Study of Neutral Lipids of *Lupinus mutabilis* Meal and Isolates

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ABSTRACT: Two types of protein isolates have been obtained from defatted *Lupinus mutabilis* meal. The isolates, MA and MB, were obtained by alkaline extraction with 0.2% NaOH and 0.25% sodium bisulfite, respectively, followed by precipitation at the isoelectric point (pH 4.8). Total associated lipids were extracted with 86% ethanol. Neutral lipids were separated in a Florisil column. The lipids in the isolates were similar to those found in the original meal. The following types of compounds were separated, identified, and quantitated: hydrocarbons, waxes, methyl esters, triacylglycerols, free fatty acids, diacylglycerols, and free sterols.
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KEY WORDS: Amino acid composition, fatty acids, hydrocarbons, isoelectric precipitation, *Lupinus mutabilis* meal, *L. mutabilis* neutral lipids, *L. mutabilis* protein isolates, methyl esters, tri- and diacylglycerols, waxes.

Lupin has great potential as a valuable oilseed crop in regions with mild climates (1). Several species of lupin, such as *Lupinus albus*, *L. angustifolius*, *L. luteus*, and *L. mutabilis*, are being cultivated in many countries because of both their oil and protein contents (2). For example, *L. mutabilis*, the major crop in the Andean regions of Bolivia, Chile, and Peru, is currently being exploited for the production of edible oil (3).

The composition and nutritional properties of lupin's proteins are well documented (2,4–6) However, use in human foods and livestock is limited, mainly due to the presence of some toxic quinolizidine alkaloids in the lupin seeds (1). The major alkaloids of the lupin seed are lupanine, isolupanine, hydroxylupanine, and sparteine. A minor part of these alkaloids, which are extracted together with the oil, could be removed during the refining process. However, the major part of these alkaloids are retained in the defatted meal. The toxicity of the alkaloids restricts use in foods and feed formulations. Breeding and cultivation of suitable varieties of "sweet lupin" with low alkaloid contents are still at the experimental stage (7). Therefore, the development of an efficient process for the removal of alkaloids from defatted meals of common "bitter lupin" is necessary to increase the use of lupin proteins in the food industry. The production of lupin protein isolates can overcome this problem because alkaloids are water-soluble and would be removed during preparation of the isolates. However, protein isolates were always associated with a low content of lipid. Lupin oil is an excellent source of unsaturated fatty acids [25–30% of which are polyunsaturated (8–12)]. Oxidation of unsaturated fatty acids can lead to the formation of hydroperoxides and their secondary degradation products, such as *n*-alkanals and alk-2-enal. These compounds react with some amino acid side-chains of proteins, leading to some quality deterioration during storage by the formation of nondigestible products (13–15). Knowledge of the nature and the quantity of the lipids associated with the isolates could be helpful in the prevention of such deterioration. This paper describes the isolation and characterization of lipids associated with lupin protein isolates obtained by alkaline extraction and isoelectric point (pI) precipitation of *L. mutabilis* meal.

EXPERIMENTAL PROCEDURES

Materials. Meals from prepress, hexane-extracted, partially dehulled *L. mutabilis* seeds were used. Hydrocarbons and normal alcohols were obtained from olive oil unsaponifiables (16). Pure octadecyl octadecanoate was a gift from Dr. H.K. Mangold (Münster, Germany). Pure triacylglycerols were obtained from olive and sunflower oils by preparative thin-layer chromatography (TLC) on silica gel. 1,2- and 1,3-Diacylglycerols and cholesterol were commercial samples (Nu-Chek-Prep, Elysian, MN). A qualitative mixture of methyl ester fatty acids with different degrees of unsaturation was prepared by transesterification of an equilibrated blend of coconut oil and linseed oil.

General methods. Standard methods for fiber, alkaloids, and nitrogen (17) were used. The total protein content was calculated as total nitrogen × 6.25.

