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# Composition and structure of carob (*Ceratonia siliqua* L.) germ proteins

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**Abstract**

This study was focused on the analysis of the chemical composition of defatted carob germ flour and the protein isolate. The amino acid composition and the nature of the subunits that compose carob germ proteins were also studied. Isolate was obtained by alkaline extraction followed by isoelectric precipitation of proteins. Results showed that an isolate of 96.5% of protein content was obtained. A high amount of amino acids like glutamic acid, aspartic acid and arginine was detected. Carob proteins were found to be composed by aggregates formed by a 131 and 70 kDa subunits linked by non-covalent bonds, and other peptides strongly bounded by disulfide interactions. Both, aggregates and subunits were formed mainly by 100 and 48 kDa monomers linked by disulfide bonds. A considerable content of high molecular mass peptides (HMWP) strongly bounded were also found. Proteins became partially denatured and thermally stabilized at acid pH (pH 2). These results could be useful in the study of different functional properties of carob germ proteins, and the application of these proteins as nutritional ingredients in formulated food.


*Keywords:* Carob proteins; Chemical analysis; Amino acid composition; Protein solubility; Protein subunits; Thermal properties

## 1. Introduction

The carob tree (*Ceratonia siliqua* L.) is a typical Mediterranean plant (Avallone, Plessi, Baraldi, & Monzani, 1997; Dakia, Wathelet, & Paquot, 2007; Yousif & Alghzawi, 2000). In many Arabian countries, the fruit is used for preparing popular beverages and confectionery. In Western countries, carob powder is produced by deseeding carob pods. The kibbled carob thus obtained is finally roasted and milled (Yousif & Alghzawi, 2000). Carob pods are characterized by high sugar content (more than 50%) mainly composed of sucrose. Carob powder is a natural sweetener with flavor and appearance similar to chocolate; therefore it is often used as cocoa substitute. The advantage of using carob as a chocolate substitute resides in that carob is an ingredient free from caffeine and theobromine. In Europe several carob commercial products can be found as Carovit™ (Alimcarat S.L., Spain). Carovit™ is roasted carob flour used as a cocoa substitute in baking, cereal bars, chocolate confectionery, ice creams and light products. Other products, such as carob germ flour contains high protein content, almost 50%, with a high content of lysine and arginine. Carob germ flour is used as dietetic human food (Dakia et al., 2007) or as a potential ingredient in cereal-derived foods for celiac people (Feillet & Roulland, 1998).

The protein content of carob germ flour obtained from seeds is higher than those observed for other beans such as faba bean (*Viciafaba* L.), pea (*Pisum stivum* L.) and soybean (*Glycine max. Merr.*). Maza et al. (1989) found values of 48.4% for protein content for carob germ defatted flour, while Marcone, Kakuda, and Yada (1998) determined protein values of 18.83% and 34.35% for pea and soybean seeds, respectively. Caroubin, the water-insoluble protein

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isolated from carob bean embryo, is a mixture composed of a large number of polymerized proteins of different size (Wang et al., 2001). This protein system has been reported to possess similar rheological properties to gluten, though caroubin has a more ordered structure, with minor changes in secondary structure when hydrated.

The chemical composition of carob pods (Avallone et al., 1997; Calixto & Canellas, 1982; Yousif & Alghzawi, 2000) and carob germ meals (Dakia et al., 2007) has been studied by several authors. Nevertheless, the chemical composition in relation to structural and thermal properties of carob germ proteins has not yet been deeply studied. It is well known that carob germ proteins have a well-balanced amino acid composition. These proteins could be used as healthy ingredients in nutraceutical foods and can constitute a new food source for different population sectors. Therefore, the aim of this work is to study the chemical composition and structure properties of a carob germ powder and a carob protein isolate.

## 2. Materials and methods

### 2.1. Preparation of carob protein isolate

Carob protein isolate (CI) was prepared from commercial carob germ flour (*Caratina*) produced by Alimcarat (Alimcart S.L., Mallorca, Spain) with a composition of 46% protein, 5% fiber, 7% soluble sugars and 25% other carbohydrates, 7% lipids and 6% ash. Carob germ flour was defatted by hexane. Aliquots of 350 g of flour were inserted in cylindrical cartridges (3 cm diameter × 20 cm height) made with filter paper. All cylinders were introduced in a bag that was perfectly sealed. Samples were percolated three times at 50 °C with hexane (150 L) and then macerated during four days. Flour was put into trays and excess of hexane was eliminated by evaporation into the air at room temperature and then in air oven 48 h at 30 °C. Flour was stored at room temperature until used. Sifted (mesh size 0.9 mm) flour was dispersed in water (8% w/v). The original pH of this flour dispersion was 6.8. The dispersion was adjusted to pH 10.5 with 25% NaOH, stirred at room temperature for 2 h and centrifuged at 9000*g* for 15 min at 4 °C in a RC5C Sorvall centrifuge (Sorvall Instruments, Wilmington, DE, USA). The supernatant was then adjusted to pH 4.0 with 6 N HCl and centrifuged at 9000*g* for 10 min at 4 °C. The pellet was washed once and then resuspended with distilled water. Protein dispersion was introduced in a lab S1 spray dryer (ANHYDRO, Copenhagen, Denmark) at a 7 L/min flow rate with an intake temperature of 190 °C. The isolate was only 1 min inside the spray dryer and the temperature at the outflow was 90 °C.

