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Large scale purification of rapeseed proteins (*Brassica napus* L.)

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Abstract

Rapeseed (*Brassica napus* L.) cruciferin (12S globulin), napin (2S albumin) and lipid transfer proteins (LTP) were purified at a multi-g scale. The procedure developed was simple, rather fast and resolutive; it permitted the recovery of these proteins with a good yield, such as 40% for cruciferin and 18% for napin. Nanofiltration eliminated the major phenolic compounds. The remaining protein fraction was fractionated by cation exchange chromatography (CEC) on a streamline SP-XL column in alkaline conditions. The unbound neutral cruciferin was polished by size exclusion chromatography. The alkaline napin isoforms and LTP, adsorbed on the beads, were eluted as a whole fraction and further separated by an other CEC step at acidic pH. Napins were polished by hydrophobic interaction chromatography (HIC). The fractions were characterized by reverse phase HPLC, electrophoresis, N-terminal sequencing and mass spectrometry. All the fractions contained less than 5% of impurities.

Keywords: Purification; Preparative scale; Rapeseed; Cruciferin; Napin; LTP

1. Introduction

Rapeseed proteins are underexploited because they are only used in animal feeding. However, some potential for non-food valuable applications have been recently shown for these proteins in an European project [1]. Therefore, great quantities of proteins were needed to study potential applications as functional additives. Preparative purification processes of seed storage proteins were described for 11S and 7S globulin-type proteins in pea and soya [2–8]. In the case of rapeseed storage proteins which are composed of 11S globulin- and 2S albumin-type proteins, only analytical purifications were developed, except procedures based on ammonium sulphate selective precipitation of proteins [9]. Size exclusion chromatography (SEC) was largely used to achieve the separation of 11S and 2S proteins [10], and cation exchange chromatography (CEC) for the separation of 2S isoforms [11]. These chromatographic methods led to highly purified proteins but in low amounts incompatible with functional studies. For this purpose, our aim was to develop a purification procedure at a large scale. The preparative processes optimized for soya or pea proteins cannot be used for rapeseed because of its specificities (i) great quantities of pigments and polyphenols which can bind proteins, and (ii) great amounts of albumins. Consequently, a specific process must be developed for the purification of rapeseed proteins, taking into account these particularities.

Rapeseed protein meal contains two predominant classes of seed storage proteins: 12S globulin (cruciferin) which represents 25–65% of its protein content [12] and 2S albumin (napin). It contains also some minor proteins of interest, such as thionins, trypsin inhibitors and a lipid transfer protein (LTP).

Cruciferin, as a member of the 11S globulin family, shares structural features with soya glycinin which is organized into hexamers as revealed by its crystal structure [13]. The mean molecular mass of cruciferins was estimated to be around 300,000 Da in their native conformation and their isoelectric point (p*I*) about 7.2 [14]. The native conformation is stable at neutral pH and high ionic strength. At extreme pH and in urea solutions, the protein totally dissociates into six subunits [15], each of them being composed of two polypeptide chains (α and β) of about 30,000 and 20,000 Da linked by a

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disulfide bond [10]. Ten isoforms of the cruciferin precursor are described in Swiss-Prot/TrEMBL for *Brassica napus*. The hexameric structure resulting from a combination of several isoforms leads to a high complexity and high protein diversity [10].

Napins belong to albumin storage proteins; in the seeds of recent varieties, they are present in lower quantities than cruciferins. Mature napins are highly basic proteins (p*I* around 11) and exhibit molecular weights between 12,500 and 14,500 [11]. They comprise two polypeptide chains held together by two disulfide bonds: a small (4500 Da) and a large one (10,000 Da). They are encoded by a multigenic family, initially synthetised as a precursor [16] which is proteolytically cleaved to generate mature napin chains. Only four napin genes were sequenced [17–22] among 10–20 napin genes attended. Such a large number of genes result in a multiplicity of isoforms among those five were identified by Monsalve and Rodriguez [11].

Among the minor proteins of rapeseed, LTP are of potential interest: the biological role of these proteins is still unknown, but some hypothesises have been proposed, such as contribution in the cutin layer synthesis [23] and defensive role against pathogens [24] or environment stresses [25–27]; moreover, the LTP present some properties which are desirable for cosmetic or food additive purposes, such as stabilizing beer foam after brewing process in the case of barley LTP1 [28]. Another topic concerning LTP is that plant LTPs have been recently suspected of being allergenic: for example, maize [29], peach [30], wheat [31] and even *Brassica* species [32]. The LTPs are present in many vegetable sources. They are basic proteins with a p*I* comprised between 9 and 10. They are coded by a multigenic family which leads mainly to two different groups of different MW. The first one, named LTP1, is composed of 9 kDa proteins, the second one (LTP2) of 7 kDa proteins [33]. Only the 9 kDa family has been revealed in rapeseed. Four isoforms have been observed and three of them sequenced [34]. The different rapeseed LTPs show a great homology between themselves (at least 82%) but they share only 40–50% with cereal LTPs. As other plant LTPs, rapeseed LTPs contain eight cystein residues, all of them engaged in four disulfide bridges. Wheat LTP structure consists in a very hydrophobic cavity fixed by four α-helix stabilized by the disulfide bridges, and able to fix lipids or other hydrophobic molecules [35].

