Neutral Lipids of Chickpea Flour and Protein Isolates

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ABSTRACT: The neutral lipids composition of defatted chickpea flour and two types of protein isolates has been studied. The main compounds in neutral lipids are triacylglycerols, free fatty acids, and diacylglycerols. Other compounds present are wax esters, free fatty alcohols, and free sterols. The main fatty acids in neutral lipids are $C_{18:2}$ and $C_{18:1}$ among the unsaturated, and $C_{16:0}$ and $C_{18:0}$ among the saturated acids. Free and esterified alcohols range from $C_{16:0}$ to $C_{28:0}$, the majority being those with an even number of carbon atoms. Sterols observed are β-sitosterol, campesterol, stigmasterol, and δ-5-avenasterol. Triacylglycerols are partially hydrolyzed, and the amounts of unsaturated sterols and unsaturated fatty acids are reduced as a result of the chemical treatment during production of the protein isolates.

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KEY WORDS: Chickpea, flour, neutral lipids, protein isolates.

Plant proteins are increasingly being used as an alternative source of dietary protein in human nutrition. Chickpea has been considered a good source of protein because it has outstanding protein quality, equivalent to that of soybean (1,2). It is used as a substitute for traditional protein sources (milk, eggs, or meat) for the preparation of different foods (3,4). Chickpea flour can also be used to produce protein isolates for adding to human foods as functional ingredients, or for improving the protein composition (5).

Lipids, even in small amounts, are an important component of protein isolates because of the role that fats play in the physicochemical properties, nutritional quality, and acceptability of the product. Most of these lipids are not originally associated with proteins, but, as a result of the rupture of cells and organelles during production of the flour and protein isolates, they could interact with other components, such as proteins (6). Lipid interaction with proteins may create exposure of some protein domains and protection of others, and alter the conformational pattern and, thereby, the functional characteristics of the proteins.

Boatright and Hettiarachchy (7) reported that the solubility of soybean protein isolates is greatly reduced by lipids associated with them, and Arêas (8) has studied the beneficial effect of residual lipids present in lung protein isolates on thermoplastic extrusion. Unsaturated fatty acids are easily oxidized, releasing chemical compounds that could react with proteins, decrease their biological value, and affect flavor (9,10). Therefore, a knowledge of the nature and quantity of the lipids associated with the isolates could be of interest for preventing these changes in the final product.

Neutral lipids associated with protein isolates have been studied in various crops, such as sunflower or lupine; the main compounds observed were triacylglycerols, free fatty acids, and diacylglycerols (11,12). However, although several studies on total lipid composition of chickpea seeds have been reported (13–15), no data on lipid composition in chickpea protein isolates are available in the literature. The objective of this paper was to resolve, identify, and quantitate the neutral lipids of defatted chickpea flour and protein isolates.

EXPERIMENTAL PROCEDURES

Plant material. Chickpea seeds (c.v. Athenas), cultivated in the experimental fields of Carmona (Sevilla), were a gift from Koipesol Semillas, S.A. (Sevilla, Spain). Seeds were cleaned and freed from broken seeds, dust, and other foreign materials.

Determination of moisture, ash, nitrogen, carbohydrates, and fiber. Moisture, ash, and nitrogen were determined according to AOAC approved methods (16). Total fiber was determined according to the procedure described by Lee *et al*. (17). Total carbohydrates were calculated as the difference between 100% and the totals of moisture, ash, fiber, protein, and lipid.

Production of protein isolates. Chickpea flour was defatted for 9 h with boiling hexane in a Soxhlet system. Two types of protein isolate were prepared from the flour. Isolate A was obtained by dispersing defatted chickpea flour in 0.2% NaOH solution (pH 12) at the ratio of 1:10 (wt/vol) and extracting by shaking for 1 h. After centrifugation at $8000 \times g$ for 15 min, two additional extractions were carried out with half the volume of alkaline solution. Supernatants were pooled, and the pH was adjusted to 4.3 (the isoelectric point of chickpea proteins). After centrifugation at $8000 \times g$ for 15 min, the precipitate was washed with water at pH 4.3, frozen at –20°C,

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and lyophilized. Isolate B was prepared as above but with a 0.25% Na_2SO_3 solution at pH 10.5 for the extraction of proteins. Supernatants were pooled, and the pH was adjusted to the isoelectric point of chickpea proteins as for isolate A. The precipitate was successively washed with water (pH 4.3), 96% ethanol, and acetone, and dried at room temperature.