### 2.2. Determination of protein isoelectric point (IEP)

For determination of the IEP of germ flour proteins, aqueous flour dispersions (0.96 g protein/40 mL) were prepared and pH of different aliquots were adjusted with 6 N NaOH to pH 6, 8, 10 and 12; and with 6 N HCl to pH 2. Dispersions were centrifuged and supernatants analyzed (%N × 6.25) using a LECO CHNS-932 nitrogen micro analyzer (Leco Corporation, St. Joseph, MI, USA) (Etheridge, Pesti, & Foster, 1998). Percentages of soluble protein in the supernatants in relation to the total protein extracted were plotted vs. pH. We assume the IEP as the minimum protein solubility.

### 2.3. Chemical composition of carob flour and isolate

Protein, lipid, moisture and ash contents were determined using AOAC (1990) approved methods. Protein content of carob germ flour, carob protein isolate and aqueous flour dispersions was determined as %N × 6.25 using a LECO CHNS-932 nitrogen micro analyzer (Leco Corporation, St. Joseph, MI, USA) (Etheridge et al., 1998). Soluble sugars and polyphenols were measured using a method described previously by Maza et al. (1989). Standard curves of glucose and chlorogenic acid were used. Total fiber was determined according to the procedure described by Lee, Prosky, and De Vries (1992).

### 2.4. Protein composition of carob germ flour and carob protein isolate

#### 2.4.1. Protein comparison of carob germ flour and isolate (SDS–PAGE)

Protein composition was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Carob germ flour (CF) and carob protein isolate (CI) proteins were extracted for 2 h at 20 °C with a buffer containing 0.025 M Tris–HCl and 0.1% SDS, 5% β-mercaptoethanol at pH 8.4. Electrophoresis was performed according to the method of Laemmli (1970) using a 20% gel and a 5% stacking gel prepared with 30% Acrylamide/Bisacrylamide solution.

#### 2.4.2. Analysis of carob protein isolate under different extraction conditions

*2.4.2.1. Extraction of proteins.* Proteins present in carob isolate were extracted and analyzed by electrophoresis under different denaturing (method A), denaturing and reducing (method B) and native (method C) conditions. Isolate was extracted for 2 h at 20 °C with a 0.086 M Tris-base, 0.045 M mM glycine, 2 mM EDTA, 0.5% SDS pH 10 buffer in method A; while in method B, proteins were dissolved in the same buffer containing 1% DTT. In method C, proteins were extracted at pH 2 with 2 N HCl and at pH 10 with sodium borate buffer (0.1 M). With the aim of knowing the species that form proteins extracted at these extreme pH values, these extracts were also treated with β-mercaptoethanol. Dispersions were centrifuged at 10,000*g* for 10 min at 15 °C.

*2.4.2.2. SDS–PAGE.* These assays were performed using a 10% continuous running gel with a 4% stacking gel. A

dissociating buffer system was used, containing 1.5 M Tris-base, 0.5% SDS, pH 8.8 for the separating gel and 0.125 M Tris-base, 0.96 M glycine, and 0.5% SDS, pH 8.3, for the running buffer. Electrophoresis was performed in a Mini Protean III at a constant voltage of 60 V (stacking gel) and 120 V (continuous gel) with a Power-Pack 300 (Bio-Rad, Richmond, CA, USA). Low MW markers (Pharmacia calibration kit) used included phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

*2.4.2.3. Native-PAGE.* Electrophoretic mobility of proteins extracted by method C was analyzed by native electrophoresis. Native electrophoresis was performed in a 7% polyacrylamide running gel with a 3.5% polyacrylamide stacking gel. A non-dissociating buffer system containing 1.5 M Tris-base, pH 8.8 for the separating gel and 0.125 M Tris-base, 0.96 M glycine pH 8.3, for the running buffer was used. Soybean proteins isolate (SPI) and soybean 11S globulins (11S), prepared according to Puppo and Añón (1999), were used as standards.

