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Detailed Physicochemical Characterization of the 2S Storage Protein from Rape (*Brassica napus* L.)

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Chromatographic, chemical, and spectroscopic techniques were used to characterize the physicochemical properties of napin purified by preparative chromatography. The molar extinction coefficient was determined ($\epsilon = 0.56$), and static and dynamic light scattering measurements enabled the average molecular weight ($M_w = 13919$), the second virial coefficient ($A_2 = 23.95 \times 10^{-5}$ mol cm^3 g^{-2}), and the hydrodynamic radius ($R_H = 1.98$ nm) to be determined. No conformational changes were observed by fluorescence and circular dichroism measurements in different buffers at pH 3, 4.6, 7, and 12, confirming the high pH stability of this protein. From MALDI-TOF analysis and after enzymatic digestion, it was found that this purified sample, extracted from the rapeseed variety Express, contained mainly isoform 2SS3_BRANA.

KEYWORDS: **Rapeseed; napin; isolation; isoform identification; light scattering; mass spectrometry**

INTRODUCTION

Rapeseed (*Brassica napus* L.) is mainly produced for its high oil content (45−50%). After oil extraction, a meal is obtained containing most of the proteins (30−40%) (*1*). Originally, its use was limited to animal feeding because of the presence of undesirable substances (glucosinolates, erucic acid). To extend the use of rape oil to human food consumption and to exploit the functional properties of rape proteins in food applications, varieties called "double zero", without these undesirable molecules, have been developed.

In *B. napus*, there are two major families of storage proteins termed cruciferins and napins, which provide nitrogen and sulfur during germination (*2*, *3*). Cruciferins belong to the 12S globulin class of proteins. These neutral proteins, with a high molecular weight, are composed of several subunits and constitute 26−65% of the total rape seed protein content, depending on the variety (*4*). Napins belong to the 2S albumin class of proteins (and are hence water soluble) and represent 15−45% of the total rape seed protein content depending on the variety (*4*).

Napins are low molecular weight proteins (12500−14500). They belong to a multigene family encoded by 10−16 genes (*5*) and are expressed during seed development as precursors of 21 kDa (*6*, *7*). After their maturation, two peptide chains of 4.5 and 10 kDa result, linked by two disulfide bonds (*8*, *9*). The large chain includes two additional intrachain disulfide bonds, which reinforce the stability of the proteins (*10*−*12*). Napins are characterized by their strong basicity (isoelectric point, p$I \sim 11$) mainly due to a high amidation of amino acids (*13*).

Many isoforms of napin exist because of the large number of napin genes and differences in proteolytical cleavages. Five isoforms have been first identified according to their molecular weights (*10*, *14*). One of them (isoform BnIb, called 2SSI_BRANA in the Swiss-prot databank nomenclature) has been totally sequenced and its three-dimensional structure determined by NMR (*15*). More recently, their sequences and structures were compared from data available from databanks (*16*). The study clearly identified nine isoforms, but many others exist, which can influence properties such as the hydrophobicity (*17*). Their separation therefore appears to be complex.

The first method of purification developed for these proteins used precipitation by ammonium sulfate (at 40−80% saturation). This method allowed the production of large quantities of proteins, but pigments or contaminants were not totally removed from the protein fractions (*18*, *19*). Chromatographic methods were then developed using either size exclusion chromatography (SEC) (*20*) or ion exchange chromatography (IEC) (*10*), which led to highly purified proteins but only on an analytical scale. To study the functional properties of different varieties of napin, a larger scale purification procedure was developed to produce larger amounts of purified napins with most of the contaminating pigments removed (*21*).

Very few studies deal with the physicochemical characterization of napin because of these difficulties to purify and isolate

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the isoforms. Data interpretation may be rendered difficult by the differences observed between the isoforms, even if they are minor. Recently, deconvolution of circular dichroism (CD) spectra indicated about 25% of α-helix and 38% of β-sheet structure (*8*), whereas other results led to 40−45% of α-helix and 16−20% of β-sheet structure (*22*). The structural stability in solution was also studied by microcalorimetry; the study found transition temperatures of 101 and 88 °C at pH 6 and 3, respectively (*8*). It confirmed the high heat stability of napin, which is probably due to its very stable tertiary structure (*22*, *23*).

The present paper describes the physicochemical characterization of a napin sample obtained from large-scale purification by preparative chromatography. This study used mainly chromatographic, chemical, and spectroscopic methods in order to determine the sample composition (nitrogen, water, and sugar contents) and its solubility and general features of the protein (molecular weight, molar extinction coefficient, hydrodynamic radius).

MATERIALS AND METHODS

Isolation and Purification of Napin. The isolation and purification process was based on the method developed by C. Malabat (*21*). Rapeseed meal (Express variety) was purchased from CETIOM (Bordeaux, France), where the seeds have been dehulled, crushed, and defatted by hexane extraction.

After protein extraction from the meal with an extraction buffer (50 mM Tris, 1 M NaCl, 15 mM sodium disulfite, 5 mM EDTA, pH 8.5) and centrifugation at 17360*g* for 20 min at 20 °C, pigments were eliminated by SEC on a Cellufine GH-25 column (Amicon, 100 × 930 mm, 7.5 L) equilibrated in buffer A, 50 mM Tris, 5 mM EDTA, and 15 mM sodium disulfite, pH 8.5. Proteins were eluted with the same buffer. Napins were then fractionated by IEC on a Streamline SP-XL gel (Pharmacia, 55 × 500 mm) equilibrated with buffer A. The elution of the bound fraction, composed of napins, was performed using an increasing gradient of buffer B (buffer A + 1 M NaCl). The fraction previously recovered was purified by hydrophobic interaction chromatography on a phenyl Sepharose 6B Fast Flow column (Pharmacia, 50 × 125 mm, 200 mL) equilibrated with buffer C (buffer A + 1 M sodium sulfate). Elution was performed using a decreasing gradient of buffer C.

The eluted fractions were then purified on a Cellufine GH-25 column (90 × 400 mm, 5L) equilibrated with 0.1% ammonium carbonate to eliminate the remaining impurities. Finally, the samples were dialyzed against water before being freeze-dried.