In summary, cruciferin exhibits very different characteristics from other proteins: high molecular weight, neutral p*I*; but aggregation could occur during the purification process. On the opposite, napin and LTP show rather close characteristics, that could complicate the purification process: molecular weights and p*I*.

The approach developed in the present paper wants to meet two main objectives, (i) the scaling-up for producing rather large amounts of purified napin, cruciferin and LTP and (ii) the quantitative removal of the contaminating pigments. Consequently, we chose a combination of membrane and chromatographic techniques. We describe a comparison between analytical- and preparative-scale purification.

2. Experimental

All the chromatographic devices and columns used were from Amersham Biosciences, except the desalting column GH 25 and the RP-HPLC columns.

2.1. Materials

Rapeseed meal (var. Express) was obtained at the pilot scale using CETIOM facilities (Pessac, France). The seeds were dehulled, crushed and defatted by hexane extraction under mild thermal conditions.

2.2. Analytical scale

Proteins were extracted from 100 mg of rapeseed meal slurried in 1 ml of 50 mM Tris–HCl buffer, pH 8.5, containing 750 mM NaCl, 5 mM EDTA and 0.3% sodium bisulphite during 1 h. The suspension underwent centrifugation for 10 min at 15,000 × *g*; the supernatant was recovered and the extraction was repeated on the pellet in the same conditions. Both supernatants were pooled and stored at −20 °C. Pigments were removed by chromatography using a HiTrap desalting column (1.6 cm × 2.5 cm, 5 ml) on a Äkta Purifier system. The column was equilibrated in a 50 mM Tris–HCl buffer pH 8.5 containing 1 M NaCl at a flow rate of 4 ml/min. Five hundred microliters of the centrifuged protein extract were loaded on the column and the depigmented protein extract was recovered in the excluded peak.

The purification process at the analytical scale involved a CEC step followed by a SEC step for 12S purification, and a hydrophobic ion chromatography (HIC) for 2S purification.

The CEC step involved a Mini S column (4.6 mm × 50 mm, 0.8 ml) equilibrated in buffer A (50 mM Tris–HCl, pH 8.5, 5 mM EDTA, 0.3% sodium bisulphite). The elution was performed at 0.5 ml/min by a linear gradient from buffer A to buffer B (buffer A added with 1 M NaCl) in 10 min.

The unbound fraction obtained was then purified by a SEC step using a Sephacryl S-300 column (1 cm × 30 cm, 24 ml). Elution with buffer C (50 mM Tris–HCl, pH 8.5, containing 1 M NaCl) was carried out at 0.2 ml/min.

The bound fraction obtained after CEC was purified by hydrophobic interaction chromatography (HIC). This fraction was added with sodium sulphate at 1 M final concentration and stirred overnight at 7° C. After centrifugation for 10 min at 20,000 × *g*, the supernatant was loaded on a HiTrap Phenyl Sepharose 6 Fast Flow (1 ml) equilibrated in buffer D (50 mM Tris–HCl pH 8.5, 1 M sodium sulphate, 5 mM EDTA, 0.3% sodium bisulphite). Elution was performed at 1 ml/min by a decreasing non-linear gradient of buffer D mixed with buffer A in four steps at 60, 35, 10 and 0% of D. The eluted frac-

tions were pooled, desalted on the HiTrap desalting column and freeze-dried.

2.3. Extract preparation at a preparative scale

The meal (3.50 kg) was dispersed for 1 h in 100 l of buffer B at room temperature. The suspension was separated with an industrial continuous centrifuge clarifier Westfalia SA 14 (Château-Thierry, France) at a maximum acceleration of 6400 × *g*. The supernatant (100 l) was then concentrated and desalted by nanofiltration on a home-made module equipped with Pall-Exekia 1 kDa membranes (Bazet, France): an initial concentration step to 30 l, then a diafiltration step with deionised water and a final concentration to 20 l. Finally, the retentate was freeze-dried.

2.4. Preparative cation exchange chromatography

A first CEC was performed at alkaline pH on a Streamline 50 column (5.5 cm × 100 cm) containing 300 ml of Streamline SP-XL gel previously equilibrated in buffer A, in expanded mode. The depigmented protein extract was loaded by fractions of 60 g diluted in 1.8 l of buffer A and centrifuged for 20 min at 10,000 × *g*. The Streamline SP-XL dynamic binding capacity of lysozyme and recommended velocity for the elution step were equal to 70 mg/ml gel and 50–150 cm/h respectively (Amersham Biosciences). We chose a 50 ml/min flow-rate corresponding to a velocity of 126 cm/h, during the different stages: loading, washing and elution. Total cycle production took 133 min: 10 for gel expansion, 36 for loading, 23 for washing, 4 for lowering the valve, 47 for elution and 13 for regeneration. After recovering of the unbound fraction (2.5 l, labeled F_0), the elution of the bound fraction was performed on the gel in packed mode by a gradient of B buffer. The gradient was established in two steps at 35 and 100% of B buffer. The fraction F_1 corresponding to the 35% step was recovered and splitted in three parts. Each of them was desalted on a Cellufine GH-25 column (Millipore Corporation, 9 cm × 40 cm, 5 l) equilibrated in 0.1% ammonium carbonate and eluted at 80 ml/min. The fractions containing the proteins were pooled and freeze-dried.