### 2.5. Amino acid analysis

Samples containing 2 mg of protein were hydrolyzed using 6 N HCl at 110 °C for 20 h under inert nitrogen atmosphere. Derivatization of amino acids was performed at 50 °C during 50 min with an excess of diethyl ethoxymethylenemalonate. Amino acids were analyzed by reversed-phase high-performance liquid chromatography (HPLC) using D,L-α-aminobutyric acid as internal standard as described (Alaiz, Navarro, Giron, & Vioque, 1992). Tryptophan was analyzed by HPLC after basic hydrolysis according to Yust et al. (2004).

### 2.6. Thermal analysis of carob proteins

The extent of thermal denaturation of carob germ proteins was evaluated by differential scanning calorimetric (DSC) measurements (Puppo & Añón, 1999). Dispersions (8% w/v) of isoelectric isolate (CI4) were adjusted to pH 2 (CI2) and pH 10 (CI10) with 2 N HCl and 2 N NaOH, respectively; and then freeze-dried. Aqueous dispersions of carob flour (CF) and isolates (CI2, CI4, CI10) at 20% w/v were analyzed. Tests were performed in duplicate in hermetically sealed aluminum capsules, where sample and reference were gradually heated between 20 and 130 °C, at 10 °C $min^{-1}$. Capsules were punctured after each run and kept overnight in an oven to determine dry matter content. Assays were performed in a Rheometric Scientific DSC calorimeter (Polymer Laboratories, UK). The equipment was calibrated using indium, lauric acid and stearic acid as standards (Rheometric Scientific, Polymer Laboratories, UK). Denaturation temperature (Td) and transition enthalpy ($\Delta H$) were obtained by analyzing the thermograms with a Software Plus V5.41 (UK).

## 3. Results and discussion

### 3.1. Chemical composition of carob germ flour and carob isolate

A protein isolate may be an attractive ingredient to use in the production of human food, as it has reduced levels of undesirable components, like fiber, lipids or darkening agents (Maza et al., 1989). Compositional data for both carob flour and isolate are shown in Table 1. The isolate and the flour have a protein content of 96.5% and 48.6%, respectively. The results for the protein isolate are in concordance with the criteria of the minimum protein content used in obtaining a protein isolate (higher than 85%). In the case of other legumes, like soybean, a protein concentrate must have at least a protein content of 70%, while a protein isolate should have at least 85% (dry basis, N × 6.25) (Pearson, 1983, chapter 20). Different factors, such as the high percentage of protein obtained or the drop in the content of lipid, ash, humidity, soluble sugars and total fiber, show that the procedure based on alkaline solubilization and isoelectric precipitation was an effective way to get a carob isolate with a high protein content. This method was used previously for other legumes, such as soybean, lupine and beach pea (Chavan, McKenzie, & Shahidi, 2001; Pozani, Doxastakis, & Kiosseoglou, 2002; Puppo & Añón, 1999) and in a pseudo cereal with a protein content similar to that of legumes, as amaranth (Martínez & Añón, 1996).

### 3.2. Solubility profile of carob proteins

The solubility profile of carob germ flour was obtained in order to know the pH corresponding to the isoelectric point of carob proteins. The knowledge of the minimum protein solubility is essential to carry out the isolation process. Flour dispersion (8% w/v) has a pH of 6.8. Fig. 1 shows that the isoelectric point (IEP) of carob germ proteins is located near pH 4.0. This means that the proteins are more soluble at acidic (pH < 2.5) and especially at alkaline pH values (pH > 7.0). Solubility is an important property when functional properties as gelation, emulsification or foaming are considered.

Table 1
Chemical characterization of defatted flour and the isolate obtained from carob germ meal[a]

| | Flour | Isolate |
|---|---|---|
| Protein content (%) | 48.2 ± 0.24 | 96.5 ± 0.71 |
| Lipids (%) | 2.26 ± 0.13 | 0.58 ± 0.01 |
| Moisture (%) | 5.76 ± 0.32 | 2.81 ± 0.03 |
| Ash (%) | 6.34 ± 0.15 | 1.61 ± 0.23 |
| Polyphenols (%) | 0.45 ± 0.01 | 0.56 ± 0.01 |
| Soluble carbohydrates (%) | 2.92 ± 0.03 | 0.27 ± 0.03 |
| Total fiber (%) | 24.3 ± 0.09 | 0.95 ± 0.02 |

[a] Results are expressed as the mean ± standard deviation of two determinations.