Lipid extraction. Lipids in the defatted chickpea flour and protein isolates were extracted with 86% ethanol in a proportion of 1:20 wt/vol for 36 h at room temperature (18). The ethanol solution was transferred to a separatory funnel, and a 1:1 mixture of chloroform and 0.5% NaCl aqueous solution was added. The chloroform phase was extracted according to Singh and Privett (19) to remove nonlipid compounds.

Fractionation of lipids. A preliminary separation of lipids was performed by the method of Singh and Privett (19) in an acid-treated Florisil (Merck, Germany) column. The different lipid components were fractionated with chloroform, chloroform/acetone (1:1), and methanol to elute neutral lipids, glycolipids, and phospholipids, respectively. The elution was monitored by thin-layer chromatography (TLC) with silica gel 60G (Merck) 0.25-mm layer plates. Definitive separation and isolation of lipid components were achieved by TLC with the solvent systems hexane/diethyl ether/formic acid (95:5:1) for wax esters, hexane/diethyl ether/formic acid (70:30:1) for triacylglycerols and free fatty acids, and hexane/diethyl ether/formic acid (50:50:1) for free fatty alcohols, free sterols, 1,2-diacylglycerols, and 1,3-diacylglycerols. Lipids were visualized with iodine vapor.

Quantitation of lipid components. Waxes, triacylglycerols, free fatty acids (as methyl esters), and diacylglycerols were quantitated by the hydroxamic method (20). Fatty acid esters were reacted with hydroxylamine and Fe^{3+} to produce fatty acid derivatives with an absorption peak at 520 nm. A calibration curve of myristic acid methyl ester was used.

Free fatty alcohols were quantitated by gas–liquid chromatography (GLC), with $C_{21:0}$ fatty alcohol as internal standard. Free sterols were quantitated according to Huang *et al*. (21), based on the colorimetric reaction (absorption peak at 550 nm) between the sterols and a chemical reagent that is comprised of sulfuric acid/acetic acid/acetic anhydride (1:3:6) and 2% sodium sulfate. A calibration curve of β-sitosterol was used.

GLC. A Hewlett-Packard GC 5890 model series II, fitted with a flame-ionization detector and HP 3390A integrator (Palo Alto, CA), was used. Hydrogen at 12 psi column head pressure and 1 mL/min flow was employed as carrier gas. Nitrogen was used as auxiliary gas. Fatty acids were determined as methyl derivatives with a Hewlett-Packard 20 M 25 m × 0.2 mm × 0.2 μm capillary column. Fatty acids were derivatized with 2.5% H_2SO_4 in anhydrous methanol/benzene (9:1). Injector, detector, and oven temperatures were maintained at 225, 250, and 170°C, respectively. Sterols and fatty alcohols were derivatized with a mixture of pyridine/hexamethyldisilane/trimethylchlorosilane (9:3:1) at room temperature. GLC analyses were performed with a TRB-1 30 m × 0.25 mm × 0.25 μm capillary column (Supelco, Bellefonte, PA). Injector and detector temperatures were maintained at 300°C. Oven temperature was maintained at 265°C for sterol analyses. For fatty alcohol analyses, the column temperature was held at 200°C for 6 min, increased to 280°C (3°C/min), and maintained for 15 min. For the analyses of wax esters, samples were hydrolyzed into fatty acid methyl esters and free fatty alcohols with 2.5% H_2SO_4 in anhydrous methanol/benzene (9:1), purified by TLC with hexane/ethyl ether/formic acid (50:50:1), and analyzed by GLC as above.

Structural analysis of triacylglycerols. Triacylglycerols were hydrolyzed with pancreatic lipase to obtain β-monoglycerides whose structures were determined. With the composition in total and position β fatty acids, the possible types of triacylglycerols were calculated according to Van der Wal formulae (22).

