedades funcionales.

Se obtuvieron hidrolizados proteicos de la harina del altramuz y de la globulina α -conglutina purificada y se estudiaron sus propiedades funcionales. La hidrólisis con alcalasa durante 60 minutos produjo hidrolizados con grados de hidrólisis entre el 4% y el 11% para la harina y entre el 4% y el 13% para la α -conglutina. Se estudió en un hidrolizado con un 6% de grado de hidrólisis la solubilidad proteica, absorción de aceite, capacidad y estabilidad espumante y actividad y estabilidad emulsificante. Los hidrolizados proteicos mostraron mejores propiedades funcionales que las proteínas originales. Más aún, la solubilidad de los hidrolizados de α -conglutina y la harina se incrementó en un 43% y 52% respectivamente. Así pues, hidrolizados de proteínas de semilla de lupino presentan mejores propiedades funcionales y podrían usarse en la elaboración de productos como pan, dulces, salsas o cremas.

PALABRAS-CLAVE: Alcalasa - Hidrólisis proteica - *Lupinus angustifolius* - Propiedades funcionales.

SUMMARY

Production of *Lupinus angustifolius* protein hydrolysates with improved functional properties

Protein hydrolysates were obtained from lupin flour and from the purified globulin α -conglutin, and their functional properties were studied. Hydrolysis with alcalase for 60 minutes yielded degrees of hydrolysis ranging from 4% to 11% for lupin flour, and from 4% to 13% for α -conglutin. Protein solubility, oil absorption, foam capacity and stability, emulsifying activity, and emulsion stability of hydrolysates with 6% degree of hydrolysis were determined and compared with the properties of the original flour. The protein hydrolysates showed better functional properties than the original proteins. Most importantly, the solubility of the α -conglutin and *L. angustifolius* flour hydrolysates was increased by 43% and 52%, respectively. Thus, lupin seed protein hydrolysates have improved functional properties and could be used in the elaboration of a variety of products such as breads, cakes, and salad dressings.

KEY-WORDS: Alcalase - Functional properties - *Lupinus angustifolius* - Protein hydrolysis.

1. INTRODUCTION

Besides nutritional value, functional properties are very important characteristics of foods. Thus, chemical modification has been extensively used to improve the functionality of proteins. Among the various chemical modification techniques, acetylation with acid

anhydrides has been widely employed with proteins from different plant sources (Kim and Kinsella, 1987). Although succinylated proteins were functionally superior to unmodified proteins, incorporation of chemically modified proteins was of nutritional concern because succinylated bonds were not hydrolyzed by gastric and pancreatic proteases (Shukla, 1982). In contrast, enzymatic modification of proteins is not likely to be a safety concern, and thus enzymatic modification may be a viable alternative to chemical modification (Kim et al., 1990).

Enzymatic hydrolysis of proteins has a great potential for modifying functional properties of food proteins. The production of enzymatic protein hydrolysates has undergone considerable development in recent years. Protein hydrolysates can be classified according to their degree of hydrolysis. Protein hydrolysates with low degree of hydrolysis (<10%) frequently have better functional properties than the original proteins and are used as food ingredients (Vioque et al., 2000). Protein hydrolysates with variable degrees of hydrolysis are used as flavorings in soups, sauces, and meat products (Weir, 1986). Extensive protein hydrolysates are used as protein supplements or in special medical diets, such as hypoallergenic foods (Frokjea, 1994). Investigations on the effect of proteolysis on the emulsifying properties of plant proteins such as soy bean (Kim et al., 1990) and faba beans (Krause y Schewenke, 1995) have been carried out. Other legumes such as peas (Periago et al 1998), and chickpeas (Clemente et al., 1999) are becoming increasingly important as a source of edible protein hydrolysates with good functional and nutritional properties (Guegón, 1991). Additional sources of plant protein hydrolysates are the under-utilized by-products of the oil extraction industry, including sunflower (Villanueva et al., 1999) and rapeseed (Vioque et al., 2000) defatted flours.

In the present paper we describe the production of *L. angustifolius* protein hydrolysates and the characterization of their functional properties (solubility, fat absorption, emulsion capacity and stability, foaming capacity and stability) in order to determine the potential application of these hydrolysates in food processing.