Ultrapure water obtained from a Milli-Q system (Millipore) was used for all experiments and buffer preparations.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed using 90 × 90 × 0.75 mm slabs according to the Laemmli procedure (*24*). Migration and stacking gels contained 18 and 6% of polyacrylamide, respectively. Electrophoresis was performed under nonreducing and reducing conditions, the latter being obtained by incubation of the sample for 1.5 min at 100 °C with 2-mercaptoethanol 5% (v/v). Molecular weights were estimated in reference to three standard proteins (insulin chain A, cytochrome *c*, carbonic anhydrase).

Nitrogen Content. The total amount of nitrogen (protein and nonprotein) was evaluated according to the Kjeldahl method using a nitrogen−protein conversion factor of 5.7 for rape (*25*). One hundred milligrams of protein powder was then dissolved in 5 g of 10% trichloroacetic acid (w/v) and stirred for 1 h at room temperature in order to make the nonprotein nitrogen solubilize selectively. After centrifugation for 20 min at 2500*g*, the supernatant was recovered and its nonprotein nitrogen content determined according to the Kjeldahl method. The protein nitrogen content was quantified from the difference between the total amount of nitrogen and the amount of nonprotein nitrogen.

Isoelectrofocalization. Isoelectrofocusing (IEF) was performed in individual immobilized pH gradients (IPG) gel strips, (3 × 130 mm) with a pH range of 6−11 (Immobiline Drystrips, Amersham Pharmacia Biotech). It was operated in nonreducing and reducing conditions with the parallel use of a p*I* calibration kit (p*I* 3−10, Pharmacia) containing well-characterized p*I* markers. IPG dry strips were rehydrated to their original thickness (0.5 mm) with a solution containing 8 M urea, 2% (w/v) CHAPS, bromophenol blue (traces), and distilled water. The protein sample (40 μg) and the p*I* calibration kit were dissolved in 250 μL of a solution containing 1 mL of rehydration stock solution added to 5 μL of IPG buffer 6−11 and 0.2% dithiothreitol (DTT) if operating under reducing conditions. The samples were deposited onto the surface of the gel strips, which were placed in IPG strip holders (IPGphor) onto the plate of an IEF chamber. After 12 h of rehydration, a low-voltage gradient was applied across the gel, 500 V for 1 h for better sample entry, 1000 V for 1 h, and, finally, 8000 V until a steady state was obtained.

After IEF to steady state, the IPG strips were equilibrated for 30 min in a solution of 20% trichloroacetic acid. The strips were then soaked in a solution of glutaraldehyde 0.2% (v/v) in 30% (v/v) ethanol and 0.2 M sodium acetate. Finally, the polypeptides were stained with Coomassie Brilliant Blue R250 0.025% (v/v) in 40% methanol and 7% acetic acid.

Size Exclusion Chromatography. Gel filtration was performed on a Superdex 75 column (10 × 300 mm, 24 mL, Pharmacia) at different pH values. The column was equilibrated either in a 50 mM phosphate buffer (pH 6, 7.5, 8.5) or in a 50 mM sodium acetate buffer (pH 3, 4.5) or in a 50 mM sodium phosphate solution adjusted to pH 12 with NaOH. The separation was optimized by adding 150 mM NaCl to the elution buffer. The calibration was performed at the different pH values cited above with four protein standards covering the molecular weight range from 6500 to 67000. Calibration was found to be the same whatever the pH. Five hundred microliters of the sample at 1 mg/mL was loaded on the column, and elution was carried out at a flow rate of 0.5 mL/mn. The proteins were detected at 220 and 280 nm.

Reversed phase high-performance liquid chromatography (RP-HPLC) was carried out on a Waters system controlled by Breeze software. One hundred microliters of the sample at 3.2 g/L was loaded on a C_{18} Symmetry column (4.6 × 250 mm, Waters) maintained at 45 °C. The column was initially equilibrated with 80% of solvent A [0.1% trifluoroacetic acid (TFA) in water] and 20% of solvent B (0.08% TFA in acetonitrile). Elution was performed using a gradient of acetonitrile (1%/min) at a flow rate of 1 mL/mn. The proteins were detected at 220 and 280 nm.

Fourier Transform Infrared Spectroscopy (FTIR). The infrared spectra were recorded between 400 and 4000 cm^{-1} at 4 cm^{-1} intervals on a Fourier transform spectrometer Vector 22 (Brücker) in transmission mode. The samples were prepared by grinding 1 mg of protein powder with 99 mg of potassium bromide (KBr) powder and pressed into a pellet (diameter = 13 mm). The spectrum was analyzed in the amide I band region (1720−1580 cm^{-1}) with a corrected baseline and normalized data. The experiment was also performed with a solution of napin in 148 mM phosphate buffer (pH 7) at 5 mg/mL using an attenuated total reflection (ATR) cell. The spectrum resulting from 10 scans was corrected from solvent contribution and analyzed by using principal component analysis (PCA), a multivariate statistical treatment.

Ultraviolet (UV)−Visible (Vis) Spectroscopy. UV spectroscopy was performed on a UV−vis Shimadzu UV-1605 spectrophotometer to quantify both the solubility of the protein as a function of pH and the molar extinction coefficient.

Determination of the Molar Extinction Coefficient. The molar extinction coefficient was determined by establishing the Beer−Lambert law for napin: $A = \epsilon lc$, where A is the absorbance (AU), ϵ the molar extinction coefficient ($L \cdot mol^{-1} \cdot cm^{-1}$), l the path length (cm), and c the analyte concentration ($mol \cdot L^{-1}$).

Napin solutions in water without pH adjustment were prepared at different concentrations (0.05−2 mg/mL) by dissolving protein powder in Millipore water. The protein concentrations were calculated from the determination of the dry matter content corrected by the content in nonprotein nitrogen, water, and sugar previously determined. Absorbance was measured at 280 nm and corrected for turbidity determined

between 320 and 360 nm; absorbance values were then plotted as a function of protein concentration in order to determine the slope corresponding to the molar extinction coefficient. The experiment was repeated five times. Further to the determination of the molar extinction coefficient, all sample concentrations will be based on protein content determined by UV spectroscopy.