RESULTS AND DISCUSSION

Neutral lipids compositions of defatted chickpea flour and two types of protein isolates have been studied. Although chickpea flour was defatted previously with hexane, lipids were not completely removed and ranged from 1% in isolate B to 3.4% in isolate A (Table 1). Isolate B had lower amounts of lipids, probably because the washes with ethanol and acetone partially eliminated remaining lipids.

Lipids that remained in the defatted flour and protein isolates can be divided into glycolipids, phospholipids, and neutral lipids according to the fractionation method of Singh and Privett (19). Although qualitatively important, neutral lipids constituted a minor fraction, ranging from 11.6% in isolate A to 13.4% in the defatted flour (Table 2). Triacylglycerols, free fatty acids, and diacylglycerols were the main neutral lipids detected. Wax esters, free fatty alcohols, and free sterols were present in minor amounts (Table 3).

Triacylglycerols were the main components of neutral lipids in defatted chickpea flour and protein isolates, their amounts being higher in the flour than in the protein isolates. The amounts of diacylglycerols, particularly 1,2-diacylglycerol, in both protein isolates were higher than in the flour. These results suggest that triacylglycerols are partially hydrolyzed during formation of the protein isolates. A similar hydrolysis of triacylglycerols has been observed during wet

TABLE 1
Chemical Composition (%) of Defatted Chickpea Flour (DCF) and Protein Isolates[a]

Constituents	DCF	Isolate A[b]	Isolate B[c]
Moisture	8.1 ± 0.1	3.3 ± 0.2	5.5 ± 0.1
Ash	3.4 ± 0.1	2.8 ± 0.3	4.1 ± 0.2
Protein[d]	22.7 ± 1.7	75.0 ± 2.5	83.3 ± 0.9
Fiber	17.3 ± 2.0	3.7 ± 0.3	3.0 ± 0.1
Lipid	1.4 ± 0.3	3.4 ± 0.4	1.0 ± 0.2
Carbohydrates	47.1 ± 10.5	11.8 ± 1.5	3.1 ± 0.3

[a]Data are the mean ± SD of three determinations.
[b]Chickpea protein isolate obtained with NaOH.
[c]Chickpea protein isolate obtained with Na_2SO_3.
[d]Nitrogen × 6.25.

TABLE 2
Lipid Composition (%) of DCF and Protein Isolates[a]

	DCF	Isolate A[b]	Isolate B[c]
Neutral lipids	13.4 ± 2.2	11.6 ± 1.6	11.9 ± 0.3
Polar lipids	86.6 ± 2.2	88.4 ± 1.6	88.1 ± 0.3

[a]Data are the mean ± SD of three determinations. See Table 1 for abbreviation.
[b]Chickpea protein isolate obtained with NaOH.
[c]Chickpea protein isolate obtained with Na_2SO_3.

TABLE 3
Neutral Lipid Composition (%) of DCF and Protein Isolates[a]

	DCF	Isolate A[b]	Isolate B[c]
Wax esters	trace	trace	trace
Triacylglycerols	64.1 ± 1.3	32.7 ± 1.1	45.2 ± 1.3
Free fatty acids	11.9 ± 1.5	5.0 ± 1.2	4.2 ± 1.0
1,3-Diacylglycerols	14.6 ± 2.2	18.8 ± 2.5	16.0 ± 2.3
1,2-Diacylglycerols	6.4 ± 1.0	23.2 ± 2.5	29.8 ± 1.2
Free fatty alcohols	1.0 ± 0.1	1.2 ± 0.1	0.8 ± 0.1
Free sterols	2.0 ± 0.2	19.1 ± 2.3	4.0 ± 0.9

[a]Data are the mean ± SD of three determinations. See Table 1 for abbreviation.
[b]Chickpea protein isolate obtained with NaOH.
[c]Chickpea protein isolate obtained with Na_2SO_3.

fractionation of oat flour (23). These authors suggest that mechanical damage during dehulling, in combination with wet heat treatment, increases the free fatty acid levels in dry grains and the possibility of further triacylglycerol hydrolysis upon processing.