2. MATERIALS AND METHODS

Materials

L. angustifolius seeds were a gift from Koipesol Semillas, S.A. (Sevilla, Spain). The seeds were ground and extracted with hexane in a soxhlet extractor for 9 h. The resulting defatted lupin flour was used as the starting material. Trinitrobenzenesulfonic acid (TNBS) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Proteolytic enzyme

Alcalase 2.4 I (Novo nordisk, Bagsvaerd, Denmark) was used for protein hydrolysis. A main component of this commercial preparation is subtilisin A, a serine protease from *Bacillus licheniformis* with endopeptidase activity. The specific activity of alcalase is 2.4 Anson Units (AU) per gram. One AU is the amount of enzyme that, under standard conditions, digests hemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product that gives the same color with the Folin reagent as 1 meq of tyrosine released per minute.

Total nitrogen

Total nitrogen was determined in 0.1 g samples by the micro-Kjeldahl method (AOAC, 1975). Crude protein content was estimated using a conversion factor of 5.8.

Purification of α -conglutin

L. angustifolius seeds (100 g) were ground in a blender (particle size 0.2-0.5 mm) and extracted under stirring with 1,000 ml 0.1 M sodium borate buffer (pH 8.3) at room temperature for 1 h, according to the method of Singh et al. (1988). The extract was centrifuged at 8,000 g for 15 min. The supernatant was dialyzed against 25 mM sodium citrate buffer (pH 4.6) at 4 °C for 15 h and centrifuged as before. The supernatant and pellet corresponded to the albumin and globulin fractions, respectively. The globulin precipitate was dissolved in 0.1 M sodium borate (pH 8.3) containing 0.2 M sodium chloride buffer, and dialyzed against 25 mM sodium citrate buffer (pH 4.6) at 4 °C for 15 h and then centrifuged. The supernatant was discarded, and the precipitated legumin fraction was freeze-dried. The legumin fraction was dispersed by shaking in 0.1 M sodium borate (pH 8.3) containing 0.2 M sodium chloride buffer (1:20 w/v), and insoluble materials were eliminated by centrifugation at 8,000 g for 15 min. The supernatant was eluted with 0.19 M sodium phosphate and 19 mM sodium citrate buffer (pH 7.0) in a PD-10 column (Pharmacia LKB) in order to

remove non-protein compounds, and loaded in a protein Pak DEAE 8 H 1000 A 8 m anionic exchange column (Waters). Protein purification was carried out using a binary gradient system. The solvents used were as follows: buffer A, 0.19 M sodium phosphate, 19 mM sodium citrate (pH 7.0); buffer B, 0.19 M sodium phosphate, 19 mM sodium citrate, 0.5 M sodium chloride (pH 7.0). Solvents were delivered to the column as follows: time 0-10 min, elution with 100% buffer A; 10-25 min, linear gradient from 0 to 28% buffer B; 25-35 min, elution with 28% buffer B; 35-45 min, linear gradient from 28% to 100% buffer B; 45-55 min, elution with 100% buffer B; 55-60 min, linear gradient with 100% buffer A. Flow rate was 1 ml/min, and injection volume and sample concentration were 10 ml and 18 mg of protein/ml, respectively. α -Conglutin enriched fractions were pooled for further purification.

α -Conglutin was further purified by gel filtration chromatography using a Superose 12 column (Pharmacia). Elution was carried out using 25 mM sodium phosphate buffer (pH 7.0) containing 0.5 M sodium chloride, and a flow of 0.5 ml/min. Volume injection and sample concentration were 500 μ l and 8-10 mg of protein/ml, respectively.

Hydrolysis

L. angustifolius α -conglutin and defatted flour (5% w/v in water) were hydrolyzed in a reactor vessel equipped with a stirrer, thermometer, and pH electrode. Hydrolysis was carried out for 60 min at room temperature, pH 8, with an enzyme to substrate ratio of 0.01 AU/g. Hydrolysis was stopped by heating at 85 °C for 10 min. Hydrolysates were clarified by filtering to remove insoluble material, and the filtrate was freeze-dried for further use.

Degree of hydrolysis

The degree of hydrolysis, defined as the percentage of peptide bonds cleaved, was calculated by determining free amino groups by reaction with TNBS according to Adler-Nissen (1979). The total number of amino groups was determined in a sample that had been 100% hydrolyzed at 110 °C for 24 h in 6 N HCl (10 mg sample in 4 ml HCl).

Determination of the isoelectric point (*I*_p)

For determination of the *I*_p, 15 g of defatted flour were extracted twice with 300 ml 0.2% NaOH (pH 12) and centrifuged at 8,000 g for 20 min. Aliquots (40 ml) of the supernatant were titrated with 0.5 N HCl to various pH values, ranging from 2.5 to 6.5, and centrifuged as above. The percentages of total

nitrogen that were found in the supernatants were plotted versus pH in order to determine the Ip.