F_1 desalted fraction was further purified by CEC on a FPLC preparative system (P 6000 pumps) equipped with a Source 30S column (5 cm × 15 cm, 250 ml) equilibrated in buffer E (50 mM MES buffer pH 5.3). The Source 30S dynamic binding capacity of lysozyme was equal to 80 mg/ml gel (Amersham Biosciences). F_1 was solubilized in buffer E, loaded onto the column and eluted at a flow-rate of 20 ml/min (corresponding to a five bars pressure drop, maximum for our column and apparatus) with a salt gradient from buffer E to buffer F (buffer E + 700 mM NaCl) in steps: 0% buffer F for 5 min, from 0 to 25% buffer F over 15 min, from 25 to 40% buffer F over 45 min, from 40 to 100% buffer F over 3 min and a step at 100% F over 12 min.

2.5. Preparative hydrophobic interaction chromatography

The enriched 2S fraction F_{12} issued from the CEC Source 30S step was purified by HIC in the same conditions than at the analytical scale, except for the volume of HIC gel (200 ml) and the flow rate (50 ml/min). The eluted fractions were pooled, desalted on a Cellufine GH-25 desalting column (90 mm × 400 mm, 5 l) with 0.1% ammonium carbonate and freeze-dried.

2.6. Preparative size exclusion chromatography

Freeze-dried F_0 fraction (2 g) was solubilized in 100 ml of buffer C. The solution was centrifuged at 20,000 × *g* for 10 min. The supernatant was loaded on a Sephacryl S-300 column (50 mm × 920 mm, 1.8 l) equilibrated in buffer C. Elution was performed at 10 ml/min during 5 h. The eluted major fractions were pooled, desalted and freeze-dried.

2.7. Characterization of the fractions

The protein contents were measured by the Kjeldahl method.

Polyphenols were quantified in the extracts by measuring the absorption at 325 nm [36].

For determining the proportions of the rapeseed proteins and the recovery yields of the purified fractions, the depigmented protein extract was fractionated by SEC. Five hundred microliters of extract were loaded on a Superdex 75 HR column (1 cm × 30 cm, 24 ml) equilibrated with 50 mM Tris–HCl buffer pH 8.5 containing 1 M NaCl. Elution was performed during 45 min at a 0.5 ml/min flow rate.

SDS-polyacrylamide gel electrophoresis was performed using 90 mm × 90 mm × 0.75 mm slabs according to the Laemmli procedure [37]. Migration and stacking gel contained respectively, 15 and 6% (w/v) of polyacrylamide. Electrophoresis was performed in reducing and non-reducing conditions. Reducing conditions were obtained by incubation of the sample 5 min at 100 °C with 5% (v/v) of β-mercaptoethanol.

N-terminal amino acid sequencing was performed by Edman degradation on a model 477A gas-phase sequencer. The phenylthiohydantoin amino acids were analysed online by RP-HPLC using a 120A analyser (Applied Biosystems, Foster City, CA).

Reverse phase high performance liquid chromatography (RPC) was carried out on a Gilson system controlled by Unipoint software (Gilson). Fifty microliters of the samples diluted at 1 mg/ml in water containing 0.06% of trifluoroacetic acid (TFA) were loaded on a C18 nucleosil column (450 mm × 250 mm, 3.1 ml, Nucleosil), equilibrated in water containing 0.06% of TFA. Proteins were eluted by a gradient of acetonitrile (0–80% in 20 min) and detected at 220 and 280 nm.

The conformation of the 12S globulin was followed by ultracentrifugation. The protein fractions were dispersed in

0.1 M sodium phosphate buffer pH 7 at 8 mg/ml) and centrifuged at 20,000 × *g* for 20 min. Supernatants were layered on the top of an isokinetic sucrose gradient (5–20%) established in phosphate extraction buffer, and centrifuged for 16 h at 164,500 × *g* in a Beckman L-65B preparative ultracentrifuge using a SW41Ti rotor. The protein separation was detected by UV detector (Amersham Biosciences, with a 280 nm filter). Lysozyme (1.9S), bovine serum albumin (4.4S), globulin (7S) and catalase (11.2S) obtained from Sigma were used as standards for the determination of the sedimentation coefficients.

Electrospray mass spectra of the intact proteins were recorded on a ion trap mass spectrometer equipped with an electrospray ionisation source at atmospheric pressure (electro-spray mass spectrometer, ES-MS) LCQ Advantage, Thermo-Finnigan. The purified and freeze dried proteins were dissolved in water:acetonitrile 1:1 (v/v) containing 0.5% of formic acid. Sample was continuously infused at a flow-rate of 5 μl/min and mass spectrum was recorded in the range of 200–2000 *m/z*.

The lipid binding activity used the enhancement of fluorescence induced by the binding of Pyr-GPG with LTP. Pyr-GPG (hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol), in solution in phosphate buffer forms small unilamellar vesicles which are not fluorescent. The change in fluorescence was measured at 25 °C with a Fluoromax-Spec (Jobin Yvon, France) using an excitation wavelength set at 340 nm. Ten microliters of hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (Pyr-GPG) previously solubilized at 10 mg/ml in 10% ethanol were added to 1 ml of 50 mM phosphate buffer pH 7 and the fluorescence intensity recorded at 375 nm. After few seconds, 10 μL of protein solution were added to this solution and fluorescence intensity was recorded versus time.