Free lipids are referred to those extracted with hexane under continuous stirring for six hours. Associated lipids were obtained, following the method of Nash *et al.* (18), by extracting with 86% ethanol at room temperature for 37 h and removing the nonlipid material according to Singh and Privett (19).

pIs were determined as follows: 10 g of meal was extracted three times with 200 mL water, pH 8.5, and a 40-mL aliquot

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of the extract was titrated with 0.5 N HCl to various pH values, ranging from 2.0 to 7.0. The formed precipitate was separated by centrifugation for 30 min at 8000 × *g*. The supernatant was decanted, and the volume and protein content were determined. The percentages of protein precipitated were plotted vs. pH to determine the pI.

Preparation of isolates: isolate MA. Meal (100 g) suspended in 1 L of 0.2% NaOH solution, pH 8.5, was extracted under stirring for 2 h. After centrifugation at 8000 × *g*, two additional extractions were carried out with half of the volume of alkaline solution. The supernatants were pooled and the volume was measured and analyzed for nitrogen. The residue was air-dried, and oven-dried at 50°C in vacuum, weighed, and analyzed for nitrogen. The pooled supernatant was taken to the pI (pH 4.8), and the precipitate was recovered by centrifugation at 8000 × *g*. The precipitate was washed with distilled water (pH 4.8), air-dried, and then dried under vacuum at 50°C (20). The final color was dark brown.

Preparation of isolates: isolate MB. Meal (100 g) was extracted as above but with 0.25% Na_2SO_3 at pH 11.0 for the extraction to avoid darkening of the final product. The precipitate obtained at the pI was successively washed with water at pH 4.8, ethanol, and acetone, and dried as before. The obtained isolates were almost white.

Fractionation of the lipids. A preliminary separation into the different types of compounds was achieved by the method of Singh and Privett (19) with a column (2 × 23 cm) of acid-washed Florisil (80–100 mesh; E. Merck, Darmstadt, Germany). A chloroform solution of the lipids was poured into the column. The elution was carried out with the following solvents (to obtain the different kinds of compounds indicated): (i) chloroform for neutral lipids; (ii) chloroform/acetone (1:1) for glycolipids; and (iii) methanol for phospholipids. The separation of the different compounds was monitored by TLC on silica gel. Hydrocarbons and waxes were separated with pentane as developing solvent. The other neutral lipids (i.e., methyl esters, triacylglycerols, free fatty acids, diacylglycerols, sterols) were separated with hexane/diethyl ether/formic acid (70:30:1). The lipids were visualized either by iodine vapors or by charring after spraying with 50% sulfuric acid. Glycolipids were separated with chloroform/methanol/7N ammonia (100:15:2) and detected with the abovementioned reagents. Phospholipids were developed with *n*-butanol/acetic acid/water (40:7:32) and detected with the reagent of Vaskovsky *et al.* (21).

Quantitative determination of the different compounds by TLC flame-ionization detection. Samples of lipid compounds (15 µg/µL) were applied to a rod. Rods were developed in a tank containing benzene/acetyl acetate/acetic acid (95:3:2) for 40 min (22). The lipid compositions were analyzed with an IATROSCAN analyzer MK-5 (Iatro Co., Ltd., Tokyo, Japan). Removing the nonpolar triacylglycerols led to an important improvement of the separation process.

The relative proportion of the different fatty acids, hydrocarbons, fatty alcohols, and sterols was determined by gas–liquid chromatography (GLC) after hydrolysis of samples obtained from TLC plates, as has been described previously. Waxes were hydrolyzed by boiling the sample for 5 min in a 0.2 N NaOMe solution. The methyl esters and free alcohols formed were separated by TLC with hexane/diethyl ether (8:2, vol/vol), scraped off the plate, and analyzed by GLC. Other esters (tri- and diacylglycerols) were hydrolyzed in two steps—first, the sample was boiled with a few mL of methanolic 0.2 N NaOMe, and, second, the resulting solution was neutralized (phenolphthalein) with a methanol/4% HCl mixture and boiled for 5 min. The obtained methyl esters were analyzed by GLC.

