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Functional Assessment and Subunit Constitution of Lentil (*Lens culinaris*) Proteins during Germination

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ABSTRACT

Germination is of major importance in improving nutritional attributes of legumes for human consumption. In this study, we attempted to investigate the effect of germination on globulin subunits and functional properties of lentil proteins. Germination caused a notable decrease in protein content and significant increase in non-protein nitrogen. Results from gel electrophoresis showed that proteolytic cleavage occurred in acidic subunit of legumin and the largest subunit of vicilin in early stages of germination. Germinated lentil proteins had improved solubility and water and fat adsorption properties. Foaming capacity of germinated lentil proteins was higher than that of non-germinated seeds, while the foam stability decreased progressively. These improved functional properties make germinated lentil proteins a useful product to be used in food formulations in order to enhance their quality.

Key Words: Lentil; Globulins; Legumin; Vicilin; Germination; Functional properties

INTRODUCTION

Although traditional animal sources of protein are superior for their nutritional and functional properties, but they cannot continue to meet adequately the growing need for food proteins (Marcone, 1999). The current increased interest in utilization of legume grains stems not only from the fact that they typically possess two to four times the quantity of protein than traditional cereal grains, but that their proteins are typically of higher nutritional quality (Adams *et al.,* 2004; Dogan *et al.,* 2005). They are also of increasing importance in the manufacture of various pharmaceutical and nutraceutical/functional food products (Mandal & Mandal, 2000). Among leguminous species, lentil (*Lens culinaris* Medik.) is consumed in appreciable amounts in Middle East countries. Lentil proteins, composing 22-31% of the seed dry weight, have an amino acid composition similar to those of other legumes (Frias *et al*., 1995). Even though it may have all prerequisites to be nutritionally superior, a protein will have no effect on human nutrition unless it has the functional properties necessary for its successful incorporation into food systems (Koyoro & Powers, 1987). So, it is necessary to investigate the functional properties of plant proteins and if necessary, incorporate desirable functional properties in the protein through modification (Bora, 2002). Germination of legume seeds has been documented to be an effective treatment to reduce anti-nutritional factors and improve the nutritional quality by increasing the level of some amino acids, vitamins and minerals (Urbano *et al*., 1995; Vidal-Valverde *et al*., 2002). Germination causes important changes in the biochemical, nutritional and sensory characteristics of legume seeds, due mainly to enzyme activity in moist seeds, which is engaged in protein and starch hydrolysis (Sadowska *et al*., 1999). Increased enzymatic activities in the germinating seeds are usually accompanied by inter-conversion and production of new compounds (Wanasundara *et al.,* 1999; Ahmed *et al.*, 2003).

There are some reports about the effect of germination on the nutrient and anti-nutrient contents of some legumes (Bartolome *et al*., 1997; Kuo *et al*., 2004), but very little information is available on changes in functional attributes and subunit structures of lentil proteins. With these in sight, the purpose of the present work was to study the effect of germination on protein subunits and functional properties of Persian lentil proteins.

MATERIALS AND METHODS

Materials. Lentils of small seeded Persian type (*Lens culinaris* Medik. cv. Gachsaran) produced in the south western area of Iran (Gachsaran) were obtained from Gachsaran Agricultural Research Center, Gachsaran, Iran.

Determination of the isoelectric point (IEP). Fifteen grams of lentil flour were extracted twice with 300 mL 0.2% NaOH and centrifuged at 8000 × *g* for 15 min. Pellet

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was further extracted for another hour with half the volume of alkaline solution. Aliquots (40 mL) of the pooled supernatants were titrated with 0.5 N HCl to various pH values, ranging from 3 to 7 and precipitates were separated by centrifugation as above. A 20 mL volume of the clear liquid was transferred to Kjeldahl tubes for sub-sequent nitrogen determination. Percentages of soluble nitrogen in the supernatants in relation to the total nitrogen extracted were plotted vs. pH to determine the IEP (Sanchez-Vioque *et al*., 1999).