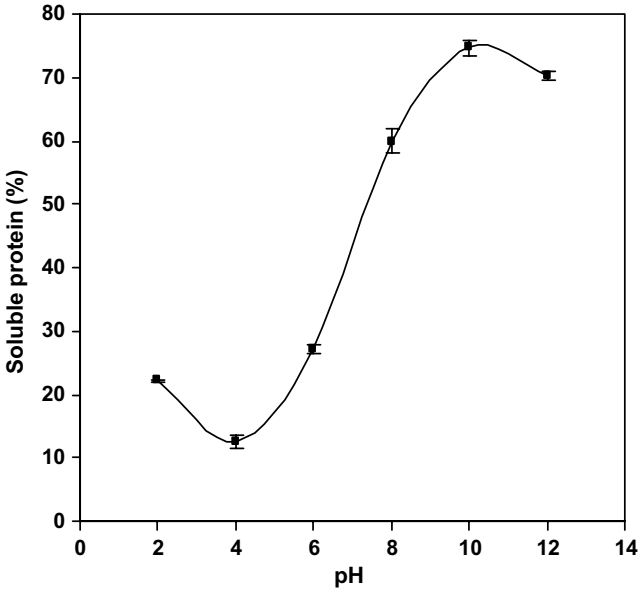


Fig. 1. Solubility profile (Solubility, % vs. pH) of carob germ flour.

As a result, the carob protein isolate was obtained by precipitation at pH 4.0 before spray drying.

### 3.3. Protein composition of carob germ flour and carob protein isolate

#### 3.3.1. Protein profile

*3.3.1.1. Comparison between flour and isolate. SDS–PAGE.* Treatment with denaturing and dissociating agents (SDS + β-mercaptoethanol) made it possible to extract from both the flour and the isolate four protein fractions (131, 102, 48 and 24 kDa), with the 131 and the 48 kDa being the more abundant fractions (Fig. 2). There were no qualitative or quantitative differences between the protein fractions of the flour or the isolate. The 48 kDa band of carob proteins possesses a molecular weight similar to those presented by the subunit of 11S globulins present in vegetable proteins, as soybean conglycinin, pea legumin, fababean, lupine globulin and globulin-P of amaranth (Abdellatif et al., 2005; Martınez & Añón, 1996; O'Kane, 2004, chapter 5; Puppo & Añón, 1999). Nevertheless, more data are needed to confirm that this subunit belongs to an 11S-type globulin. The 11S globulins are formed by subunits of approximately 50 kDa linked by non-covalent bonds (AB subunits). These subunits are known to be constituted by acidic (A polypeptide) of 30 kDa and basic (B polypeptide) of 20 kDa polypeptides linked by disulfide bonds (Puppo & Añón, 1999). Therefore, in dissociation and reducing conditions, the AB subunit should be absent, and a great proportion of A and B polypeptides should be found. Due to the absence of the 30 and 20 kDa polypeptide, the 24 kDa band could be a new kind of low molecular mass polypeptide, that is not present in other legumes. Similar electrophoretic profiles were obtained by other authors for carob seed germ proteins (Dakia et al., 2007) and caroubin (Feillet & Roulland, 1998).

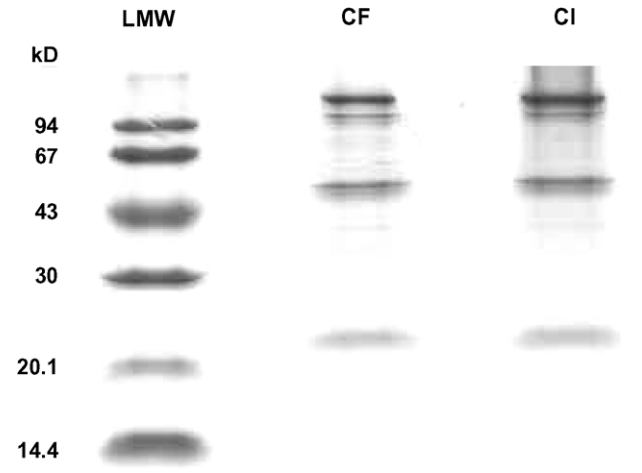


Fig. 2. SDS–PAGE of carob germ flour and carob isolate proteins analyzed under reducing conditions: CF, carob flour; CI, carob isolate; LMW: low molecular weight markers.