Structural analysis of triacylglycerols. The structures of these compounds were established by the pancreatic lipase method (23). This requires the determination of the total fatty acid composition and the fatty acid distribution in the β-position of the acylglycerol. With these data, the possible types of triacylglycerols were calculated by the Van der Wal formulae (24).

Gas-chromatographic methods. Fatty acid methyl esters were prepared by the abovementioned method and analyzed in an HP 5710-A (Hewlett-Packard, Palo Alto, CA) apparatus with a column of EGS 5% on Supelcoport (Supelco, Bellefonte, PA) 80–100 mesh, 2 m × 2.5 mm, oven temperature 160°C, and detector and injector temperature 250°C. Hydrocarbons, alcohols, and sterols were analyzed in a Carlo Erba (Milan, Italy) Chromatograph, with a column of OV-17, 2.5% on Supelcoport 80–100 mesh, 2 m × 2.5 mm, oven temperature 270°C, and detector and injector temperature 300°C.

Amino acids. The sample (20–30 mg) was hydrolyzed with 6M HCl overnight (25). The analysis was carried out in a Bio-Rad analyzer (Bio-Rad Laboratories, Richmond, CA), after adding 0.5 M Na–citrate buffer, pH 2.2, to the hydrolyzate (26) and using 2-aminobutyric acid as internal standard (27).

RESULTS AND DISCUSSION

Table 1 shows the recovery of protein during the process of preparing the two different *L. mutabilis* isolates. The main fraction (68%) was recovered during the first extraction. As shown, three extraction processes with 0.2% NaOH or 0.25% sodium bisulfite solution are sufficient to recover 80.0 or 85.3%, respectively, of the initial protein content. Further extractions are not effective from a practical point of view. It is also shown that pI precipitation is not exhaustive, and some components remain soluble in the supernatant. This soluble fraction represents 30.7 or 17.8% of initial protein content, depending on the extraction process, and is mainly composed by proteins with an acidic pI (<4.0) and with an alkaline pI (data not shown).

Table 2 shows the chemical compositions of the original meal and protein isolates. These data show that most of the fiber and alkaloids are eliminated during the isolation process (precipitation at the pI), which yields products with a protein concentration in the range of 82–89% (Table 2). The content of the associated lipids is much lower in isolate MB (0.39%) than in isolate MA (0.91%). The isolation and quantitation

TABLE 1
Protein Distribution During the Preparation of Protein Isolates from *Lupinus mutabilis*

	Extraction process (% of initial protein)	Precipitation at pI[a] (% of initial protein)	
		Precipitate	Supernatant
Isolate MA			
First extract	68.0	41.9	26.1
Second extract	12.0	9.3	2.7
Third extract	5.3	3.4	1.9
	(85.3)	(54.6)	(30.7)
Wash water	0.8	—	—
Residue	5.2	—	—
Isolate MB			
First extract	68.1	52.8	15.3
Second extract	9.4	7.6	1.8
Third extract	2.9	2.2	0.7
	(80.4)	(62.6)	(17.8)
Wash water	0.2	—	—
Residue	5.1	—	—

[a]Isoelectric point (pI) 4.8

(by TLC) of neutral lipids from the meal, and the two isolates are shown in Table 3. These data reveal two main differences: (i) the free fatty acid content in isolate MB is 9.5% lower than in isolate MA; and (ii) the triacylglycerol content in isolate MB is 10.2% higher than in isolate MA. The other types of compounds are in similar proportions.

Figure 1 shows a thin-layer chromatogram of the total lipids from *L. mutabilis* meal and from isolates MA and MB. These data show that, with the solvent system used (hexane/diethyl ether/formic acid, 70:30:1), the hydrocarbons and waxes run with the solvent front, and the glycolipids and phospholipids remain at the origin, resulting in a suitable system for the resolution of neutral lipids. The R_f values for the main lipids were 0.8 for triacylglycerols and 0.4 for free fatty acids.