Preparation of lentil protein isolate and enriched protein fractions. Vicilin and legumin were purified by a non-denaturing fractionation procedure adapted from the method of Koyoro and Powers (1987) and O'Kane *et al*. (2004). Salt-soluble proteins were extracted (1:10) into a 100 mM Tris-HCl buffer, pH 8.0 and the extract was collected by centrifugation (12000×g, 10°C, 25 min). Isoelectric precipitation, pH 4.5 was used to isolate the globulin proteins. Precipitated protein was left for 2 h, 4°C before it was collected by centrifugation. The pellet was suspended in the extraction buffer, pH 8.0 (10 mg mL^{-1}) and dialysed at 4°C for 24 h against McIlvaine's buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid, containing 0.2 M NaCl), pH 4.8. Centrifugation of the sample (19000×g, 4°C, 25 min) collected a precipitated fraction (referred to as legumin-rich) and a clear supernatant. This supernatant was desalted by further dialysis at 4°C against McIlvaine's buffer, pH 4.8, with no salt. Centrifugation of the sample obtained a second precipitated fraction (referred to as vicilin-rich). These fractions were freeze-dried.

Germination. Seeds were germinated in a semi-pilot scale. 500 g of lentil seeds were pre-treated with 2500 mL of 0.07% sodium hypochlorite solution for 30 min at room temperature to remove surface contamination. Seeds were then drained, washed to neutral pH and then soaked in distilled water for 5.5 h. Finally, imbibed seeds were transferred to Petri dishes lined with wet filter paper and germinated in a seed germinator (Memmert 854, Schwa Bach, Germany) in the dark at 20°C. Seeds were sprayed daily by distilled water and harvested at different stages of germination (imbibed seeds, 1st, 3rd & 5th day). Germinating seeds were frozen at -18°C for 12 h to stop the germination process, then freeze-dried and ground to pass through a 40 mesh sieve (Kuo *et al*., 2004). For peptide extraction, seed flour was homogenized in a 10-fold volume of 25 mM citrate-phosphate buffer, pH 5, containing 2 mM β-mercaptoethanol, filtered, centrifuged (12000×g, 20 min, 4°C) and the supernatants were freeze dried (Chrispeels & Boulter, 1975).

Nitrogenous compounds of germinated lentils. Soluble protein, insoluble and non-protein nitrogen were measured according to the method described by Periago *et al*. (1996).

Gel electrophoresis. SDS gel electrophoresis of protein fractions and germinated lentils was performed according to the method of Laemmli (1970) with minor modifications. Samples were solubilized in a buffer consisting of 50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 5% β-mercaptoethanol. Equal amounts of total nitrogen (20 µg) were loaded in each lane. Electrophoresis was performed on discontinues 5%-stacking and 13%-resolving gels and gels were fixed and stained with 0.2% coomassie brilliant blue R-250 in methanol: acetic acid: water (5:4:1 v/v/v). Molecular weight of the protein bands was estimated from their relative mobility in gel (Ferguson Plot) (Copeland, 1994).

Functional Properties

Nitrogen solubility index (NSI). The nitrogen-solubility index of each sample was determined according to the method of Wanasundara and Shahidi (1994).

Water and fat adsorption. Water and fat adsorption were studied according to the methodologies described by Wanasundara and Shahidi (1994).

Foaming capacity and stability. Foaming capacity and stability of the samples were studied according to the method of Kim *et al*. (2004). The foaming capacity was calculated according to the following equation:

$$\text{Foaming capacity (\%)} = \frac{V_2 - V_1}{V_1} \times 100$$

V_2: Volume after whipping (mL).

V_1: Volume before whipping (mL).

Foam stability was expressed as the percentage of foam remaining after 60 min.

Statistical analysis. All results were expressed as the mean ± standard error (n_{min} = 3). The statistical analysis of data was performed using statistical software system (SAS institute, Cary, NC, USA). Student *t*-test was used to compare the averages.

RESULTS AND DISCUSSION

Isolation and partial purification of lentil globulin fractions. Total nitrogen content of lentil flour and protein isolate was 3.7% and 13.6%, corresponding to 23.1% and 85% total protein, respectively. The final protein recovered from lentil flour was 56% in lentil protein isolate. The isoelectric point of the lentil proteins was determined in order to optimize the production of protein isolate. The lowest protein solubility was achieved at pH 4.5 (Fig. 1).

Legumin and vicilin-rich fractions contained 17.6% and 16.03% nitrogen, respectively. Derbyshire *et al.* (1976) reported a nitrogen content of 18.04% for pea legumin and 17.40% for vicilin. The electrophoretic patterns of the protein fractions are shown in Fig. 2.