In spite of the hydrolysis of triacylglycerols, the percentage of free fatty acids in the protein isolates decreased with respect to the flour. A possible explanation is that free fatty acids are removed during production of the isolates and/or are quickly oxidized, undergoing degradation. Free fatty alcohols and sterols were more abundant in protein isolate A, indicating that the ethanol and acetone washes used in the production of isolate B probably contributed to the removal of these compounds.

The fatty acid composition of neutral lipids was studied (Table 4). As has been observed in other Leguminosae (13), including several chickpea varieties (15), unsaturated fatty acids, mainly $C_{18:1}$ and $C_{18:2}$, predominate in triacylglycerols and diacylglycerols from chickpea flour. The fatty acid composition of diacylglycerols in the protein isolates differed from that observed in the defatted flour, with higher average contents of $C_{16:0}$ in the isolates. In contrast, the amounts of unsaturated fatty acids, mainly $C_{18:1}$, were lower in the protein isolates. This selective loss of esterified unsaturated fatty acids has also been reported during the processing of oat flour (23) and could be attributed to degradation by oxidation of unsaturated compounds. Fatty acids from wax esters are characterized by the predominance of saturated compounds with even numbers of carbon atoms, the main ones being $C_{16:0}$ and $C_{18:0}$. A similar composition has been observed in wax esters of sunflower (11), *Calotropis gigantea* (24), and *Coincya* sp. (25). The differences in fatty acid composition between waxes and triacylglycerols could exist because wax esters comprise less-reactive saturated fatty acids as a result of their susceptibility to environmental factors. In contrast, triacylglycerols, with predominance of unsaturated fatty acids, are stored in oil bodies, where they are protected from the environment of the cell.

TABLE 4
Fatty Acid Composition (%) of Neutral Lipids of DCF and Protein Isolates[a]

	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Wax esters						
DCF	3.0 ± 0.1	50.0 ± 1.4	24.3 ± 0.9	16.2 ± 0.7	6.5 ± 0.1	trace
Isolate A[b]	5.6 ± 0.1	54.4 ± 1.2	24.3 ± 0.3	14.5 ± 0.5	1.2 ± 0.1	trace
Isolate B[c]	2.9 ± 0.1	59.9 ± 1.6	19.6 ± 1.3	12.0 ± 0.7	5.6 ± 0.7	trace
Triacylglycerols						
DCF	1.0 ± 0.1	14.4 ± 0.1	2.3 ± 0.1	26.2 ± 0.1	53.6 ± 0.1	2.5 ± 0.1
Isolate A	1.5 ± 0.1	13.4 ± 1.1	3.0 ± 0.1	23.5 ± 0.3	55.9 ± 0.6	2.7 ± 0.2
Isolate B	trace	15.9 ± 0.1	1.8 ± 0.1	23.7 ± 0.1	56.7 ± 0.1	1.9 ± 0.1
Free fatty acids						
DCF	2.7 ± 0.5	25.2 ± 3.7	7.5 ± 2.5	24.1 ± 0.1	38.7 ± 7.1	1.5 ± 0.2
Isolate A	7.0 ± 0.3	32.6 ± 0.2	13.2 ± 0.6	25.6 ± 1.4	21.6 ± 1.5	trace
Isolate B	2.1 ± 0.1	25.7 ± 0.8	5.1 ± 0.1	15.8 ± 0.7	51.3 ± 1.3	trace
1,3-Diacylglycerols						
DCF	2.0 ± 0.2	21.8 ± 0.6	6.1 ± 0.8	37.5 ± 0.8	31.6 ± 0.3	1.0 ± 0.1
Isolate A	3.2 ± 0.1	48.6 ± 0.8	7.0 ± 0.3	13.5 ± 1.1	27.7 ± 0.1	trace
Isolate B	1.2 ± 0.1	41.0 ± 1.0	4.9 ± 0.1	15.3 ± 0.3	36.6 ± 0.8	1.0 ± 0.1
1,2-Diacylglycerols						
DCF	trace	29.9 ± 0.2	5.0 ± 0.2	29.4 ± 0.4	34.7 ± 0.4	1.0 ± 0.1
Isolate A	1.5 ± 0.1	54.4 ± 0.2	6.9 ± 0.1	14.8 ± 0.3	22.4 ± 0.1	trace
Isolate B	trace	57.6 ± 0.1	4.3 ± 0.1	11.8 ± 0.1	26.3 ± 0.1	trace

[a]Data are the mean ± SD of three determinations. See Table 1 for abbreviation.
[b]Chickpea protein isolate obtained with NaOH.
[c]Chickpea protein isolate obtained with Na_2SO_3.