Gas-chromatographic analysis of the hydrocarbons from olive oil, used as standard, demonstrated the presence of C14

TABLE 2
Chemical Composition of *Lupinus mutabilis* Meal and Protein Isolates[a]

	L. mutabilis meal (%)	Isolate MA (%)	Isolate MB (%)
Dry matter	92.3	86.5	91.9
Moisture	7.7	13.5	8.1
Ash	4.2	1.8	1.7
Crude protein (N × 6.25)	44.6	82.0	89.1
Fiber	8.2	0.5	0.2
Associated lipids	2.1	0.9	0.4
Alkaloids	2.5	0.03	0.05
NFE[b]	30.7	1.8	0.5

[a]Results are expressed as percent of dry matter.
[b]Nitrogen-free extract; calculated by difference.

TABLE 3
Weight Distribution of Neutral Lipids of Meal and Protein Isolates[a]

Sample	Wax + methyl esters (%)	TG (%)	FFA (%)	Diglycerides (%)		Polar lipids (%)
				1,3-	1,2-	
Meal	0.1	16.0	16.3	9.8	5.6	51.4
Isolate MA	0.7	10.6	35.7	7.6	2.1	43.7
Isolate MB	0.9	21.2	26.2	8.4	2.3	41.1

[a]Results are expressed as percent of the total content of neutral lipids. TG, triglycerides; FFA, free fatty acids.

to C28 hydrocarbons in lupin isolates. The results obtained are shown in Table 4.

The composition of the different acylglycerols present in the meal and in the protein isolates are shown in Table 5. These data reveal that in the original sample (*L. mutabilis* meal) the main triacylglycerols were diunsaturated and triunsaturated (39.5 and 51.1%, respectively). However, in isolate MA, the main triacylglycerol type was monounsaturated (37.9%), and in isolate MB, the main triacylglycerol type was diunsaturated (41.5%). Table 5 also shows that in the original sample, as well as in isolates MA and MB, only a small percentage of trisaturated triacylglycerols are present.

Table 6 shows the fatty acid composition from waxes and methyl esters and the free fatty acid content of *L. mutabilis* meal and protein isolates MA and MB. Methyl esters were present in all samples, and their fatty acid composition is similar to that of the free fatty acid. The free fatty acid composition of the original meal is quite different from those of isolates MA and MB.

The isomers 1,3- and 1,2-diacylglycerols represent 9.8 and 5.6%, respectively, of the total neutral lipids in the meal (see Table 3), which roughly represents a proportion of 2:1. In the isolates, the content of these isomers is slightly lower, 1–2% for the 1,3-diacylglycerols and 3% for the 1,2-diacylglycerols

TABLE 4
Hydrocarbons Present in Lupin Meal and Isolates[a]

Sample	Chainlength (%)							
	C14	C16	C18	C20	C22	C24	C26	C28
Meal	6.9	8.0	—	13.8	18.4	16.1	—	36.8
Isolate MA	10.2	8.8	7.4	15.4	16.2	14.1	14.3	13.7
Isolate MB	6.8	7.6	7.7	—	17.3	17.2	—	43.4

[a]Results are expressed as percent of total hydrocarbon content of the sample.

TABLE 5
Triacylglycerol Components of Meal and Protein Isolate[a]

Sample	S_3[b] (%)	S_2U (%)	SU_2 (%)	U_3 (%)
Meal	0.7	8.7	39.5	51.1
Isolate MA	2.4	37.9	35.2	24.5
Isolate MB	5.1	27.2	41.5	26.0

[a]Results are expressed as percent of total triacylglycerol components in the sample.
[b]S_3, Trisaturated; S_2U, monounsaturated; SU_2, diunsaturated; U_3, triunsaturated.