Subunits of 40 and 20 kDa have been reported to be the acidic and basic subunits of pea legumin, respectively (Derbyshire *et al.,* 1976). Several minor subunits having the molecular weight of 9, 25, 55-70 and 91 kDa can also be observed. Hurkman and Beevers (1980) reported a 93 kDa polypeptide in legumin, which may correspond to the most cathodic (largest molecular weight) polypeptide in the

Table I. Effect of germination on total, insoluble, protein and non-protein nitrogen content of lentil

Nitrogen (g/100 g DM)	Non-germinated lentil	Germinated lentil		
		1st day	3rd day	5th day
Total	3.99 ± 0.01[a]	4.01 ± 0.01[a]	4.14 ± 0.02[a]	4.44 ± 0.01[a]
Insoluble	0.32 ± 0.02[a]	0.35 ± 0.02[a]	0.25 ± 0.02[a]	0.21 ± 0.03[a]
Soluble protein	3.09 ± 0.02[a]	2.84 ± 0.03[b]	2.01 ± 0.04[c]	2.59 ± 0.03[c]
Soluble non-protein	0.52 ± 0.03a	0.67 ± 0.04a	1.05 ± 0.03b	1.49 ± 0.02c

DM, dry matter; N, nitrogen

*Values are mean ± standard deviation (n = 3), the same superscript letter in the same row indicated no significant difference (P<0.05)

Table II. Water and fat adsorption capacities of lentil proteins before and after germination

	Water adsorption (%)	Fat adsorption (%)
Lentil flour (non-germinated)	197.3 ± 6.2[a]	144.4 ± 1.3[a]
1st day of germination	204.6 ± 5.0[ab]	156.6 ± 4.1[b]
3rd day of germination	234.7 ± 4.9[c]	176.4 ± 4.0[c]
5th day of germination	223.3 ± 6.6[bc]	218.1 ± 0.9[d]

* Values are mean ± standard deviation (n = 3). The same superscript letter in the same column indicated no significant difference (P<0.05)

legumin fraction. Major subunits of 50, 30-35 and 19 kDa along with a minor lower MW polypeptide (approx. 12 kDa) have been reported for vicilin fraction (Koyoro & Powers, 1987). Subunits of 59-64 kDa are enriched in the vicilin fraction. However, these and several other polypeptides are also found in the legumin fraction. The cross contamination of legumin and vicilin fraction prepared by isoelectric precipitation is common.

Germination. Assayed non-germinated lentil flour had an averaged 3.70 ± 0.1 g of total nitrogen/100 g of dry matter, of which 13.2% corresponded to soluble non-protein nitrogen, 77.3% to soluble protein nitrogen and 8.1% was insoluble at the basic pH condition used for extraction (Table I).

Insoluble nitrogen content decreased during the germination period. The insoluble nitrogen residue has been described as proceeding from non-covalent interactions or from disulfide bonds between different proteins (Urbano *et al*., 2005). The decrease in the level of insoluble nitrogen as a result of the germination process found in the present experiment was similar to that reported by Wanasundara *et al*. (1999) in flax seeds. Soluble non-protein nitrogen progressively increased throughout the germination periods. SDS-electrophoresis patterns of extracts of lentil that had germinated for various lengths of time are shown in Fig. 2. In germinated lentils there were several protein bands resistant to proteolysis. As judged by the band width and intensity, the four original subunits (35, 30, 12, 11 kDa) of vicilin and the basic subunit of legumin remain intact at the 5th day of germination. On the other hand, the acidic subunit of legumin and the largest subunit of vicilin (55 kDa) were degraded after 3 days of germination. Their break down was accompanied by generation of new subunits of approximately 28-32, 20 and 10 kDa, as well as a large

Fig. 1. Solubility curve for lentil proteins as a function of pH. Solubility of the proteins is expressed by measuring the relative percentages of nitrogen soluble at various pH values