Solubility According to pH. The solutions were prepared at different pH values (pH 2−11) at a concentration of 1 mg/mL. The pH values of these solutions were adjusted by adding HCl or NaOH at concentrations of 0.01, 0.1 or 1 M. The samples were then centrifuged at 15000*g* for 15 min. The supernatant was recovered and the absorbance recorded at 280 nm to determine its protein concentration.

Circular Dichroism (CD). Far-UV CD spectra were recorded under nitrogen flow at 25 °C in the wavelength range of 185−260 nm at 0.5 nm intervals using a CD6 Spex Dichrograph (Jobin-Yvon, France). Cuvettes of 0.1 mm path length were used. Protein concentrations in the samples were determined after filtration on 0.2 μm filters (Anotop, Alltech, France) using the value $\epsilon = 0.56$ determined in this study. Concentrations were 0.295 ± 0.02 mg/mL in citrate buffers (pH 3 and 4.6), phosphate buffer (pH 7), and a sodium phosphate solution adjusted to pH 12 by NaOH. Each spectrum was the mean of six scans and was corrected for the solvent contribution.

The ellipticity data (in deg) were converted into mean residue ellipticity (in $deg \cdot cm^2 \cdot dmol^{-1}$), which was calculated by assuming a mean residue molar mass of napin of $M_r = 112$ g/mol. CD spectra were analyzed in terms of α-helix content by using the dicroprot software, a package freely available online (http://dicroprot-pbil.ibcp.fr) that was developed by Deléage (*26*). The self-consistent method, which is an iterative refinement of the CD spectrum of a protein chosen as an initial guess, was retained to assess the percentage of α-helices. In this method (*27*), the prediction for the secondary structure content was calculated from four reference databases.

Fluorescence Spectroscopy. Fluorescence spectra of napin were obtained using a Fluoromax-Spex spectrofluorometer (Jobin-Yvon, France) at 25 °C. The excitation wavelength was set to 295 nm for selective excitation of tryptophan (W), whereas the emission spectra were recorded from 300 to 500 nm; 5 nm slits were used for both excitation and emission. Concentrations were 0.295 ± 0.02 mg/mL in citrate buffers (pH 3 and 4.6), phosphate buffer (pH 7), and a sodium phosphate solution adjusted to pH 12 by NaOH and determined as previously for CD. The spectra were corrected for solvent contribution, and the maximum emitted fluorescence intensity (I_{em}) was normalized by the protein concentration.

Mass Spectrometry (MS). Matrix-assisted laser desorption ionization (MALDI) MS analyses were performed on a Bruker Biflex III time-of-flight (TOF) mass spectrometer (Bruker-Daltonic, Bremen Germany) equipped with a Scout 384 probe ion source. Samples were desorbed/ionized from the probe tip with a 337 nm pulsed nitrogen laser (model VSL-337i, Laser Science Inc., Boston, MA) having a repetition rate of 3 Hz and a pulse width of 2 ns. All of the experiments were performed in positive ion mode. In linear mode, the ions were accelerated under delayed extraction conditions with an acceleration voltage of 20 kV. A 5.75 kV potential difference between the target and the extraction lens was applied with a time delay of 2 ns. In reflectron mode, the ions were accelerated with a 19 kV acceleration voltage with a 0.5 ns delayed extraction and a 3.5 kV potential difference between the target and the extraction lens. The voltage of the reflectron was set at 20 kV. A Lecroy 9384C, 1 GHz digital storage oscilloscope was used for data acquisition (Lecroy Corp., Chestnut Ridge, NY). The detector signals were amplified in two stages, digitized, and transferred to the XACQ program on a SUN work station (Sun Microsystems Inc., Palo Alto, CA).

The instrument was calibrated externally with a mixture of bovine pancreas insulin (Sigma-Aldrich Co., St. Louis, MO) and horse skeletal muscle myoglobin (Sigma-Aldrich Co.). 3,5-Dimethoxy-4-hydroxycinnamic acid, called sinapinic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemie GmbH) (5 mg/mL) were used as matrix and prepared separately in CH_3CN/H_2O/TFA (80:20:0.1). Each protein sample was prepared in H_2O/TFA (100:0.1) in order to achieve a final concentration of 10^{-7} mol/mL. Ten microliters of this solution was mixed with 100 μL of sinapinic acid and spotted onto the probe slide. The amount of protein loaded on the probe slide was ∼10 pmol. The samples were then dried at room temperature and atmospheric pressure.

Enzymatic digestion of the overall sample was performed in 50 μL of trypsin solution [trypsin, proteomics sequencing grade (Sigma-Aldrich Chemie GmbH)] using the Millipore montage In-gel $Digest_{96}$ Kit (Millipore Corp., Bedford, MA). An amount of 1 μL of tryptic digest solution was spotted directly onto the MALDI-TOF MS target using a Zip Tip SCX pipet tip (Millipore Corp.) with the procedure described in the user guide.

Static Light Scattering (SLS). Light scattering measurements were performed using an ALV-5000 multibit, multi-tau correlator in combination with a 4700 Malvern goniometer (Malvern Instruments, Orsay, France) and a Spectra-Physics laser emitting vertically polarized light at 532.5 nm. Samples were prepared in a 148 mM phosphate buffer, pH 7, at different concentrations ranging from 1.4 to 14 mg/mL. Each solution was put into a glass measuring vial (Malvern, France), which was kept at a temperature of 25 °C in a toluene bath.

The time-averaged intensity of the scattered light was measured at one observation angle, $\theta = 90°$, as a function of protein concentration and represented as

$$\frac{Kc}{R_\theta} = \frac{1}{M_w}[1 + 2M_w A_2 C] \tag{1}$$

where $M_w A_2 C \ll 1$ because the molecules are much smaller than the wavelength of the incident light. This allows the molecular weight M_w and the second virial coefficient A_2 at only one observation angle to be determined. C in eq 1 is the concentration of the sample ($g \cdot cm^{-3}$), R_θ the Rayleigh ratio ($R_\theta = 2.86 \times 10^{-5}$ for toluene at $\lambda = 532.5$ nm), and K an optical constant. K was calculated using the equation

$$K = \frac{4\pi^2 n^2}{\lambda_0{}^4 N_A}\left(\frac{dn}{dc}\right)^2 \tag{2}$$

where n is the refractive index of the solvent ($n = 1.3346$ for 148 mM phosphate buffer, pH 7), N_A is the Avogadro number ($N_A = 6.022 \times 10^{23}$), λ is the wavelength of the polarized light (cm), and dn/dc is the refractive index increment of the solute. A value of 0.229 for napin was determined by the slope of the line representing the refractive indices measured for each sample with a refractometer (Brice-Phoenix, France) as a function of concentration.