3. Results

3.1. Analytical purification of rapeseed proteins

The extraction of rapeseed proteins reached 77% in 50 mM Tris–HCl buffer, pH 8.5 containing 0.75 M NaCl but numerous pigments were dragged with. Because of their color and their potential interactions with proteins, their elimination is a prerequisite for further purification. We were able to remove 95% of them by using a HiTrap desalting column chosen for its exclusion limit of 5000 Da: proteins were eluted in the void volume whereas polyphenols (MW comprised between 300 and 1000 Da) were eluted near and after the total volume of the column because of interactions with the matrix.

The composition of the protein extract as determined by SEC on Superdex 75 (Fig. 1) revealed four peaks including the two major protein families, cruciferin (peak 1) and napin (peak 3). The second peak was not pure (mixture of cruciferin and napin), and the fourth peak was shown by electrophoresis to contain LTP (results not shown). From the extinction coefficients of the proteins [38], the proportions of cruciferin and napin in the protein extract of rapeseed meal variety Express were estimated at 58.3 and 34.9%, respectively. The proportion of LTP could not be determined as the fourth peak contains other proteins than LTP.

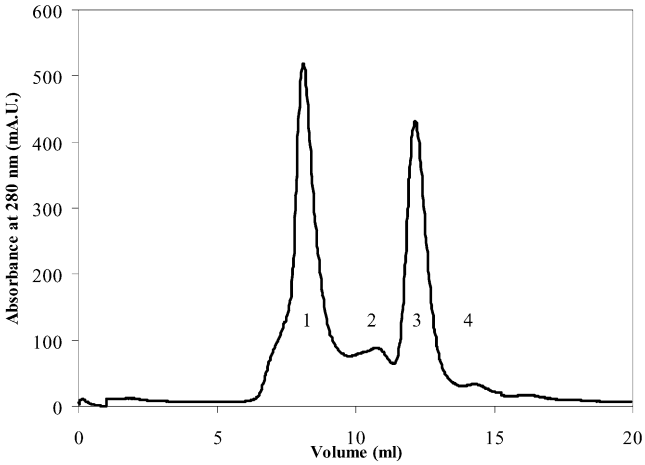

Fig. 1. Analytical separation of rapeseed proteins by SEC on a Superdex 75 column. Elution was performed in buffer C (50 mM Tris–HCl pH 8.5 containing 1 M NaCl) at 0.5 ml/min.

At the analytical scale, cruciferin was purified as the unbound fraction of CEC at pH 8.5 (Mini S column) further polished by a SEC step (S 300 column) to remove aggregates and LMW proteins. Napin and LTP, bound in the first CEC step were further separated by a HIC step on a Phenyl Sepharose 6B FF. After addition of sodium sulphate and centrifugation, five fractions were separated by hydrophobic chromatography. All these fractions corresponded to several isoforms of napin (proved by RPC and SDS–PAGE assays, results not shown) except the last fraction which corresponded to an other protein with a molecular weight around 20 kDa. The purified napin was gathered in the four first fractions.

3.2. Extraction and capture of the rapeseed proteins at a preparative scale

The process scaled-up (Fig. 2) involved the same extraction buffer than at analytical scale, but different separation tools: industrial and continuous centrifuges and nanofiltration membranes. From 3.5 kg of rapeseed meal (39% of proteins), 1036 g of enriched and depigmented protein extract (82.8% of proteins and 0.5% of salts) were obtained. The protein extraction yield was slightly lesser than at analytical scale (63% versus 77%).

The efficacy of nanofiltration technique for desalting proteins and removing pigments reached 98.6% for the desalting effect and 88.5% for the decolouration. This pigment depletion can reach 91% after an additional chromatographic step on a Hitrap desalting column. These results close to those obtained at the analytical scale prove that (i) the pigments

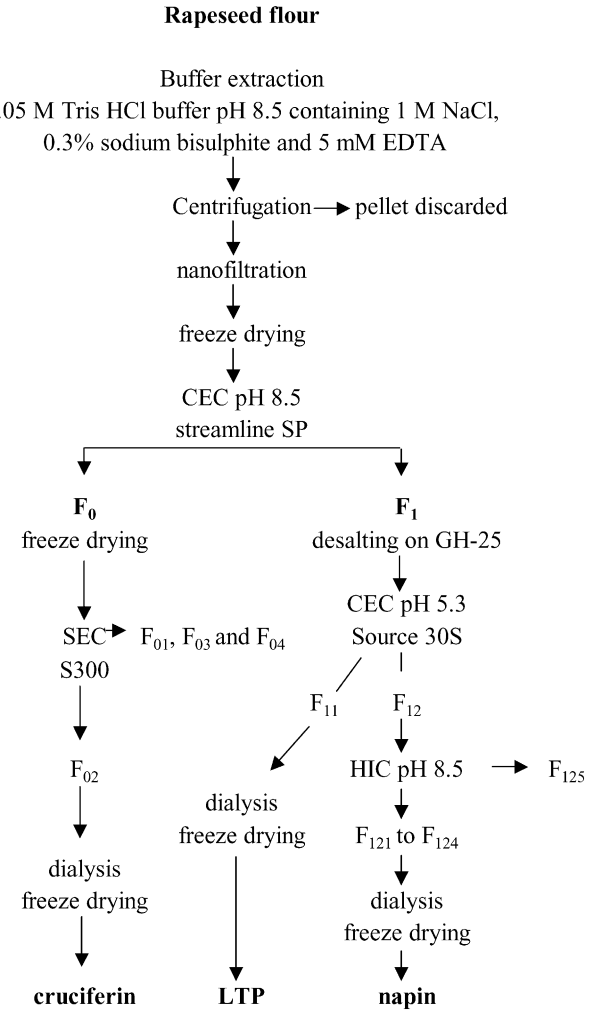

Fig. 2. General purification process.

permeated through the 1 kDa NF membrane and (ii) almost no covalent linkages were created between polyphenols and proteins. Such linkages would have been found if phenol oxidation had occurred during the process.