*3.3.1.2. Analysis of the carob proteins under different extraction conditions*

*3.3.1.2.1. SDS-PAGE. Extraction with SDS.* Electrophoretic profile of isoelectric carob proteins (p$I = 4$) extracted at pH 10 with SDS and in the absence and presence of a reducing agent (DTT) are shown in Fig. 3a. Soluble aggregates of high molecular mass (HMMA) (>138 kDa) and the 131 kDa polypeptide previously described was observed. A polypeptide of 70 kDa and small amount of the 48 kDa protein were also detected (Fig. 3a-1). The incorporation of DTT (Fig. 3a-2) produced the rupture of disulfide bonds and therefore the proportion of HMMA diminished considerably. Proteins of HMMA were therefore strongly aggregated by covalent disulfide linkages. These aggregates were mainly composed of monomer subunits of 100 and 48 kDa mainly linked by disulfide bonds. Moreover, a high amount of the aggregates of high molecular mass were retained in the stacking gel. The disappearance of the 70 kDa band and the appearance of the 48 kDa subunit indicate that the 70 kDa protein would be a dimmer protein stabilized by the 48 and 24 kDa subunits (the last one did not appear in the gel) linked by disulfide bonds. A polypeptide of 70 kDa was also obtained in an amaranth protein isolate extracted at pH 10, that belongs to the 11S globulin fraction (Martínez & Añón, 1996). Amaranth is a cereal-like crop with high seed protein content composed mainly by globulin-P, globulin 11S and glutelins. In a guava seeds protein isolate, analyzed under dissociating conditions, a 70 kDa polypeptide was also observed (Bernardino-Nicanor, Scilingo, Añón, & Dávila-Ortiz, 2006). In reducing conditions, this peptide was dissociated in several peptides including 48 kDa and 24 kDa monomers.

On the other hand, carob proteins presented a high amount of HMMA formed mainly by the 100 kD