Superose 12 gel filtration chromatography

Lyophilized samples (1 g) were dissolved in 10 ml 0.1 M sodium borate buffer (pH 8.3) containing 0.2 M sodium chloride. Gel filtration was carried out in an FPLC system equipped with a Superose 12 HR 10/30 column from Pharmacia LKB Biotechnology (Sweden). Volume injection and concentration of the samples were 200 μ l and 1.6 mg of protein/ml, respectively. The eluent was borate buffer as above at a flow rate of 0.4 ml/min. Elution was monitored at 280 nm. Approximate molecular masses were determined using blue dextran 2000 (2,000 kDa), β -amylase (200 kDa), bovine serum albumin (67 kDa) and ribonuclease A (13.7 kDa) as molecular weight standards.

Amino acid analysis

Samples containing 2 mg of protein were hydrolyzed in 6 N HCl at 110 °C for 24 h and derivatized with diethyl ethoxymethylenemalonate. Amino acids were determined by reversed-phase high-performance liquid chromatography using D,L- α -amino butyric acid as internal standard and a 300 x 3.9 mm id. reversed phase column (Nova Pack C18, 4 μ m, Waters). A binary elution gradient of 25 mM sodium acetate (pH 6.0) and acetonitrile was used and the column was maintained at 18 °C (Alaiz et al., 1992). Amino acid composition was expressed as g of amino acid per 100 g of protein.

Fat absorption

The method of Lin et al. (1974) was used for determination of fat absorption. Samples (0.5 g) were mixed with 6 ml of Soya oil. After 30 min the mixture was centrifuged at 1,600 g for 25 min and the volume of the supernatant was weighed. The oil absorption capacity was expressed as the number of grams of oil retained by 100 g of material.

Emulsion activity and stability

The emulsifying activity was determined according to Naczek et al. (1985) with modifications. Samples (3.5 g) were homogenized for 30 seconds in 50 ml water using a model A Polytron homogenizer (Brinkmann, Wesbury, NY, USA) at a setting of 6 (approximately 10,000 rpm). Canola oil (25 ml) was added, and the mixture was homogenized again for 30 seconds. Then, another 25 ml of canola oil were added, and the mixture was homogenized for 90 seconds and centrifuged at 1,100 g for 5 min. Emulsifying activity was calculated by dividing the volume of the emulsified layer by the

volume of emulsion before centrifugation x 100. Emulsion stability was determined using the same samples that were prepared for determination of emulsifying activity. Samples were heated for 15 min at 85 °C according to Naczek et al. (1985), cooled, and centrifuged at 1,100 g for 5 min. The emulsion stability was expressed as the percentage of emulsifying activity remaining after heating.

Foaming activity and stability

The activity and stability of foam were determined by the method of Lin et al. (1974). 50 ml of a 3% dispersion of material in distilled water were homogenized using a model A Polytron homogenizer as above. The mixture was immediately transferred into a 250 ml graduated cylinder and the foam volume measured. The foaming activity was expressed as the percentage of volume increase. Foam stability was expressed as foam volume remaining after 20, 40, 60, and 120 min.

3. RESULTS AND DISCUSSION

*Production of *L. angustifolius* protein hydrolysates*

The goals of this research were to set the conditions for limited hydrolysis of lupin flour and α -conglutin, and to determine whether this hydrolysis results in improved functional properties. Hydrolysis of lupin flour for 60 minutes yielded protein hydrolysates with a degree of hydrolysis of up to 10% (Figure 1A). Degrees of hydrolysis of up to 13% were obtained by hydrolysis of α -conglutin (Figure 1B). Several functional properties were studied in hydrolysates with a 6% degree of hydrolysis. These hydrolysates were obtained using a very low enzyme to substrate ratio because alcalase has a very high specific activity (Mannheim and Cheryan, 1992). After several trials, an enzyme to substrate ratio of 0.01 mg/g was chosen, with a substrate concentration of 5% (w/v). Under these conditions, hydrolysates with 6% degree of hydrolysis were obtained after 17 minutes of treatment.

The molecular weight profile of the lupin flour hydrolysate yielded fractions of lower molecular weight, FI and FII, with molecular weights of 8 kDa and 2 kDa, respectively (Figure 2A). α -Conglutin obtained by gel filtration chromatography shows a main peak with 216 kDa apparent molecular weight. Hydrolysis caused the disappearance of this peak and the appearance of other peaks with molecular weights ranging from 18 to 4 kDa (FI and FII) (Figure 2B).