The average molecular weight M_w was determined after extrapolation to $c = 0$ of the linear regression of the experimental data and the second virial coefficient A_2 from the slope of the line.

Quasi-elastic Light Scattering (QELS). Dynamic light scattering measures the temporal autocorrelation function of the fluctuations of the scattering intensity resulting from the diffusive motion of the particles. The hydrodynamic radius R_H of napin and the translational diffusion coefficient were determined by QELS for the same samples as for static light scattering. The time correlation functions $g_2(t)$ were recorded with the ALV-5000 multi-tau correlator at $\theta = 90°$. They were then analyzed using the inverse Laplace transform routine REPES in order to obtain the corresponding relaxation time distribution and the diffusion coefficients of the proteins. For purely diffusive relaxation, the translational diffusion coefficient D was obtained by extrapolation to $c = 0$ of the relaxation function Γ as a function of concentration c, where q is the wave vector defined as $(4\pi n/\lambda)\sin(\theta/2)$; here $q = 2.2 \times 10^7$ m^{-1}:

$$\Gamma = q^2 D \tag{3}$$

The hydrodynamic radius of napin was then calculated using the Stokes−Einstein relationship

$$R_H = k_B T / 6\pi\eta D \tag{4}$$

where D is the diffusion coefficient of the particle, k_B the Boltzman

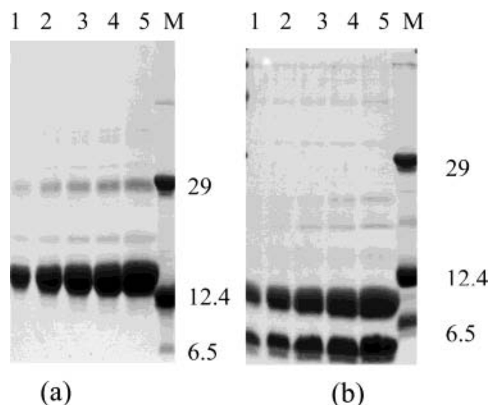

Figure 1. SDS-PAGE patterns of the napin sample in (**a**) nonreducing and (**b**) reducing conditions at different concentrations: (lanes 1) 0.36 mg/mL; (lanes 2) 0.72 mg/mL; (lanes 3) 1.08 mg/mL; (lanes 4) 1.44 mg/mL; (lanes 5) 2.16 mg/mL; (lanes M) proteins used as molecular weight standards.

constant, T the absolute temperature, and η the solvent viscosity ($\eta = 9.595 \times 10^{-4}$ Pa•s for 148 mM phosphate buffer, pH 7).

RESULTS AND DISCUSSION

Composition and Characterization of the Purified Sample. *Composition of the Sample.* The composition of the sample was determined by analyzing the content in nitrogen, water, and sugar. The amount of water in the protein powder was 8.4 wt % according to the Karl−Fisher method. The sugar content, established by quantifying colorimetrically uronic acids (as galacturonic acid) using the automated *m*-phenylphenol method (*28*), was negligible. The total percentage of nitrogen was evaluated according to the Kjeldahl method to be 86.5 wt % and the nonprotein nitrogen content to be 1.9 wt %. The protein content was thereby quantified to be 84.6 wt %. Starting with 40 g of rapeseed meal, a 2 g purified sample was obtained. The IR spectra of napin in the solid state (data shown in **Figure 6**) showed neither a stretching band around 1740 cm^{-1} characteristic of an ester nor a cluster of bands around 1050 cm^{-1} corresponding to sugars, which confirmed the absence of lipids and sugars in the sample.

The sample was then analyzed by SDS-PAGE in order to check its purity and to evaluate the molecular weight of napin. The analysis by SDS-PAGE was performed using high concentrations of napin (0.36−2.16 mg/mL) deposited on the polyacrylamide slabs in order to detect all proteins present in the sample. A very dark band at 14 kDa was seen under nonreducing conditions (**Figure 1a**) and at 5 and 9 kDa after reduction (**Figure 1b**). The bands after reduction correspond to each chain constituting the protein. There were also much less intense bands at 30 kDa and traces at 20 and 34 kDa under nonreducing conditions (**Figure 1a**). The slab was analyzed by densitometry using BIO 1D software. The results of this analysis showed that the major bands were at 14 kDa (92% of the total volume) and 30 kDa (5.8%), whereas bands at 20 and 34 kDa were present but negligible, representing, respectively, 1.5 and 0.5% of the total volume. The sample was therefore considered as pure, and the bands were attributed to napin monomer (14 kDa) and dimer (30 kDa).

Isoelectric Point (IEP). To evaluate the IEP of napins, isoelectrofocalization was performed under reducing and nonreducing conditions. Under nonreducing conditions (not shown), a band at pH 10.7 was observed, giving only an estimation of the isoelectric point of napin rather than a precise value because bands were slightly distorted. Under reducing conditions (not shown), two distinct bands were seen: one around pH 8.8, the other around pH 9.4. IEP results did not correspond exactly to those calculated from the amino acid composition and available on databanks (IEP = 10.89−12.16 for the small chain and 10.25−11.30 for the large one). It may be due not only to the fact that computation from amino acid composition does not take into account electrostatic interactions inside the protein, which modifies the pK_a of the amino acid ionizable side chains, but also to the presence of other isoforms in the purified sample.