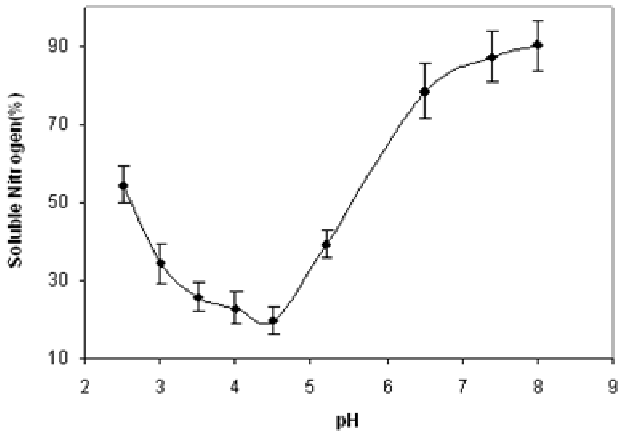
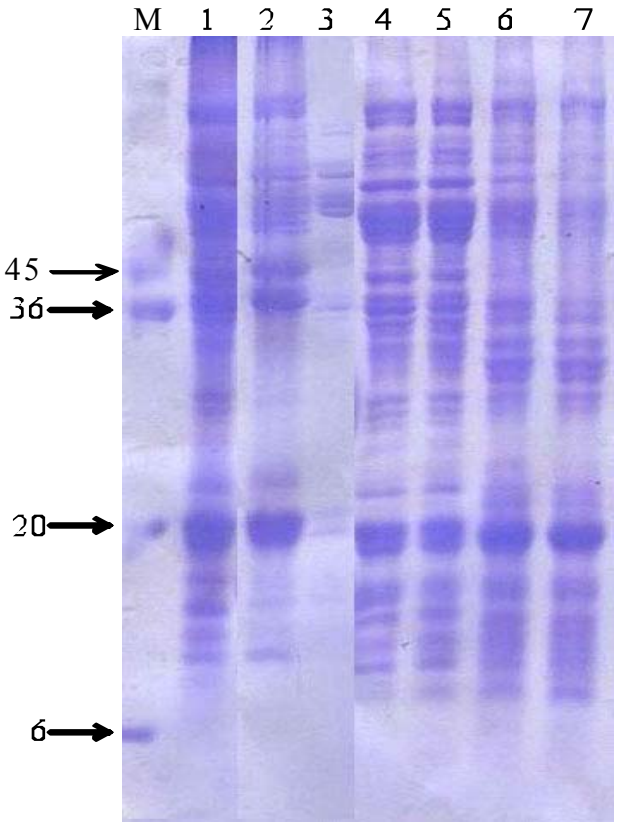


Fig. 2. SDS-PAGE profiles of lentil proteins

M; molecular weight marker, 1; Lentil protein isolate, 2; legumin-rich fraction, 3; vicilin-rich fraction, 4; imbibed seeds, 5, 6 and 7; germinated lentils at the 1st, 3rd and 5th day of germination



amount of very low MW peptides (8-12 kDa). This pattern of storage protein breakdown was similar to that reported for mung bean and pea, where the largest subunit of the major storage protein of these plants was the first to be broken down during germination (Nielsen & Liener, 1984).

Fig. 3. Nitrogen solubility of lentil flour, isolate (LPI) and germinated lentils at 1st, 3rd and 5th day of germination

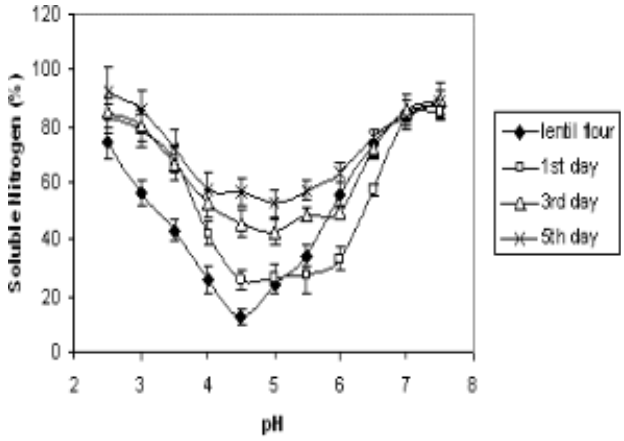
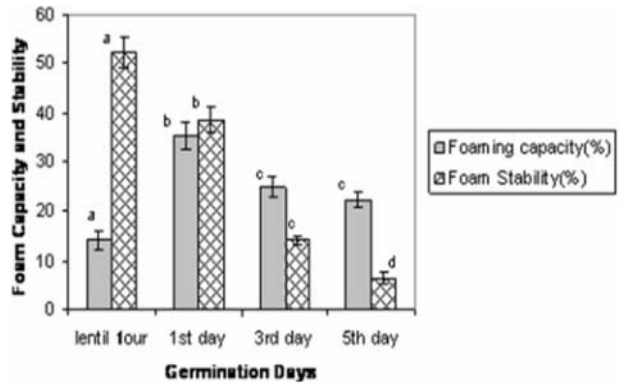


Fig. 4. Foaming capacity and foam stability of lentil protein isolate and germinated lentils at different days, results are means from 3 replicates, Different letters show significant differences (p< 0.05) between samples



Functional Properties

Nitrogen solubility index. The solubility profiles of lentil protein isolate and germinated lentils in the pH ranges of 2.5 to 7.5 are shown in Fig. 3.