Protein solubility

Hydrolysis with alcalase improved the solubility of the starting materials. Solubility increased at the

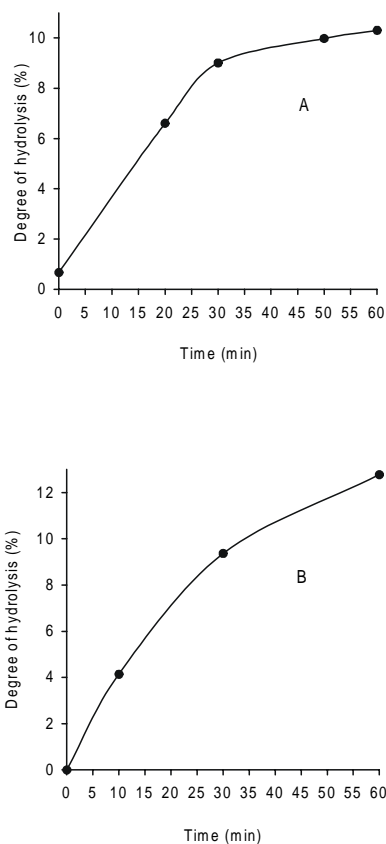


Figure 1
Kinetic of enzymatic hydrolysis of defatted lupin flour (A) and α -conglutin (B) with alcalase. Hydrolysis was carried out for 1 hour at room temperature and pH 8 with an enzyme to substrate ratio of 0.01 mg/g and a substrate concentration of 5% (w/v).

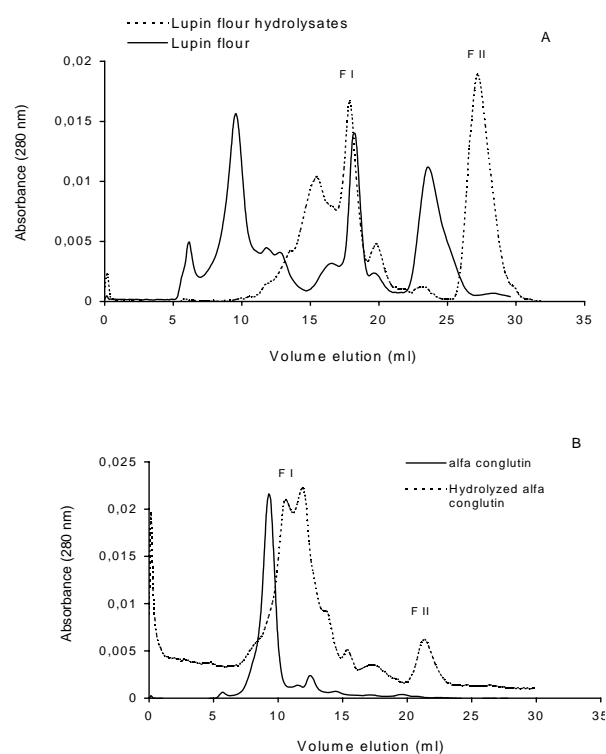


Figure 2
Superose 12 gel filtration chromatography of A) lupin flour proteins and hydrolyzed flour proteins, and B) native α -conglutin and hydrolyzed α -conglutin.

isoelectric point 4.3 by 55% and 43% for lupin flour (Figure 3A) and α -conglutin (Figure 3B), respectively. This improvement in solubility is due to decreased molecular size and an increase in amino and carboxyl groups (Deeslie and Cheryan, 1988).

Fat absorption

Fat absorption capacity is due to binding of fat by non-polar side chains of proteins. The digestion of proteins may bring out of the core of proteins non-polar side chains that bind hydrocarbon moieties of oil, contributing to increased oil absorption. Lupin flour hydrolysates had higher oil-holding capacity than the original flour (Table 1). Fat absorption was higher than in commercial preparations such as sodium caseinate or dry egg white, which have fat absorptions of 150 to 200 g/100 g (Ahmedna et al., 1999). The fat absorption of hydrolyzed α -conglutin was also higher than in protein hydrolysates of the lupin flour and the native α -conglutin (Table 1).

Foaming capacity and stability

Some food proteins are capable of forming good foams, and their capacity to form and stabilize foams depends on the type of protein, degree of denaturation, pH, temperature and whipping methods. The foaming capacity of α -conglutin hydrolysates was higher than that of native α -conglutin (Table 1). Hydrolyzed flour also presented a higher foaming capacity than lupin flour. α -Conglutin hydrolysate had the highest foaming capacity, 179%. However, the foam stability of the hydrolysates decreased drastically with respect to lupin flour protein and α -conglutin (Table 1).

It has been reported that limited proteolysis may improve foaming capacity but decrease foam stability (Chobert et al., 1988). Lupin protein hydrolysates showed higher foaming capacity than the original protein. Apparently, hydrolysates with 6% degree of hydrolysis are capable of improving foaming as the result of the reduction in protein size. Although the peptides with relatively small molecular weight were probably capable of forming films with the air interface, it is possible that the films were not strong enough to maintain their integrity.