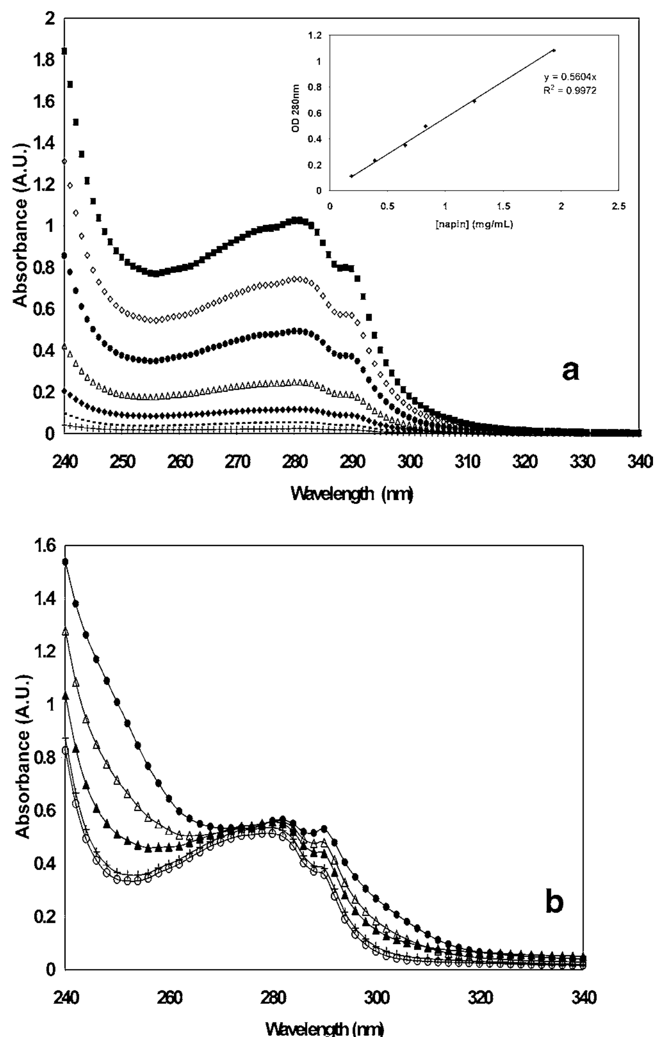

Figure 2. (**a**) UV−vis spectra of napin as a function of protein concentration: (■) 2 mg/mL; (◇) 1.5 mg/mL; (●) 1 mg/mL; (△) 0.5 mg/mL; (◆) 0.25 mg/mL; (—) 0.1 mg/mL; (+) 0.05 mg/mL. (Inset) Beer−Lambert law established for napin in water at natural pH. (**b**) UV−vis spectra of napin (1 mg/mL) as a function of pH: (+) pH 3; (○) pH 6; (▲) pH 10; (△) pH 11; (●) pH 13.

Determination of the Molar Extinction Coefficient and Solubility of Napin. The UV spectra showed the same profile whatever the napin concentration (**Figure 2a**). There were two maxima: one at 280 nm, corresponding mainly to tyrosine and tryptophan absorption, the second at 290 nm, corresponding to tryptophan absorption. The linearity of the Beer−Lambert law was checked, and the slope of the absorbance versus napin concentration line gave a value for the extinction coefficient of $\epsilon = 0.56$ in water (average of five independent experiments performed). The UV spectra (**Figure 2b**) showed similar profiles whatever the pH except above pH 10, when a shift of the maximum at 280 nm to higher wavelengths was noticed. This

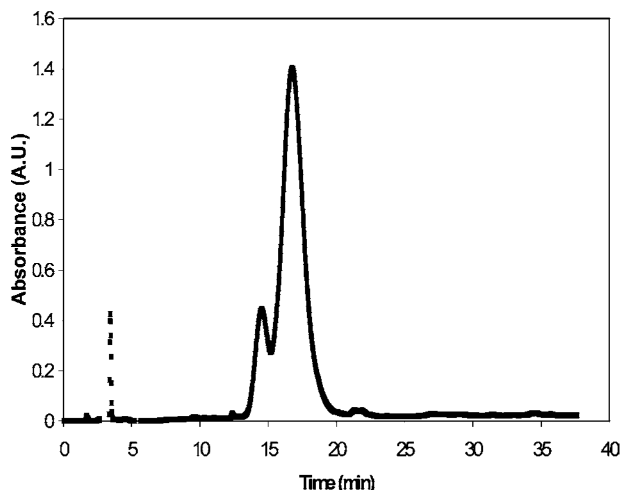

Figure 3. RP-HPLC chromatogram of the whole napin sample (100 μL) at $T = 45$ °C. Detection was performed at 280 nm and the protein eluted using a gradient of acetonitrile (1%·min^{-1}) at a flow rate of 1 mL·min^{-1}.

bathochromic effect could be attributed to a more polar environment of phenylalanine and tyrosine residues. Tryptophan absorption did not change whatever the pH as revealed by the unchanged position of the maximum observed at 290 nm.

The napin solubility was examined at different pH values ranging from 3 to 11. The absorbance was recorded at 280 nm before and after centrifugation. The protein solubility was found to be the same whatever the pH values (94%), showing that napin was highly soluble in water whatever the pH.

Hydrophobic Affinity. With the resolution of the column used for the experiment, the RP-HPLC profile (**Figure 3**) showed two peaks, one representing 15% of the total area (at 14.51 min with $A = 0.44$) and the second, 85% (at 16.76 min with $A = 1.40$). Reduced napin was also injected to compare its profile with the previous one. The profiles were not superimposed, meaning that the two peaks did not correspond to one of the two napin chains. The peaks would rather correspond to different isoforms or groups of isoforms distinguished by their hydrophobicity.

Determination of the Average Molecular Weight and Identification of Isoforms. *SEC.* The molecular weight of the protein was determined according to different complementary techniques with respect to the information they provided and the precision.

At first, gel filtration on Superdex 75 was used to evaluate the distribution in the weight of the sample. The calibration curve, established by injecting protein standards on the Superdex 75 column, allowed the determination of the molecular weight of napin. The elution pattern (**Figure 4**) displayed a major elution peak at $V_e = 13.5-13.9$ mL as a function of pH corresponding to a molecular weight of 12500−14500. It was noticed that the peaks collected from the napin solution at acidic pH values were 2 times more intense than those collected from solutions at alkaline pH. This could come from the formation of aggregates for an ionic strength of 200 mM in basic conditions. Following this assumption, aggregates would be retained on the filter at the top of the column and explain the recovery after chromatography of 92% (w/w) of initial weight of injected protein. The results in **Figure 4** confirmed this hypothesis, as peaks corresponding to dimers ($V_e = 12$ mL, 26 kDa) or to aggregates ($V_e = 9.3$ mL, 67 kDa) were observed at pH 7.5, 8.5, and 12.