Following germination, the solubility at pH values below the isoelectric point increased and approached that of 90% solubility at 5th day of germination. Germinated lentils showed 2.1, 3.7 and 4.6-fold more solubility at 1st, 3rd and 5th day of germination, respectively, compared to non-germinated lentil.

Water and fat adsorption. The data on the water and fat adsorption capacities of lentil flour and germinated lentil are shown in Table II.

Germination increased water adsorption capacity of lentil proteins from 197.3 to 223.3% after 5 days germination. Fat adsorption capacity has also increased gradually during germination.

Foaming properties. Germinated lentils possessed higher foaming capacity than non-germinated seeds (2.5, 1.8 & 1.6 fold in 1st, 3rd & 5th days of germination, respectively, compared to non-germinated lentil), while foam stability decreased throughout the germination period.

Discussion. The alkaline extraction and sub-sequent precipitation of the proteins at the isoelectric point is the most usual way to prepare protein isolates in the food industry. The isoelectric point of 4.5 is in accordance with the isoelectric points previously described for other members of the legume family (Koyoro & Powers, 1987; Sanchez-Vioque *et al*., 1999; Bora, 2002). The molecular size of the globulins has always been indicative of the existence of a subunit structure and this has been substantiated by data from dissociation studies and N-terminal amino acid analysis (Derbyshire *et al.,* 1976). Electrophoretic analysis of lentil proteins revealed and confirmed the presence of subunit structure in these major storage proteins. At the onset of germination stored proteinases have to become active in order to initiate storage protein mobilization (Muntz *et al*., 2001). The observed increase in soluble non-protein nitrogen implied that the germination in legumes is accompanied by an increase in proteolytic activity in the storage organs. In electrophoretic patterns, as germination proceeded, there was a gradual disappearance of protein bands and a concomitant increase in components with low molecular weights, suggesting that the cleavage of proteins might be due to the activation of endoproteases at the early stages of seedling growth. Using SDS-PAGE to study changes in germinating lentil proteins, Hsu *et al.* (1982) observed a progressive decrease in large protein subunits and formation of small fragments as germination progressed.

Solubility of lentil protein isolate was very low because under the usual conditions for isolation of soybean globulins, part of the 7 S fraction rapidly insolubilizes. After the initial acidification, this fraction and also the 2 S and >15 S fractions, slowly, but progressively lose solubility (Nash & Wolf, 1976). Germinated lentils are more soluble particularly at the isoelectric point of the native protein. This increase in solubility is due to the smaller size of peptides and higher hydrophilicity of the proteins after proteolytic treatment. In germinating seeds, the storage proteins are hydrolysed to soluble peptides and free amino acids, which serve as precursors for the synthesis of new proteins and other nitrogen-containing compounds in the seedling (Marcone, 1999). The increased solubility may be useful for the supplementation of drinks (with acidic pH) to improve their nutritional quality.

The increase in water adsorption capacity is mainly due to a reduction in the molecular weight and increase in the number of polar groups upon germination. Swelling and expansion of particles by adsorbing water are an important functional property in foods such as processed meat, where protein mix with water without dissolution and impart thickening power and viscosity to the food (Wanasundara & Shahidi, 1994).

Fat adsorption capacity of proteins may be affected by conformation and environmental factors; conformational

changes in the protein molecules during germination may expose previously enclosed non-polar amino acid side chains thereby making them available to interact with hydrocarbon moieties of oil, contributing to increased oil adsorption (Paredes-Lopez *et al.*, 1991; Nielsen, 1997).

As germination proceeded, severe hydrolysis of large peptides resulted in small peptides and amino acids, which were not ideal in forming the protein-protein interactions that give rise to stable foams. This may explain why the non-germinated lentil presented very low foaming capacity, but its foam remained quite stable over time, when compared to the germinated lentils.

Overall, the results from this study confirmed that germination process can be considered as a natural and safe process of enzymatic modification to develop functional, as well as nutritional properties of lentil seeds.

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