TABLE 5
Triacylglycerol Composition (%) of DCF and Protein Isolates

DCF		Isolate A[a]		Isolate B[b]	
$C_{18:2}C_{18:1}C_{18:2}$	25.1	$C_{18:2}C_{18:1}C_{18:2}$	16.7	$C_{18:2}C_{18:1}C_{18:2}$	30.6
$C_{18:2}C_{16:0}C_{18:2}$	9.9	$C_{18:2}C_{18:2}C_{18:2}$	14.7	$C_{18:2}C_{18:1}C_{16:0}$	13.9
$C_{18:2}C_{18:1}C_{18:1}$	9.7	$C_{18:2}C_{18:1}C_{18:1}$	8.4	$C_{18:2}C_{18:2}C_{18:2}$	10.7
$C_{18:2}C_{18:2}C_{18:2}$	9.4	$C_{18:2}C_{16:0}C_{18:2}$	8.4	$C_{18:2}C_{16:0}C_{18:2}$	7.7
$C_{18:2}C_{18:1}C_{16:0}$	8.3	$C_{18:2}C_{18:2}C_{18:1}$	7.4	$C_{18:2}C_{18:1}C_{18:1}$	5.9
$C_{18:2}C_{18:0}C_{18:2}$	4.3	$C_{18:2}C_{18:1}C_{16:0}$	5.4	$C_{18:2}C_{18:2}C_{16:0}$	4.9
$C_{18:2}C_{16:0}C_{18:1}$	3.8	$C_{18:2}C_{18:2}C_{16:0}$	4.8	$C_{18:2}C_{16:0}C_{16:0}$	3.5
$C_{18:2}C_{18:2}C_{18:1}$	3.6	$C_{18:2}C_{16:0}C_{18:1}$	4.2	$C_{18:2}C_{18:0}C_{18:2}$	3.2
$C_{18:2}C_{16:0}C_{16:0}$	3.3	$C_{18:2}C_{18:0}C_{18:2}$	3.9	$C_{18:3}C_{18:1}C_{18:2}$	2.4
$C_{18:2}C_{18:2}C_{16:0}$	3.1	$C_{18:2}C_{16:0}C_{16:0}$	2.7	$C_{18:2}C_{18:2}C_{18:0}$	2.1
Others	19.5	Others	23.4	Others	15.1

[a]Chickpea protein isolate obtained with NaOH.
[b]Chickpea protein isolate obtained with Na_2SO_3. See Table 1 for abbreviation.

The structure of triacylglycerols in the defatted flour and protein isolates was calculated knowing the composition in total and position β fatty acids of the triacylglycerols (data not shown). The main triacylglycerol in defatted flour and protein isolates was $C_{18:2}C_{18:1}C_{18:2}$, ranging from 16.7% in isolate A to 30.6% in isolate B. Other abundant triacylglycerols were $C_{18:2}C_{16:0}C_{18:2}$, $C_{18:2}C_{18:1}C_{18:1}$, $C_{18:2}C_{18:2}C_{18:2}$, and $C_{18:2}C_{18:1}C_{16:0}$ (Table 5). The triacylglycerol compositions of defatted flour and protein isolates agree with those reported for the seed (13).

In free and esterified fatty alcohols, those with an even number of carbon atoms predominate, as has been observed in *Zea mays* (26) and *Coincya* sp. (27). The main alcohols of defatted flour and protein isolates ranged from $C_{16:0}$ to $C_{28:0}$, with the highest amounts being $C_{16:0}$ and $C_{18:0}$ (Table 6). Fatty alcohols, like fatty acids in wax esters, are characterized by the predominance of less-reactive saturated molecules. Probably because of this, there were no significant differences in alcohol composition between the defatted flour and protein isolates, except in the content of $C_{18:1}$ fatty alcohol, which was lower in the protein isolates, perhaps because $C_{18:1}$ alcohol is more sensitive to the chemical treatment used to obtain the protein isolates.