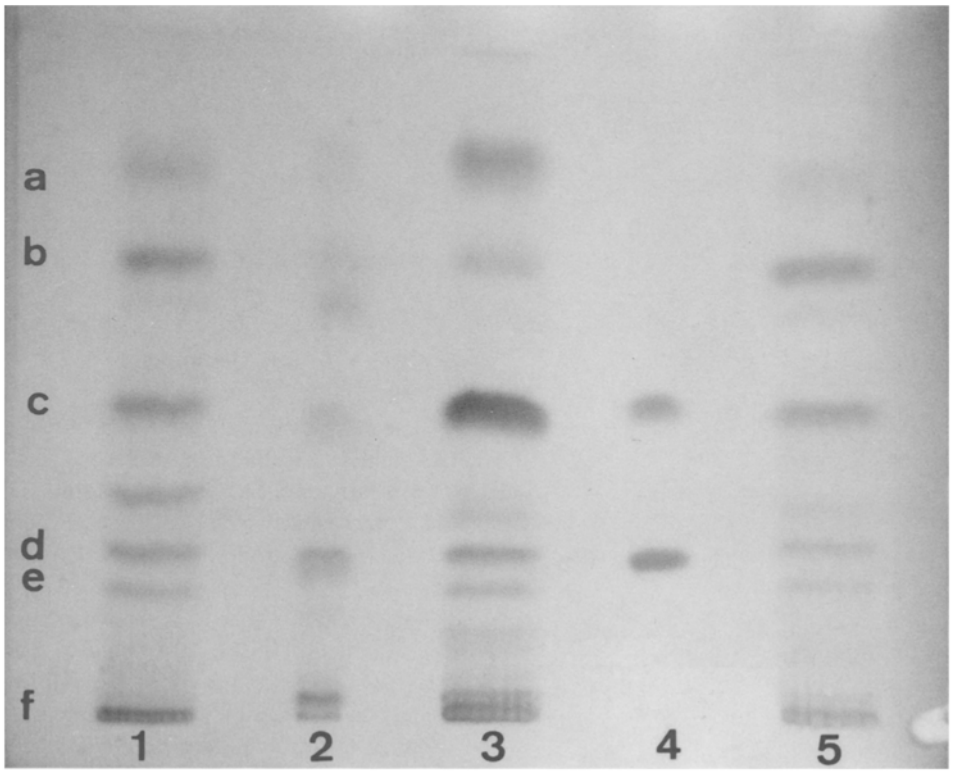

FIG. 1. Thin-layer chromatographic separation of neutral lipids of *Lupinus mutabilis,* meal and protein isolates MA and MB on Silica Gel G. (1) Lipids of original meal; (2) standard mixture, from top to bottom: methyl esters, triacylglycerols, free fatty acids, 1,3- and 1,2-diacylglycerols, monoacylglycerols, glycolipids and phospholipids; (3) associated lipids of isolate MA; (4) cholesterol and free fatty acids; and (5) associated lipids of isolate MB. Letters on the figure refer to the identified compounds: (a) waxes and methyl esters; (b) triacylglycerols; (c) free fatty acids; (d) sterols and 1,3-diacylglycerols; (e) 1,2-diacylglycerols; and (f) glycolipids and phospholipids. Eluent: hexane/diethyl ether/formic acid (70:30:1). Detection: Charring after spraying with 50% sulfuric acid.

(see Table 3). However, in both isolates the isomers are in approximately the same proportion, 4:1. They probably originated from the hydrolysis of the triacylglycerols. The relatively high proportion of free fatty acids (16.3% in the meal, 35.7 and 26.2% in the isolates) (Table 2) is not in accordance with the proportion of diacylglycerols (about 15% in the meal and 10% in the isolates). Monoacylglycerols were not detected in any sample. The two isomers were easily separated by TLC on silica gel G plates, and after their transesterification they were analyzed by GLC. Table 7 shows the results obtained for the fatty acid composition of both types of diacylglycerols in the samples.