Static and Dynamic Light Scattering. Linear regression of the concentration dependence of the light scattering gave the following equation: $(Kc/R_\theta) = 4.79 \times 10^{-4}\ c + 7.18 \times 10^{-5}$. Extrapolation to $c = 0$ from this equation led to an average molecular weight M_w of 13920. This value was in accordance with those obtained by the above-mentioned results. The second virial coefficient A_2 was 2.4×10^{-4} mol·cm^3·g^{-2}. From QELS measurements, linear regression of the concentration dependence of the relaxation function Γ gave the equation $\Gamma = -5.39 \times 10^5 c + 5.55 \times 10^4$. The resulting translational diffusion coefficient D was of 1.15×10^{-10} m^2·s^{-1}, corresponding to a hydrodynamic radius R_H of 1.98 nm.

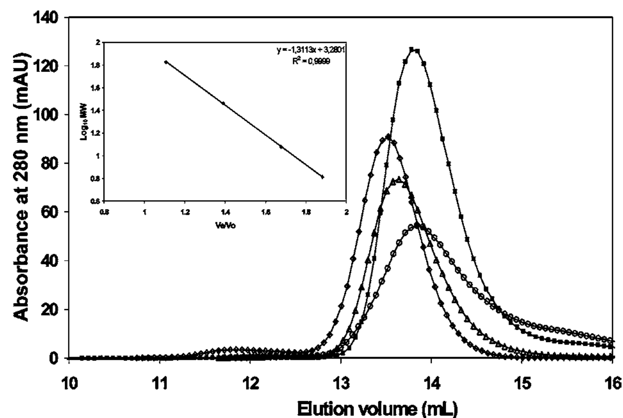

Figure 4. SEC (Superdex 75 column, Pharmacia) of napin sample (100 μL, 1 mg·mL^{-1}) as a function of pH: (■) pH 4.5; (○) pH 7.5; (△) pH 8.5; (◇) pH 12. (Insert) Calibration curve: $\text{Log}_{10}\ \text{MW} = f(V_e/V_0)$.

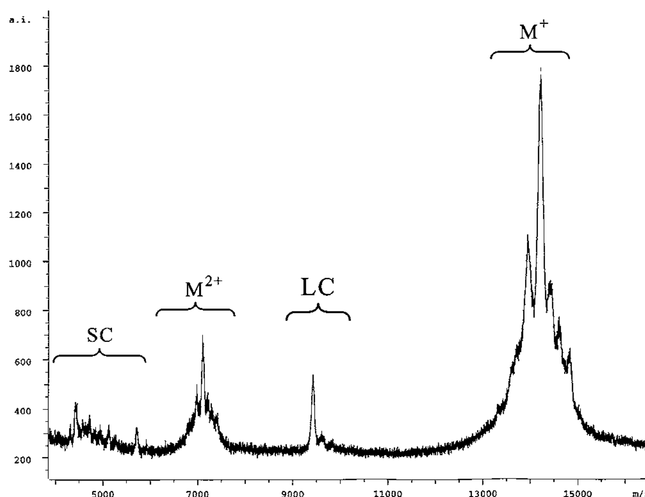

Figure 5. Positive ion mode MALDI mass spectrum of napin sample performed in linear mode. The annotations correspond to (i) monocharged species ($[M + H]^+$ denoted M^+), (ii) dicharged species ($[M + 2H]^{2+}$ denoted M^{2+}), (iii) small chain fragments (denoted SC), and (iv) large chain fragments (denoted LC).

MS. To specify the molecular weight of the whole sample and identify more precisely the isoforms present with the amino acid sequence, the sample was analyzed by MALDI-TOF. The linear mode MALDI-TOF mass spectrum of the sample (**Figure 5**) exhibits protein-ion peaks that illustrate the presence of several isoforms. Five peaks are distinguishable in a broad signal in the m/z range either of $[M + H]^+$ quasi-molecular ions and of dicharged species, denoted M^+ and M^{2+} in **Figure 5**, respectively. The mass of the detected ionic species could not be measured with accuracy due to low signal resolution of the mono- and dicharged pattern. However, the observed m/z values (from m/z 13950 u to m/z 14850 u) were in good agreement with those of the literature (*16*). The linear mode mass spectrum

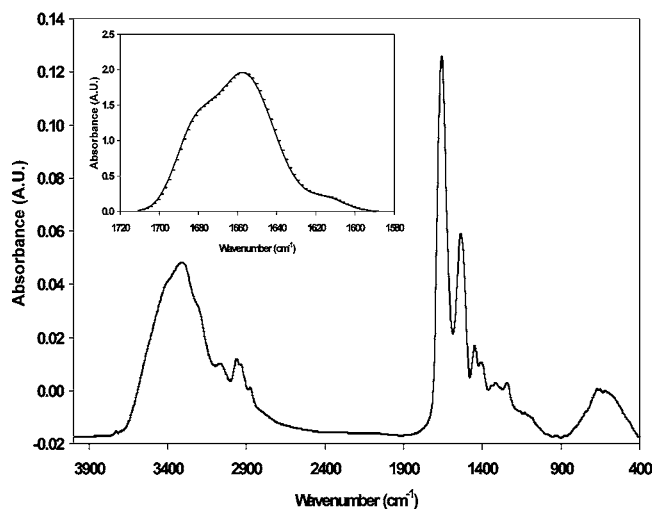

Figure 6. FTIR spectrum (10 scans) of napin (1% w/w in a KBr pellet) recorded between 400 and 3900 cm^{-1}. (Inset) Zoom of the FTIR spectrum of napin between 1580 and 1720 cm^{-1}. Data were normalized and baseline corrected.