Table I
Functional properties of lupin flour proteins (LFP), lupin flour protein hydrolysate (LFPH), α -conglutin (α -C) and hydrolyzed α -conglutin (H α -C)

	LFP	LFPH	α -C	H α -C
Fat absorption	153.5 \pm 4.5	359.9 \pm 1.9	286.1 \pm 0.2	371.6 \pm 3.2
Foaming capacity (%)	114.0 \pm 2.0	120.5 \pm 1.5	117.0 \pm 1.0	179.0 \pm 1.0
Foaming stability (%)				
at 20 min	96.3 \pm 1.4	17.6 \pm 0.4	96.8 \pm 0.8	33.0 \pm 1.5
at 40 min	93.8 \pm 2.7	11.9 \pm 0.9	95.6 \pm 0.5	22.9 \pm 2.5
at 60 min	92.5 \pm 2.3	6.0 \pm 1.4	94.9 \pm 0.9	17.3 \pm 2.0
at 120 min	90.3 \pm 1.1	3.5 \pm 0.2	94.1 \pm 0.6	12.2 \pm 1.0
Emulsion activity (%)	74.0 \pm 3.1	88.7 \pm 3.0	72.5 \pm 2.5	99.7 \pm 2.1
Emulsion stability (%)	69.4 \pm 4.1	57.9 \pm 3.1	68.8 \pm 1.3	60.9 \pm 2.4

Data are the mean \pm SD of two independent experiments.

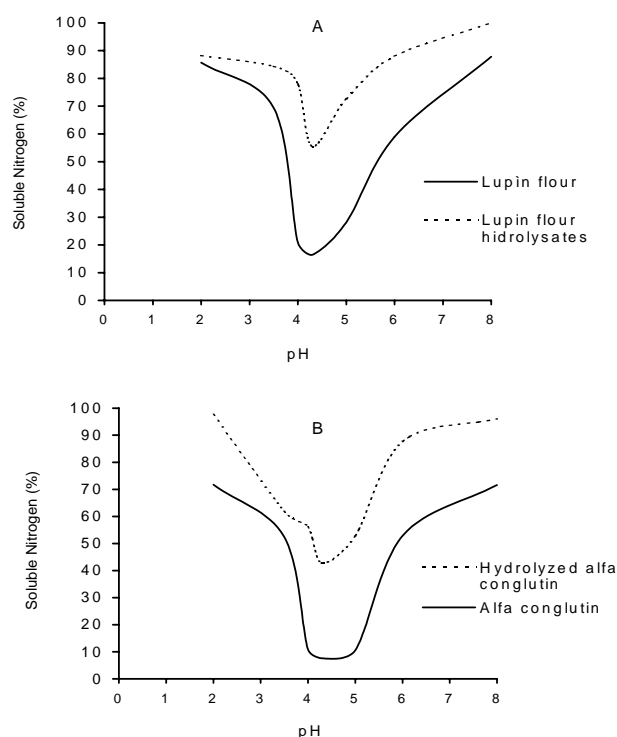


Figure 3

Solubility curve of lupin flour proteins (A) and α -conglutin (B), and their corresponding hydrolysates. Protein solubility is expressed as percentages of soluble nitrogen at various pH values.

Emulsion activity and stability

Interactions between proteins and lipids are common in many food systems, and thus the ability of proteins to form stable emulsions is important. An increase in the number of peptide molecules and exposed hydrophobic amino acid residues due to hydrolysis of proteins would contribute to an improvement in the formation of emulsions.

Hydrolysates of lupin flour and α -conglutin had better emulsifying activity than native lupin flour and α -conglutin, and hydrolyzed α -conglutin had higher emulsion activity than hydrolyzed lupin flour (Table 1). However, the emulsifying stability of both hydrolysates decreased with respect to native proteins (Table 1). Thus, considering these emulsifying properties, lupin protein hydrolysates could be used as ingredients in emulsion-based food formulations such as salad dressing and mayonnaise.

Proteolytic enzyme modification is an effective way to improve functional properties. Treatment of α -conglutin and lupin flour with alcalase produces hydrolysates with a 6% degree of hydrolysis and enhanced functional properties, while maintaining the amino acid composition of the starting material (data not shown). Hydrolysis results in increases in solubility, fat absorption, emulsifying activity and foaming capacity. Thus, these hydrolysates have potential uses in the production of foods such as

salad dressings, ice creams, mayonnaises, etc, where functional properties are especially important.

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