TABLE 6
Fatty Acids Constituents of Waxes, Methyl Esters and Free Fatty Acids[a]

	Chainlength (%)							
Sample	14:0	15:0	16:0	16:1	18:0	18:1	18:2	18:3
Waxes								
Meal	41.9	3.6	18.6	9.0	7.5	11.9	10.6	0.5
Isolate MA	53.4	2.3	15.1	4.4	6.7	8.0	9.4	0.6
Isolate MB	2.8	1.3	28.4	4.6	10.6	36.6	1.0	—
Methyl esters								
Meal	2.3	8.4	16.2	—	5.5	53.3	14.3	—
Isolate MA	—	16.2	18.2	5.0	2.2	48.3	10.1	—
Isolate MB	0.9	—	13.5	5.9	12.6	23.7	20.3	22.2
Free fatty acid								
Meal	7.5	0.3	35.7	2.5	11.4	31.0	11.0	0.4
Isolate MA	0.1	—	23.1	0.2	3.1	62.6	10.9	—
Isolate MB	5.6	2.3	47.8	4.3	12.8	20.7	5.7	—

[a]Results are expressed as percent of total fatty acids present in waxes, methyl esters and as free fatty acids.

TABLE 7
Constituent Fatty Acids of 1,3- and 1,2-Diacylglycerols[a]

		Chainlength						
Sample	Type	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Meal	1,3-	trace	19.1	trace	trace	54.5	trace	trace
	1,2-	trace	8.5	trace	trace	31.6	trace	trace
Isolate MA	1,3-	trace	11.8	trace	7.5	3.0	17.4	12.3
	1,2-	4.1	32.6	trace	13.9	24.9	8.3	0.5
Isolate MB	1,3-	1.8	11.4	45.0	5.5	9.0	22.0	trace
	1,2-	3.7	31.7	4.0	7.6	38.2	8.0	trace

[a]Results are expressed as percent of total fatty acids present in 1,3- and 1,2-diacylglycerols.

TABLE 8
Amino Acid Composition of Defatted Lupin Meal and Isolate MA and MB[a]

Amino Acids	Meal	Isolate MA	Isolate MB	FAO[b]
Aspartic acid	11.1	10.7	10.8	—
Threonine	4.7	4.1	4.0	4.0
Serine	6.0	5.6	5.7	—
Glutamic acid	25.3	23.7	25.5	—
Glycine	4.7	4.2	4.1	—
Alanine	4.3	3.3	3.3	—
Valine	4.5	3.2	3.4	5.0
Methionine[c]	1.3	0.8	0.9	3.5
Isoleucine	3.7	4.2	4.7	4.0
Leucine	6.8	6.6	6.9	7.0
Tyrosine	5.9	8.5	6.4	—
Phenylalanine	4.1	4.2	4.1	6.0
Histidine	3.2	2.9	3.0	—
Lysine	6.1	6.4	5.8	5.5
Arginine	8.2	10.7	10.9	—
Tryptophan	0.9	0.8	0.7	1.0

[a]g/16 g N. [b]Food and Agriculture Organization (FAO) (1973). [c]Methionine + cysteine.

The amino acid compositions of the proteins of meal and isolates are shown in Table 8. For the essential amino acids, the FAO requirements in the defatted meal are fulfilled for threonine, valine, isoleucine, leucine, lysine, and tryptophan, and for threonine, isoleucine, leucine, lysine, and tryptophan in isolates MA and MB. Methionine and phenylalanine contents in proteins of meal and isolates MA and MB were deficient.

The recovery of protein by this process is 54.6 and 62.6% for isolate MA and MB, respectively. These proteins are associated with lipids (0.91 or 0.39%, for isolate MA and MB, respectively). The content of mono- and polyunsaturated fatty acids can be estimated (0.36 and 0.14% for the isolate MA and MB, respectively) from data shown in Tables 2, 3, 5, 6 and 7. The content of these substances in the meal is 0.68%. Oxidation of mono- and polyunsaturated fatty acids can lead to the formation of hydroperoxides and their secondary degradation products, such as *n*-alkanal and alk-2-enal. The capacity of these compounds to react with some amino acid side chains of proteins is considered one of the main causes for the loss of quality and functionality of proteins (14,15).

The low content of oxidable products in the isolates obtained by the procedure described in this paper allows processing and storage of these products with without or a small amount of antioxidants to prevent oxidative deterioration of the proteins.

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