The sterol composition of the defatted flour and protein isolates was determined (Table 7). The main sterols in the plant kingdom are sitosterol, campesterol, and stigmasterol (28). In defatted chickpea flour and protein isolates, these compounds were also the most abundant, with β-sitosterol ranging from 76.7% in the flour to 79.2% in isolate B (Table 7). The average values of stigmasterol and δ-5-avenasterol were lower in the protein isolates than in the defatted flour, indicating that these compounds are more sensitive to the process used to obtain the protein isolates. This could be due to unsaturation in the side chain, which is absent in β-sitosterol and campesterol.

The neutral lipids, found in chickpea protein isolates, reveal a variety of compounds with different chemical characteristics. Most of these lipids are not in contact with storage proteins in the seed, but, after the flour homogenate is obtained, they can interact with proteins and affect functional characteristics and quality of the protein isolates. The high amounts of unsaturated fatty acids in neutral lipids may increase lipid oxidation, with the consequent negative effect on the protein isolates. 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. The capacity of these compounds to react with certain amino acid side chains of proteins is considered one cause for the loss of functionality and quality of proteins (7,9).

Changes in neutral lipid composition during the formation

TABLE 6
Fatty Alcohol Composition (%) of DCF and Protein Isolates[a]

	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{20.0}$	$C_{22:0}$	$C_{24:0}$	$C_{26:0}$	$C_{28:0}$
Free fatty alcohols								
DCF	24.2 ± 0.8	34.5 ± 0.9	trace	7.4 ± 0.2	14.6 ± 0.2	5.6 ± 0.3	trace	13.7 ± 1.5
Isolate A[b]	22.0 ± 2.0	34.2 ± 2.0	trace	8.8 ± 1.2	14.6 ± 1.6	5.9 ± 0.5	trace	14.5 ± 3.0
Isolate B[c]	22.4 ± 2.1	38.1 ± 2.9	trace	5.5 ± 0.3	12.6 ± 0.4	4.2 ± 0.2	trace	17.2 ± 4.9
Ester fatty alcohols								
DCF	21.4 ± 0.6	29.2 ± 1.1	12.9 ± 0.2	7.2 ± 0.2	10.3 ± 0.1	4.2 ± 0.2	2.6 ± 0.1	12.2 ± 1.7
Isolate A	20.6 ± 0.3	35.3 ± 0.1	6.0 ± 2.4	11.6 ± 0.3	13.0 ± 0.7	5.1 ± 0.3	trace	8.4 ± 1.4
Isolate B	18.3 ± 0.7	28.1 ± 0.1	9.2 ± 0.6	8.8 ± 0.1	10.8 ± 0.3	4.5 ± 0.2	3.1 ± 0.3	17.2 ± 0.1

[a]Data are the mean ± SD of three determinations. See Table 1 for abbreviation.
[b]Chickpea protein isolate obtained with NaOH.
[c]Chickpea protein isolate obtained with Na_2SO_3.

TABLE 7
Free Sterol Composition (%) of DCF and Protein Isolates[a]

	β-Sitosterol	Campesterol	Stigmasterol	δ-5-Avenasterol
DCF	76.7 ± 1.9	11.3 ± 0.1	8.5 ± 0.6	3.5 ± 0.1
Isolate A[b]	77.3 ± 0.1	13.5 ± 0.1	7.8 ± 0.1	1.3 ± 0.1
Isolate B[c]	79.2 ± 0.3	12.1 ± 0.3	7.1 ± 0.1	1.5 ± 0.1

[a]Data are the mean ± SD of three determinations. See Table 1 for abbreviation.
[b]Chickpea protein isolate obtained with NaOH.
[c]Chickpea protein isolate obtained with Na_2SO_3.

of protein isolates have been observed. The hydrolysis of triacylglycerols and the reduction in the amounts of unsaturated sterols and unsaturated fatty acids could be produced by chemical reactions.

Better knowledge of the nature and quantity of the lipids in the protein isolates will be helpful to understand the interactions between lipids and proteins and to know how these interactions could be prevented or modified, so that the quality of the protein isolate can be maintained or even improved.

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