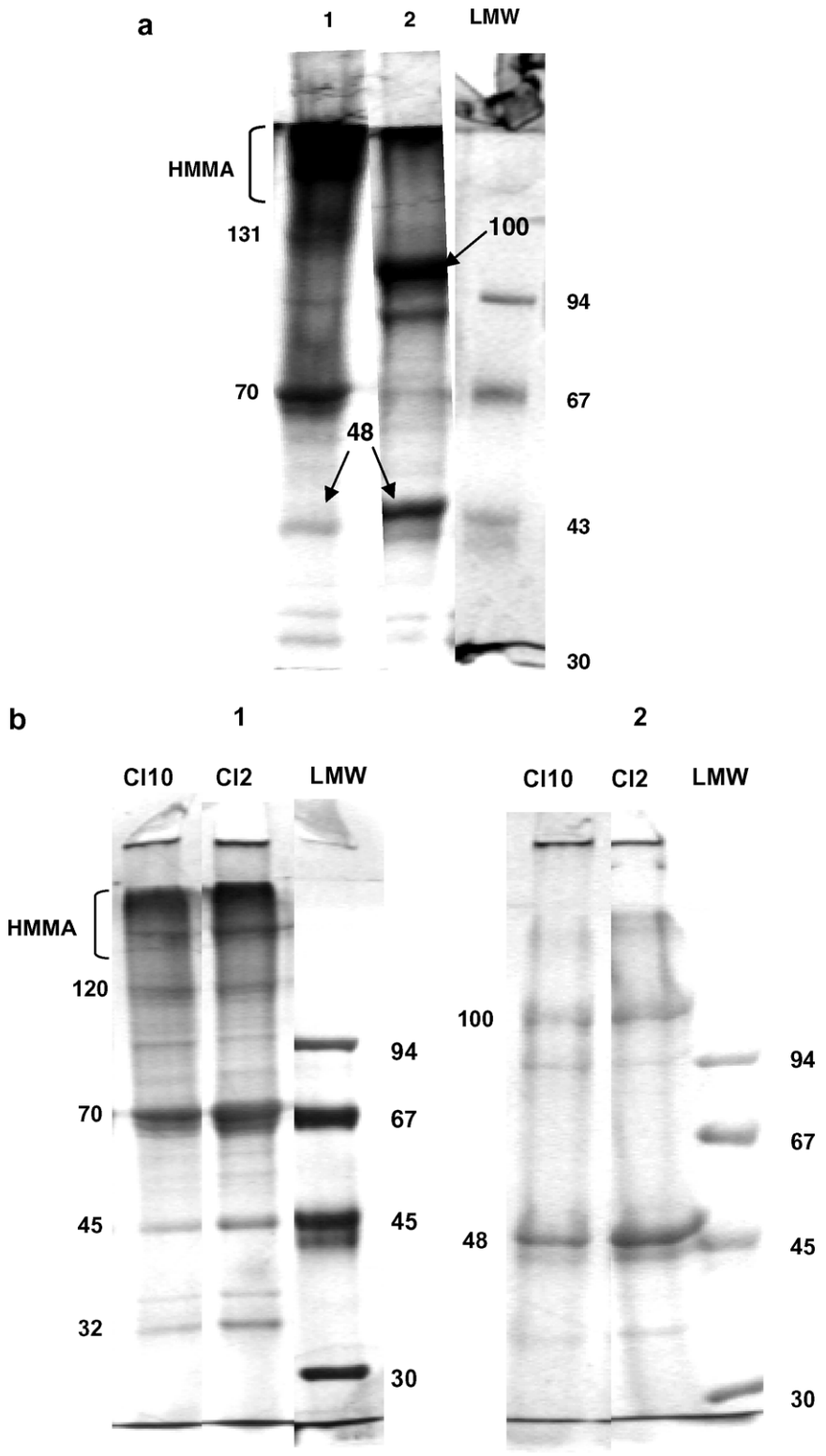


Fig. 3. SDS–PAGE of proteins extracted from carob germ isolate. (a) Proteins extracted under dissociating (SDS – lane 1) and reducing (SDS + DTT – lane 2) conditions. (b) Proteins extracted at pH 10 (CI10) and pH 2 (CI2): (gel 1) electrophoretic sample buffer with 0.5% SDS; (gel 2) electrophoretic sample buffer with 0.5% SDS + 5% β-mercaptoethanol. LMW: low molecular weight markers.

monomer. This monomer is not present in soybean, neither in amaranth and guava seeds. Therefore, carob proteins presented different structure than those observed in other seeds.

*Extraction without SDS*. Electrophoretic profile (SDS–PAGE) of carob proteins extracted without denaturing agents, like SDS, is shown in Fig. 3b-1. Proteins that are linked by non-covalent bonds such as electrostatic,

hydrogen, van der Waals and hydrophobic interactions, are able to be extracted at extreme pH. At pH values above and below the p*I*, where protein has negative and positive charge, protein unfolds interacting with water and increasing its solubility (Vodjani, 1996, chapter 2). The extraction performed at pH 10 and pH 2 (CI10 and CI2) showed a great proportion of HMMA (>120 kDa) and high amounts of the 70 kDa polypeptide. Lower amount of 120, 45 and 32 kDa subunits were observed. Extracts at extreme pH values presented the same protein profile of that observed for the SDS extract of the isoelectric isolate (Fig. 3b-1). Results suggest that a high amount of these proteins are greatly stabilized by non-covalent bonds such as hydrogen bonds, van der Waals bonds, ionic and hydrophobic interactions. Proteins of 48 and 100 kDa (Fig. 3a) present in the aggregates were only observed in the presence of the reducing agent, indicating that they would be linked by disulfide bonds.

No differences in the electrophoretic profile between proteins extracted with an SDS buffer containing β-mercaptoethanol (Fig. 3a-2) and proteins extracted only with SDS and then reduced by β-mercaptoethanol (Fig. 3b-2), were observed. Both extraction procedures showed that HMMA and peptides of 70 kDa were mainly made up of the 48 kDa subunit through disulfide bonds.

*3.3.1.2.2. Native-PAGE.* Carob protein isolate, at acid (CI2) and mainly at alkaline pH (CI10), presented only one protein fraction with lower electrophoretic mobility in comparison to fractions observed for the 11S soybean glycinin of pH 8. Soybean isolate contains low and a high electrophoretic mobility proteins corresponding to the 11S and 7S subunits, respectively (Fig. 4 – SPI, 11S). No differences were observed between CI2 and CI10 profiles. In addition, the typical 7S fraction was not present. These results indicate that proteins present in carob germ would belong to a different kind of protein group, distinct from the 11S globulins observed in other legumes.

### 3.3.2. Amino acid profile

Table 2 shows the amino acid profile of both the carob germ flour and carob protein isolate. They both show a high content of the non-essential amino acids, specially glutamic or aspartic acid and arginine. Values of glutamic and aspartic acid, for both samples (CF and CI), were higher than those obtained by Maza et al. (1989). On the other hand, in carob seed germ flours, our values were lower than those obtained by Dakia et al. (2007). Low values of sulphur amino acids, methionine and cysteine were obtained; with a Met + Cys content approximately 30% and 24% of the values prescribed by the FAO–WHO (1991) for the flour and the isolate, respectively. In addition, our values of these amino acids were lower than those observed by Dakia et al. (2007). The very low content of Met + Cys may be due to the absence of oxidation before hydrolysis in order to avoid the partial degradation of these amino

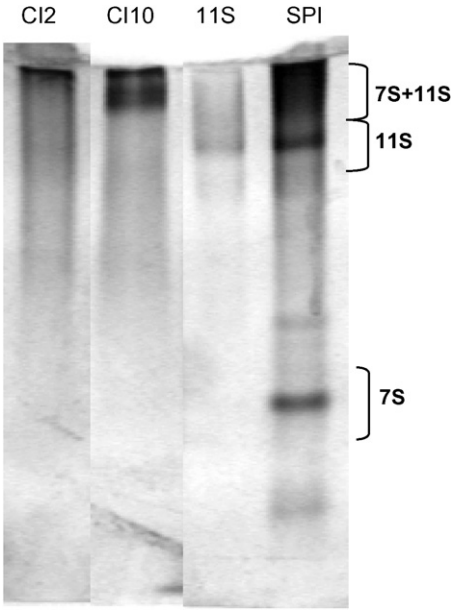


Fig. 4. Native-PAGE of isolate carob proteins extracted at pH 2 (CI2) and pH 10 (CI10). Soybean proteins isolate (SPI) and 11S globulins (11S) were used as standards.