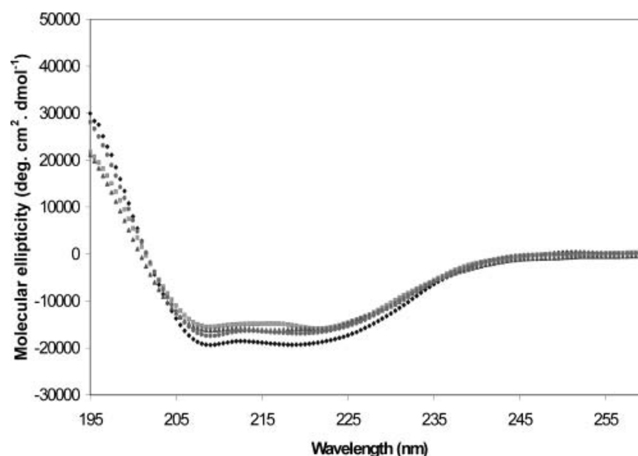

Figure 7. Far-UV CD spectra of napin (0.295 mg/mL) at pH 3 (●), pH 4.6 (◆), pH 7 (■), and pH 12 (▲).

Table 1. Assessment of the Percentage of α-Helix Content of Napin at pH 3, 4.6, 7, and 12, by Each of the Four Reference Databases Available in the Self-Consistent Method (SCM)

	pH 3	pH 4.6	pH 7	pH 12
SCM1	48.8	62	51.7	51.5
SCM2	47.7	53.4	45	44.9
SCM3	48.3	54.5	46.2	46.5
SCM4	54.5	62	51.7	51.5
mean	49.8	57.9	48.6	48.6

also shows the presence of low-mass ion signals (denoted SC in **Figure 5**) and peaks around 9400 u (denoted LC in **Figure 5**) that represent small and large napin chains, respectively. As described previously, such signals can be attributed to disulfide bond fragmentation events in the source. To confirm the characterization of the napin sample, a tryptic digest was performed on the whole sample without reductive condition, that is, by maintaining the disulfide bonds of the napin isoforms. In this case, the presence of the $[M + H]^+$ ion of 1853.71 u was detected in reflectron mode MALDI-TOF analysis (mass spectrum not shown). This measurement corresponds to the *m/z* value of the peptide 134−149 (QQQGQQGQQLQQVISR) of 2SS3_BRANA (calculated mass = 1853.96 u). Note that this characteristic peptide fragment does not contain any cysteine residue.

The major isoform was labeled 2SS3_BRANA and the second 2SS2_BRANA or 2SSB_ BRANA. The low molecular weight isoforms (2SSI_BRANA, napA, napB, and 2SS1_BRANA) seemed to be absent from the sample. According to the phylogenic rapeseed tree of Barcieszewski (*16*), these isoforms are slightly distant from the others. They are absent from our sample because they would not be expressed in the variety chosen for this study.

Characterization of the Napin Conformation. The region between 1600 and 1700 cm^{-1} of the IR spectrum (**Figure 6**) gave some information on the secondary structure of the protein. The shoulder at 1670 cm^{-1} indicated the presence of β-turns, whereas the narrow band at 1650 cm^{-1} indicated the presence of α-helices. The band at 1650 cm^{-1} was confirmed in the liquid state. The small shoulder at 1620 cm^{-1} characterized the presence of some aggregates.

To analyze the secondary structure in more detail, far-UV CD spectra were obtained at pH 3, 4.6, 7, and 12 for napin at 0.295 ± 0.02 mg/mL (**Figure 7**). A sharp positive band in the 185−200 nm range and a broad negative band located around 210−220 nm were observed. These spectra were characteristic of a protein belonging to the (α+β) class with a major α-helix content. No obvious difference was observed between the spectra at different pH values. To compare the spectra in more detail, percentages of α-helix content, assessed by using the four reference databases available in the software, were given in **Table 1**. The prediction method gave ∼48.6−57.9% α-helices and ∼7−15.3% β-sheets as a function of pH. These data were consistent with previous studies (*22*) and indicated no conformational changes in the helical region.

Recently, the secondary structure was also determined on the basis of two-dimensional ^{1}H NMR spectra of isoform BnIb, called 2SSI_BRANA in the Swiss-prot data bank nomenclature (*15*). The proposed structure of this study contains five helices and a C-terminal loop arranged in a right-handed helix. By calculating the proportion of amino acids implicated in α-helices according to the secondary structure established by Rico et al. (*15*), we found 56.6%, in accordance with the percentage of α-helices obtained in our study and also in the study by Schwenke et al. (*22*). Even if this calculation was made on isoform 2SSI_BRANA instead of 2SS3_BRANA, we can rely on this result because Barciszewski et al. (*16*) have shown the high similarity of the secondary structure of most napin isoforms.

With regard to the tertiary conformation of the protein, fluorescence spectroscopy was applied to probe the environment of tryptophan residues. The fluorescence emission spectra of napin (not shown) at 0.295 ± 0.02 mg/mL and for pH 3, 4.6, 7, and 12 showed a maximum at 333 ± 1 nm, whatever the pH value. These results indicated that no obvious changes took place with pH. Identification by molecular modeling of tryptophans in isoform 2SS3_BRANA, predominant in our sample, showed a unique tryptophan residue buried inside the protein. This feature would explain why there was no change in the tryptophan environment with pH.

As to the data obtained in this study, we have validated that large amounts of highly purified napin could be obtained from rapeseed meal (Express variety) after three successive separation stages: size exclusion, ion exchange, and hydrophobic interaction chromatography. The final fraction eluted was found to contain 84.6% of protein with no lipid and sugar residues. The protein was pure napin with an average molecular weight of

13920 and a hydrodynamic radius of 2 nm in a 148 mM phosphate buffer, pH 7. MALDI-TOF MS after digestion by trypsin indicated that the major isoform was 2SS3_BRANA and the second 2SS2_BRANA or 2SSB_BRANA. From CD spectra, 48−58% α-helices and 7−15% β-sheets were found, in good agreement with previous studies. It was also confirmed that the napin conformation was not sensitive to pH.