The desalted and decoloured protein extract was fractionated by CEC on a Streamline SP XL column at a 126 cm/h velocity. We did not optimise the velocity, because it was close to the maximum recommended by Amersham Biosciences for the elution step. On the other hand, we optimised the sample size: taking in account both the dynamic binding capacity and the amount of proteins to be bound by the matrix (34.5 g/100 g protein extract), 30–60 and 90 g of protein extract were loaded, corresponding to 50–100 and 150% of the maximum loading. At 150%, an appreciable amount of 2S albumin was not bound to the matrix, whereas the binding was complete at 100 and 50%. Consequently, 60 g of protein extract were loaded for all the preparative steps. As expected from the pH of the equilibration buffer, cruciferin was not retained on the sulfopropyl groups of the gel (Fig. 3). The electrophoretic pattern in reducing conditions (Fig. 4) clearly indicates that the unbound fraction (F_0) is composed of cruciferin with typical bands around 30 and 20 kDa, characteristic of its α and β constitutive polypeptides. In non reducing conditions, a major band corresponding to cruciferin subunits is found around 55 kDa. However, minor bands characteristic of the α and β polypeptides of cruciferins are detected as well as a slight band on the top of the gel. These observations reflect different conformation states of cruciferin in this fraction. Partially reduced forms and aggregated forms are present in this fraction. The reduction is probably due to the use of sodium bisulphite in the initial extraction buffer.

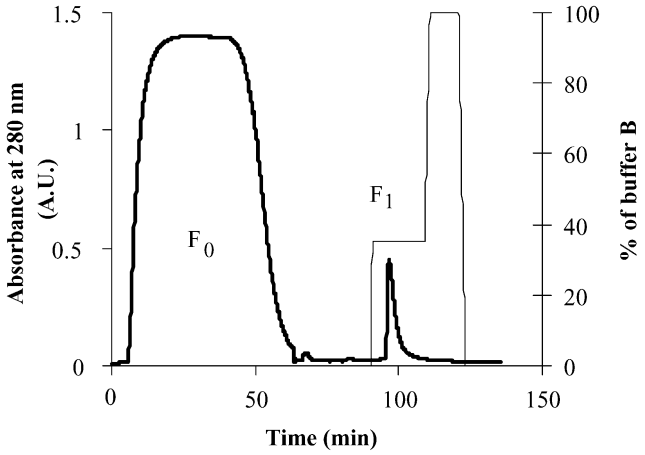

Fig. 3. Fractionation of the protein extract by CEC on a Streamline SP-XL column (55 mm × 150 mm, 300 ml). Gradient from buffer A (50 mM Tris–HCl pH 8.5 containing 5 mM EDTA and 0.3% sodium bisulphite) to buffer B (A + 1 M NaCl) in two steps at 35 and 100%. Flow rate 50 ml/min, F_0: unbound fraction, F_1: bound fraction eluted at 35% of buffer B.

Napin and LTP are bound on CEC at pH 8.5. None of the gradients tested permitted the separation of these two proteins, they were always coeluted. Therefore, we chose to elute these proteins in a single step corresponding to 35% of buffer B (F_1 on Fig. 3).

Napin and LTP were separated on a cation exchanger (Source 30S) after adjusting the pH of the fraction to 5.3. In these conditions both proteins were bound and were separated by applying a salt gradient. LTP was found in the first peak F_{11} and large quantities of napin were obtained in the second peak F_{12} (Fig. 5).

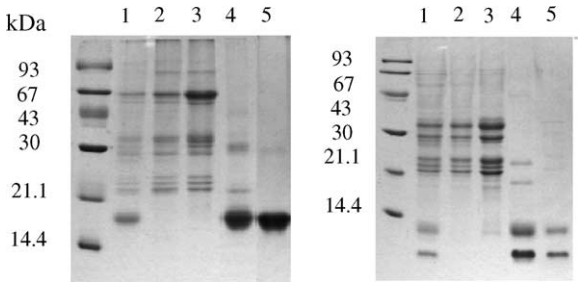

Fig. 4. SDS–PAGE in non-reducing (left part) and reducing conditions (right part) of fractions: (1) total extract; (2) F_0; (3) F_{02}; (4) F_{12}; (5) F_{122}. Migration gel contained 15% (w/v) of polyacrylamide and stacking gel 6%. Reduction of SS bridges with 5% β-mercaptoethanol.