acids. The high content of Met + Cys (3.4%) reported by the carob germ flour manufacturer (Caratina, Alimcart S.L., Mallorca, Sapin), agree with the fact that this flour is not limited in sulphur amino acids content. The content in aromatic amino acids (Phe and Tyr) was also deficient, showing values about 25% lower than the recommended ones. The amount of the amino acid lysine was slightly deficient in the isolate.

The quality of these proteins was estimated calculating the chemical score (CS) as the ratio between the percentage of the essential amino acid of the sample (CF or CI) to this amino acid in the standard (FAO–WHO (1991)). The lowest number of these ratios is the limiting amino acid. In our case, in addition to sulphur amino acids, the other limiting amino acids were found to be phenylalanine and tyrosine. Our results contrast with values obtained by Dakia et al. (2007) in which tryptophan was the limiting amino acid. On the other hand, comparing the chemical score of carob germ proteins with those obtained for other legumes, limiting amino acids of soy proteins (Pearson, 1983, chapter 20; Seligson & Mackey, 1984) were valine and mainly sulphur amino acids, while for lupin proteins (Sujak, Kotlarz, & Strobel, 2006) the first and second limiting amino acids were Met + Cys and tryptophan.

However, the high content in glutamic acid and arginine would allow the use of carob proteins as a suitable ingredient for functional foods for sportspeople. These amino acids play an important role in the nutrition of sportspeople, as they increase the muscular matter, the collagen synthesis, and the glycogen production (Flynn, Meininger, Haynes, & Wu, 2002; Varnier, Leese, Thompson, & Rennie, 1995; Wernerman, 2002).

Table 2
Amino acid composition (AAC, g amino acid/100 g protein)[c] and chemical score (CS % of FAO) of defatted flour and the isolate obtained from of carob germ meal

| Amino acids | Carob flour (CF) | | Carob isolate (CI) | | FAO–WHO (1991) standards |
|---|---|---|---|---|---|
| | AAC | CS | AAC | CS | |
| Aspartic acid | 8.75 ± 0.07 | – | 8.55 ± 0.07 | – | |
| Glutamic acid | 28.1 ± 0.07 | – | 30.2 ± 0.57 | – | |
| Arginine | 11.5 ± 0.21 | – | 13.7 ± 0.28 | – | |
| Serine | 5.05 ± 0.07 | – | 5.0 ± 0.3 | – | |
| Glycine | 5 ± 0 | – | 4.9 ± 0.1 | – | |
| Alanine | 4.4 ± 0.0 | – | 4.1 ± 0.0 | – | |
| Proline | 8.2 ± 0.3 | – | 5.1 ± 0.3 | – | |
| Histidine | 2.3 ± 0.0 | 121 | 2.4 ± 0.2 | 126 | 1.9 |
| Threonine | 3.5 ± 0.0 | 103 | 3.3 ± 0.2 | 97 | 3.4 |
| Valine | 3.05 ± 0.07 | 87 | 2.5 ± 0.3 | 71 | 3.5 |
| Isoleucine | 2.3 ± 0.0 | 82 | 2.15 ± 0.07 | 77 | 2.8 |
| Leucine | 5.9 ± 0.0 | 89 | 6.35 ± 0.071 | 96 | 6.6 |
| Lysine | 5.5 ± 0.0 | 95 | 4.9 ± 0.0 | 84 | 5.8 |
| Tryptophan | 0.9 ± 0.0 | 82 | 1.05 ± 0.07 | 95 | 1.1 |
| Phenylalanine | 2.9 ± 0.0 | 78[a] | 3 ± 0 | 78[a] | 6.3[a] |
| Tyrosine | 2 ± 0 | | 1.95 ± 0.07 | | |
| Methionine | 0 ± 0 | 32[b] | 0.05 ± 0.07 | 24[b] | 2.5[b] |
| Cysteine | 0.8 ± 0.0 | | 0.55 ± 0.07 | | |

[a] Phenylalanine + tyrosine.
[b] Methionine + cysteine.
[c] Results are expressed as the mean ± standard deviation of two determinations.