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LITERATURE CITED

(1) Schwenke, K. D. Rapeseed proteins. In *New and Developing Sources of Food Proteins*; Hudson, B. J. F., Ed.; Chapman and Hall: London, U.K., 1994; pp 281−306.
(2) Schwenke, K. D.; Raab, B.; Linow, K. J.; Pahtz, W.; Uhlig, J. Isolation of the 12S globulin from rapesees (*Brassica napus* L.) and characterization as a "neutral" protein. On seed proteins. Part 13. *Nahrung* **1981**, *25*, 271−280.
(3) Shewry, P. R.; Pandya, M. J. The 2S albumin storage proteins. In *Seed Proteins*; Shewry, P. R., Casey, R., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1999; pp 563−586.
(4) Raab, B.; Leman, H.; Schwenke, K. D.; Kozlowska, H. Comparative study of the protein patterns of some rapeseed (*Brassica napus* L.) varieties by means of polyacrylamide gel electrophoresis and high performance liquid chromatography. *Nahrung* **1992**, *36*, 239−247.
(5) Josefsson, L. G.; Lenman, M.; Ericson, M. L.; Rask, L. Structure of a gene encoding the 1.7S storage protein, napin, from *Brassica napus*. *J. Biol. Chem.* **1987**, *262*, 12196−12201.
(6) Ericson, M. L.; Rodin, J.; Lenman, M.; Glimelius, K.; Josefsson, L. G.; Rask, L. Structure of the rapeseed 1.7S storage protein, napin, and its precursor. *J. Biol. Chem.* **1986**, *261*, 14576−14581.
(7) Crouch, M. L.; Tenbarge, K. M.; Simon, A. E.; Ferl, R. cDNA clones for *Brassica napus* seed storage proteins: evidence from nucleotide sequence analysis that both subunits of napin are cleaved from a precursor plypeptide. *J. Mol. Appl. Genet.* **1983**, *2*, 273−283.
(8) Krzyzaniak, A.; Burova, T.; Haertlé, T.; Barciszewski, J. The structure and properties of napin-seed storage protein from rape (*Brassica napus* L.). *Nahrung* **1998**, *42*, 201−204.
(9) Muren, E. B.; Björk, B. I.; Rask, L. Structural comparison of the precursor and the mature form of napin, the 2S storage protein in *Brassica napus*. *Eur. J. Biochem.* **1996**, *242*, 214−219.
(10) Monsalve, R. I.; Rodriguez, R. Purification and characterization of proteins from the 2S fraction seeds of the Brassicaceae family. *J. Exp. Bot.* **1990**, *41*, 89−94.
(11) Gehrig, P. M.; Biemann, K. Assignment of the disulfide bonds in Napin, a seed storage protein from *Brassica napus*, using matrix-assisted laser desorption ionization mass spectrometry. *Pept. Res.* **1996**, *9*, 308−314.
(12) Byczynska, A.; Barciszewski, J. The biosynthesis, structure and properties of napin−the storage protein from rape seeds. *J. Plant Physiol.* **1999**, *154*, 417−425.
(13) Lönnerdal, B.; Janson, J. C. Studies on brassica seed proteins. 1. The low molecular weight proteins in rapeseed isolation and characterization. *Biochim. Biophys. Acta* **1972**, *278*, 175−183.
(14) Monsalve, R. I.; Lopez-Otin, C.; Villalba, M.; Rodriguez, R. A new distinct group of 2S albumin from rapeseed. Amino acid sequence of two low molecular weight napins. *FEBS Lett.* **1991**, *295*, 207−210.
(15) Rico, M.; Bruix, M.; Gonzalez, C.; Monsalve, R. I.; Rodriguez, R. ^{1}H NMR assignment and global fold of Napin BnIb, a representative 2S albumin seed protein. *Biochemistry* **1996**, *35*, 15672−15682.
(16) Barciszewski, J.; Maciej, S.; Haertlé, T. Minireview: Analysis of rape seed napin structure and potential roles of the storage protein. *J. Protein Chem.* **2000**, *19*, 249−254.
(17) Gehrig, P. M.; Krzyzaniak, A.; Barciszewski, J.; Biemann, K. Mass spectrometric amino acid sequencing of a mixture of seed storage proteins (napin) from *Brassica napus*, products of a multigene family. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 3647−3652.
(18) Joubert, F. S. Lupin seeds proteins. I A physicochemical study of the proteins from blue lupin seed (*Lupin angustifolius*). *Biochim. Biophys. Acta* **1955**, *16*, 370−374.
(19) Joubert, F. S. Lupin seeds proteins. II A physicochemical study of the proteins from yellow lupin seed (*Lupin luteus*). *Biochim. Biophys. Acta* **1955**, *17*, 444−445.
(20) Dalgalarrondo, M.; Robin, J. M.; Azanza, J. L. Subunit composition of the globulin fraction of rapeseed. *Plant Sci.* **1986**, *43*, 115−124.
(21) Malabat, C. Variabilité de la composition protéique de la graine de colza (*Brassica napus* L.): influence sur les propriétés émulsifiantes. Thèse de Chimie-Biologie, Université de Nantes, Nantes, France, 2002.
(22) Schwenke, K. D.; Drescher, B.; Zirwer, D.; Raab, B. Structural studies on the native and chemically modified low-moecular mass basic storage protein (napin) from rapeseed (*Brassica napus* L.). *Biochem. Physiol. Pflanzen* **1988**, *183*, 219−224.
(23) Folawiyo, Y. L.; Owusu Apenten, R. K. The effect of heat and acid treatment on the structure of rapessed albumin (napin). *Food Chem.* **1997**, *58*, 237−243.
(24) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(25) Mossé, J. Nitrogen to protein conversion factor for 10 cereals and six legumes or oilseeds. A reappraisal of its definition and detremination. Variation according to species and to seed protein content. *J. Agric. Food Chem.* **1990**, *38*, 18−24.
(26) Deléage, G.; Geourjon, C. An attractive graphic program for calculating the secondary structure content of proteins from circular dichroism spectrum. *CABIOS* **1993**, *9*, 197−199.
(27) Sreerama, N.; Woody, R. W. A self-consistent method for the analysis of protein secondary structure from circular dichroism. *Anal. Biochem.* **1993**, *209*, 32−44.
(28) Thibault, J.-F. Automatisation du dosage des substances pectiques par la méthode au méta-hydroxydiphényl. *Lebensm. Wiss. -Technol.* **1979**, *12*, 247−251.

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