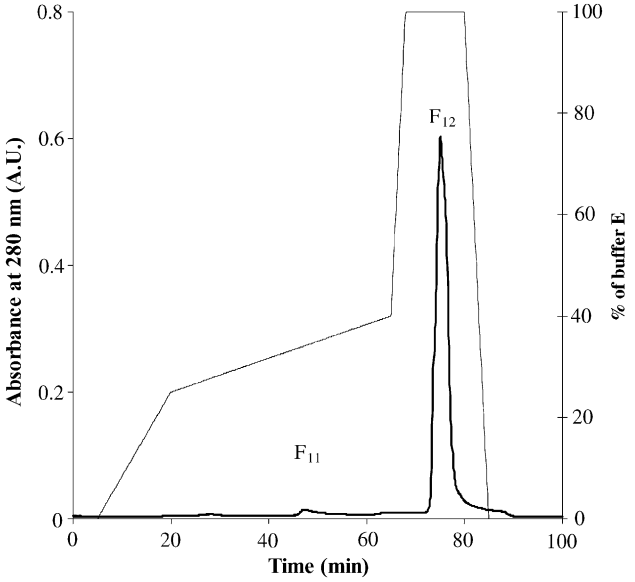

Fig. 5. Fractionation of F_1 by CEC on a Source 30 S column (5 cm × 12.5 cm, 250 ml). Gradient from buffer D (50 mM MES buffer pH 5.3) to buffer E (buffer D + 700 mM NaCl). Flow rate 20 ml/min.

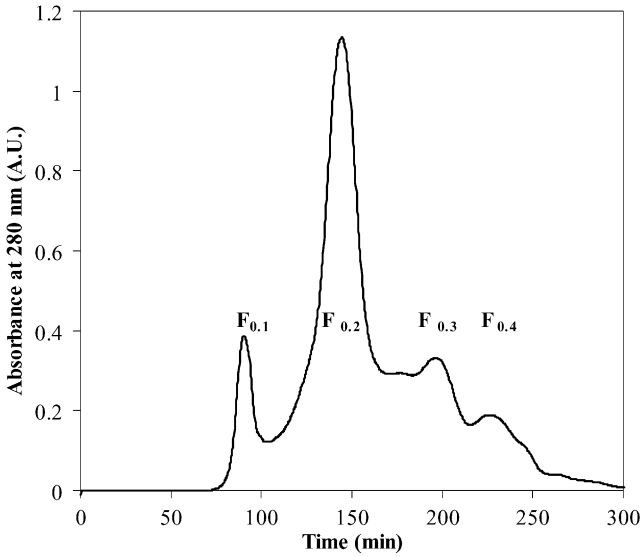

Fig. 6. Fractionation of F_0 fraction by SEC on a Sephacryl S-300 column (50 mm × 92 mm, 1.8 l) in buffer C (50 mM Tris–HCl buffer pH 8.5 containing 1 M NaCl) (flow rate 10 ml/min).

This was confirmed by the measurement of lipid binding activity of the fractions. Only F_{11} exhibited capture properties with a four-fold increase of the fluorescence. After dialysis and freeze drying, the amount of F_{11} was 5 g.

3.3. Polishing of cruciferin at a preparative scale

As shown by the electrophoretic pattern of F_0 fraction, all cruciferin was not in its native state; moreover, freeze-drying can favour the aggregation process. Consequently the freeze-dried F_0 fraction was fractionated by size exclusion on a Sephacryl S-300 in order to remove non-native forms and other small compound traces, particularly the residual pigments (Fig. 6). The peak F_{01} was composed of insoluble aggregates and made an opalescent solution. The major peak (F_{02}) was characterized by SDS–PAGE as 12S form; its native state was confirmed by ultra-centrifugation analysis (Fig. 7). Results show a major peak at $S_{W,20}$= 11.8S. Total of the surface of the minor peaks did not represent more than 5% of the total area. This proves that the quaternary structure of cruciferin was maintained during the purification process. The yield of native cruciferin was estimated after desalting and freeze-drying, it reached 40%, as high as at the analytical scale.

3.4. Polishing of napin at a preparative scale

The F_{12} fraction containing 2S protein was polished by HIC by applying the same chromatographic conditions than at analytical scale. This step allowed the separation of unknown components in the last F_{125} peak and the elimination of residual pigments which were adsorbed on the column and eluted only with 70% ethanol. Purified napin isoforms were gathered in the peaks F_{121} to F_{124}, dialyzed and freeze dried. Their purity controlled by SDS–PAGE assay (Fig. 4) was equivalent to that obtained at analytical scale: one band around 14 kDa in non reducing conditions and two bands around 10 and 5 kDa after reduction.

3.5. Characterization of LTP at a preparative scale

Fraction F_{11} containing LTP was purified by RPC (Fig. 8). The major peak F_{111} was collected and analyzed by mass spectrometry (Fig. 9). A major isoform of MW 9423.7 ± 1.6 Da was found which corresponds to the non specific lipid transfer protein 1 isoform III (MW 9424) and differs from the isoforms I and II (MW 9408) [34]. This was confirmed by sequencing some amino acids of the N terminal part of the molecule (Table 1).

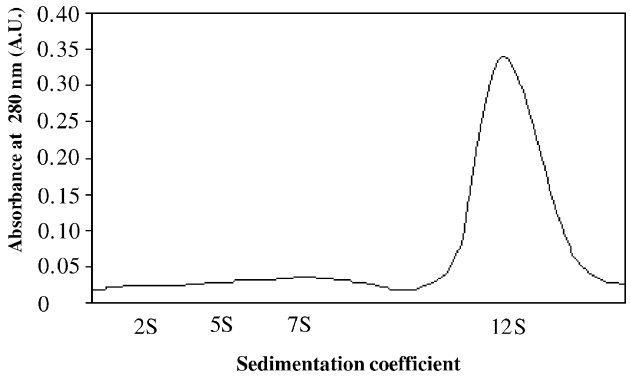

Fig. 7. Ultracentrifugation of F_{02} in a 0.1 M Na phosphate buffer pH 7 during 16 h at 164,500 × *g* (Beckman L-65B), in a 5–20% sucrose gradient.