### 3.4. Thermal analysis of carob proteins

Fig. 5 shows the thermograms for the carob flour (CF) and the isolates prepared at different pH values (CI2, CI4, CI10). Both samples, flour and isolates, presented an endotherm at different temperatures. Carob flour (pH 6.8) presented an endotherm at 105.7 ± 0.3 °C with a denaturation enthalpy of 16.6 ± 4.1 mJ/mg flour. Protein in the isolate of pH 10 (CI10) was still in native form ($\Delta H$ = 17.7 ± 0.1 mJ/mg isolate) almost at the same denaturation temperature ($T_d$ = 103.1 ± 1.2 °C) as that observed for CF. At acid pH, the carob isolate (CI2) was denatured and the endotherm was shifted to high temperatures at extreme acid pH, as it can be deduced from the enthalpy (5.0 ± 0.7 mJ/mg isolate) and the Td (115.2 ± 1.7 °C) values. This great thermal stabilization could be due to the formation of a new protein structure due to the acid pH effect. The isoelectric isolate (CI4) presented the lowest values of enthalpy and temperature of denaturation, being 3.8 ± 0.1 mJ/mg isolate and 90.5 ± 1.5 °C, respectively. The low values of $\Delta H$ of proteins at the isoelectric point have been reported previously for other legumin proteins, like soy or fababean (Arntfield, Ismond, & Murray, 1990, chapter 4). At the p*I*, there is an intense aggregation as a consequence of hydrophobic interactions. This aggregation produces an increase in the exothermic processes that would contribute to a decrease in the enthalpy. Other leguminous proteins have been reported to present, at neutral pH, endotherms in the same temperature range, like soybean (92.7 °C), fababean (91.0 °C), field pea (94.5 °C) and amaranth, a globulin-rich pseudocereal (94.0 °C) (Arntfield et al., 1990, chapter 4; Martínez & Añón, 1996; Puppo & Añón, 1999). This endotherm could be attributed to the denaturation of the 11S globulin. Nevertheless, to confirm the presence of an 11S type globulin in this isolate calorimetric assay on an 11S fraction, purified from carob germ flour, should be performed.

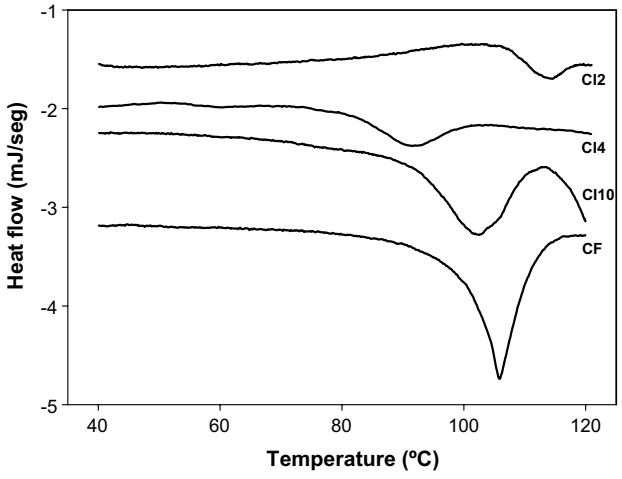


Fig. 5. DSC thermograms of aqueous dispersions (20% w/v) of carob germ flour (CF) and carob germ isolate. CI2: protein precipitated at pH 4 and dissolved at pH 2, CI4: protein precipitated at pH 4, CI10: protein precipitated at pH 4 and dissolved at pH 10.

## 4. Conclusions

An isolate with protein content higher than 95% from carob flour, applying the procedure of alkaline solubilization followed by isoelectric precipitation, can be obtained.

This isolate thus obtained presented, as in flour, high content of aspartic and glutamic acids, and arginine amino acids. According to our results, the limiting amino acids were methionine + cysteine and phenylalanine + tyrosine. The isolate presented proteins with low electrophoretic mobility and were mainly stabilized by HMMA. These aggregates were formed by the 131 and 70 kDa subunits linked by non-covalent bonds, and other peptides strongly bounded by disulfide interactions. Both, aggregates and subunits were formed mainly by 100 and 48 kDa monomers linked by disulfide bonds. A considerable content of high molecular mass peptides (HMWP) strongly bounded were also found. These HMWP were not dissociated by the combined effect of the SDS and DTT. No differences in the nature of proteins present in the acid or alkaline isolates were observed. Nevertheless, proteins presented higher denaturation temperature and became more denatured at acid pH (pH 2) than at pH 10.

Carob is a legume whose composition analysis, protein characterization and thermal properties vary from results obtained by other authors for other crops as soybean, pea, lupine, amaranth and guava. The knowledge of the nature of proteins that form carob materials like germ flours and isolates, and its thermal properties would be important from the point of view of the application of this crop as ingredient in formulated foods.

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