Table 1
Comparison between the amino acid sequence of F_{111} fraction (35 first residues) and the sequences of rapeseed LTP1 isoforms I–III[a]

	10	20	30	40
F_{111}	H_2N-ALSCGTVSGN	LAACIGYLTQ	NGPLPRGCCT	GVTNL…
Isoforms				
III	H_2N-ALSCGTVSGN	LAACIGYLTQ	NGPLPRGCCT	GVTNLNNMAR TTPDR…
II	H_2N-ALSCGTVSGY	VAPCIGYLAQ	GAPALPRACC	SGVTSLNNLA RTTPD…
I	H_2N-ALSCGTVSGY	VAPCIGYLAQ	NAPAVPTACC	SGVTSLNNMA RTTPD…

	50	60	70	80	90
III	QQACR	CLVGAANSFP	TLNAARAAGL	PKACGVNIPY	KISKSTNCNS VR
II	RQQAC	RCLVGAANAF	PTLNAARAAG	LPKACGVNIP	YKISKTTNCN SVK
I	RQQAC	RCLVGAANAL	PTINVARAAG	LPKACGVNIP	YKISKTTNCN SVK

[a] from [34].

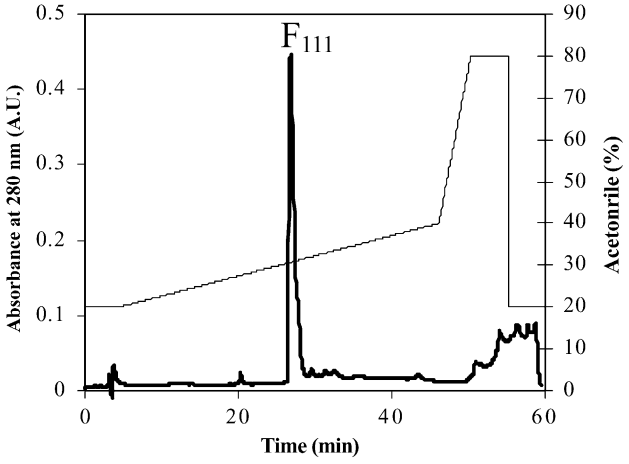

Fig. 8. RPC of F_{11} on C18 Nucleosil column. Elution from water containing 0.06% TFA to 80% acetonitrile.

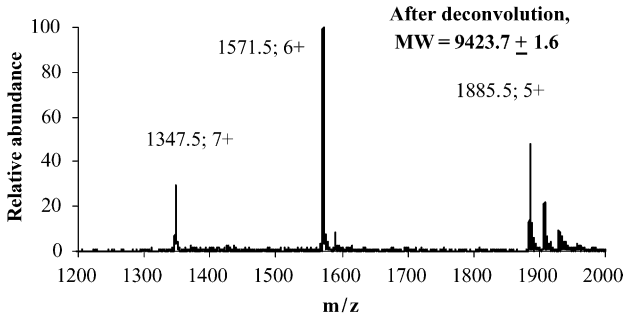

Fig. 9. ESI-MS spectrum of F_{111} dissolved in 50% acetonitrile containing 0.5% formic acid and continuously infused at 5 μl/min.

4. Discussion

Rapeseed proteins are known to be difficult to purify. One of the main difficulties is the presence of water-soluble pigments extracted with proteins. Until now the methods of purification previously described did not eliminate those pigments [10], or eliminated them partially by precipitation of the proteins [9,14,22] or fixation of polyphenols on an appropriate resin. Inconvenient of the precipitation way is the co-precipitation of the two types of proteins as complex and the partial binding of pigments to those proteins. Moreover precipitation could denature proteins, especially by forming globulin aggregates [9]. The use of resins to capture polyphenols is not a better way as they also capture proteins.

In the present work the first step of pigments elimination performed on a desalting gel at the analytical scale, is very simple and fast, preserves the structure of proteins and avoids complex fixation. Advantages of this procedure are its ease and quickness. The resulting final fractions were uncoloured in solution as well as in freeze-dried powder. By UV spectroscopy, no pigments could be detected. Moreover, the sodium bisulphite, used to avoid the phenol oxidation during extraction affects only slightly the disulfide bonds, mainly those of cruciferin. It was almost the same at the preparative scale: nanofiltration proved its efficacy to both desalt proteins and remove polyphenols. The residual polyphenols of the decoloured extract were not bound to the proteins, due to EDTA and sodium bisulphite; they were separated from the proteins in the SEC and HIC steps, for the purification of 12 and 2S proteins, respectively. Finally, the fractions recovered with the large scale procedure were as purified as fractions obtained at the analytical scale. Nevertheless, the recovery yields were slightly lower.

The ultracentrifugation performed on F_{02} confirms also that the structure of the proteins was conserved during the purification.

5. Conclusion

The method developed is rather fast and suitable for large scale purification. The techniques used were limited: nanofiltration, two steps of CEC, one step of SEC for the polishing of cruciferin and one step of HIC for the polishing of napin.

Starting with 3.5 kg of rapeseed meal, highly purified fractions (over 95% for each protein class) could be recovered: 200 g for cruciferin, 42 g for napin and 5 g for LTP; that means high recovery yields for storage proteins: 40% for cruciferin and 18% for napin.

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References

[1] Green chemicals and biopolymers from rapeseed meal with enhanced end-uses performance. European contract Enhance QLK5 CT 199901442.
[2] P.A. Howard, W.F. Lehnhardt, F.T. Orthoefer, US Patent 4,368,151 (1983).
[3] W. F. Lehnhardt, P.W. Gibson, F.T. Orthoefer, US Patent 4,370,267 (1983).
[4] V.H. Thanh, K. Shibasaki, J. Agric. Food Chem. 24 (1976) 1117.
[5] N.C. Nielsen, J. Am. Oil Chem. Soc. 62 (1985) 1680.
[6] J. Guéguen, A.T. Vu, F. Schaeffer, J. Sci. Food Agric. 35 (1984) 1024.
[7] C. Larré, J. Guéguen, J. Chromatogr. 361 (1986) 169.
[8] I. Crévieu, S. Bérot, J. Guéguen, Nahrung 40 (1996) 237.
[9] B. Raab, K.D. Schwenke, Nahrung 8 (1984) 863.
[10] M. Dalgalarrondo, J.M. Robin, J.L. Azanza, Plant Sci. 43 (1986) 115.
[11] R.I. Monsalve, R. Rodriguez, J. Exp. Bot. 41 (1990) 89.
[12] B. Raab, H. Leman, K.D. Schwenke, H. Kozlowska, Nahrung 36 (1992) 239.
[13] M. Adachi, J. Kanamori, T. Masuda, K. Yagasaki, K. Kitamura, B. Mikami, S. Utsumi, PNAS 100 (2003) 7395.
[14] K.D. Schwenke, B. Raab, K.J. Linow, W. Pähtz, J. Uhlig, Nahrung 25 (1981) 271.
[15] K.D. Schwenke, B. Raab, P. Plietz, G. Damaschun, Nahrung 27 (1983) 165.
[16] A. Kryzaniak, T. Burova, T. Haertlé, J. Barciszewski, Nahrung 42 (1998) 201.
[17] G.M. Neumann, R. Condron, I. Thomas, G.M. Polya, Biochim. Biophys. Acta 1295 (1996) 23.
[18] G.M. Neumann, R. Condron, I. Thomas, G.M. Polya, Biochim. Biophys. Acta 1295 (1996) 34.
[19] C.L. Baszczynski, L. Fallis, Plant Mol. Biol. 14 (1990) 633.
[20] A. Byczynska, J. Barciszewski, J. Plant Physiol. 154 (1999) 417.
[21] L. Rask, M. Ellerström, I. Ezcurra, K. Stalberg, P. Wycliffe, J. Plant Physiol. 152 (1998) 595.
[22] P.M. Gherig, A. Kryzaniak, J. Barciszewski, K. Biemann, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 3647.
[23] B. Hollenbach, L. Schreiber, W. Hartung, K.J. Dietz, Planta 203 (1997) 9.
[24] A. Molina, A. Segura, F. Garcia-Olmedo, FEBS Lett. 316 (1993) 119.
[25] S. Torres-Schumann, J.A. Godoy, J.A. Pintor-Toro, Plant Mol. Biol. 18 (1992) 749.
[26] M.B. Trevino, M.A. O'Connell, Plant Physiol. 116 (1998) 1461.
[27] R.S. Pearce, C.E. Houlston, K.M. Atherton, J.E. Rixon, P. Harrison, M.A. Hugues, M.A. Dunn, Plant Physiol. 117 (1998) 787.
[28] S. Jégou, J.P. Douliez, D. Mollé, P. Boivin, D. Marion, J. Agric. Food Chem. 48 (2000) 5023.
[29] E.A. Pastorello, C. Pompei, V. Pravettoni, L. Farioli, A.M. Calamari, J. Scibilia, A.M. Robino, A. Conti, S. Iametti, D. Fortunato, S. Bonomi, C. Ortolani, J. Allergy Clin. Immunol. 112 (2003) 775.
[30] G. Garcia-Casado, L.F. Pacios, A. Diaz-Perales, R. Sanchez-Monge, M. Lombardero, F.J. Garcia-Selles, F. Polo, D. Barber, G. Salcedo, J. Allergy Clin. Immunol. 112 (2003) 599.
[31] F. Battais, J.P. Douliez, D. Marion, Y. Popineau, G. Kanny, D.A. Monneret-Vautrin, S. Denery-Papini, in: The Gluten Proteins, Proceedings of the VIII Gluten Workshop, 2004.
[32] K. Toriyama, K. Hanaoka, T. Okada, M. Watanabe, FEBS Lett. 424 (1998) 234.
[33] J.P. Douliez, T. Michon, K. Elmorjani, D. Marion, J. Cereal Sci. 32 (2000) 1.
[34] J. Ostergaard, P. Hojrup, J. Knudsen, Biochim. Biophys. Acta 1254 (1995) 169.
[35] S. Tassin-Moindrot, A. Caille, J.P. Douliez, D. Marion, F. Vovelle, Eur. J. Biochem. 267 (2000) 1117.
[36] M. Naczk, R. Amarowicz, A. Sullivan, F. Shahidi, Food Chem. 62 (1998) 489.
[37] U.K. Laemmli, Nature 227 (1970) 680.
[38] C. Malabat, Ph.D. Thesis, 2002.