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Selective extraction, structural characterisation and antifungal activity assessment of napins from an industrial rapeseed meal

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ABSTRACT

This article reports an extraction–purification of napins from an industrial rapeseed meal and the assessment of their antimicrobial activity against *Fusarium langsethiae*. The best extraction conditions are observed at pH 2, 12% (w/w) of rapeseed meal after 15 min of extraction in water at room temperature. Under these conditions the extraction is highly selective, allowing a simple purification process (ammonium sulfate precipitation followed by desalting size exclusion chromatography) to get purified napins. These napins possessed significant anti-*Fusarium* activity (IC_{50} = 70 μM) and a compact secondary structure rich in α-helix, which may explain this bioactivity.

1. Introduction

Rapeseed (*Brassica napus*) is a crop cultivated worldwide, having a high lipid and protein content (40–45% and 15–30% of the dry matter, respectively) (Godon, 1996, Chap. IV). The protein fraction is mainly composed of storage proteins, divided into globulins (12S cruciferins) and albumins (2S napins) (Aider & Barbana, 2011). Cruciferins are multimeric proteins of 350 kDa having an isoelectric point of 7.2 (Schwenke, 1994). Napins (12–15 kDa) are heterodimers composed of large (9–10 kDa) and small (4–4.5 kDa) subunits held together by weak bonds and disulfide bridges (Monsalve, Lopez-Otin, Villalba, & Rodriguez, 1991). They are particularly basic (pI around 11), and account for 30–50% of the total seed proteins (Schwenke, 1994). Besides storage proteins, the rapeseed protein fraction is composed of oleosins (15–26 kDa) (Aider & Barbana, 2011) and other minor proteins, such as thionins, lipid transfer proteins (LTP) and trypsin inhibitors (Bérot, Compoint, Larré, Malabat, & Guéguen, 2005).

Rapeseed proteins exhibit a well-balanced amino acids composition (Bos et al., 2007) and great techno-functional properties, including protein dispersibility index, water absorption, fat absorption, emulsifying and foaming properties (Wu & Muir, 2008). Besides that, napins from *Brassica* species have interesting antimicrobial properties on various bacterial strains (Neumann, Condron, Thomas, & Polya, 1996; Neumann, Condron, & Polya, 1996). Antifungal activities against *Fusarium culmorum, Fusarium oxysporum* f. sp. *lycopersici, Alternaria brassicola, Botrytis cinerea* with good IC_{50} (from 20 to 200 g L^{-1}) have been reported (Terras, Torrekens et al., 1993a,b). The anti-*Fusarium* activity is of critical interest, due to the growing occurrence of contamination of cereal cultures by *Fusarium langsethiae* observed around the past decade (Torp & Niremberg, 2004). This particular species affects the cereal directly in the field, during degraded storage conditions and eventually during downstream processing such as malting for barley. This contamination is associated with agronomical losses due to cereal infection, and to economic losses and health concerns due to T2 and HT2 mycotoxins production.

Several authors assumed that the antimicrobial activity of napins is due to their high content of positively-charged amino acids and a high proportion of α-helix in their secondary structure (Terras, Torrekens et al., 1993a,b). Indeed, these properties are widely observed with numerous other antimicrobial polypeptides. Other authors pointed out a calmodulin antagonist effect and an anti-trypsic activity of napins that could limit microorganism growth (Neumann, Condron, Thomas et al., 1996a,b; Terras, Schoofs et al., 1993b).

Nowadays, rapeseed is processed at industrial scale to produce oil for human consumption and biodiesel production (Wu & Muir, 2008). The oil extraction process leaves a solid side-product particularly rich in proteins called 'rapeseed meal', widely used as cattle food. Considering their good nutritional and techno-functional properties, rapeseed proteins can also be valorised in the field of human nutrition. For this purpose, the implementation of protein

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extraction/purification processes from rapeseed meal is required. Many extractive conditions are gathered in a recent review (Rodrigues, Coelho, & Carvalho, 2012). The better extraction of total rapeseed proteins was reported under basic conditions (ranging from 11 to 14) (Aluko, McIntosh, & Katepa-Mupondwa, 2005; Pedroche et al., 2004). Proteins purification from meal extracts are achieved either by (i) isoelectric precipitation (Chabanon, Chevalot, Framboisier, Chenu, & Marc, 2007), (ii) ammonium sulfate precipitation, combined with size exclusion chromatography (SEC) (Dalgalarrondo, Robin, & Azanza, 1986) or by (iii) tangential filtration (Ghodsvali, Khodaparast, Vosoughi, & Diosady, 2005).

Antimicrobial activities shown by rapeseed napins would give a new pathway for rapeseed meal valorisation. However, such bioactivities were observed with napins extracted from seeds in low-denaturing conditions. Furthermore, napins purification is reported to be time and cost consuming, involving a nanofiltration step and several chromatography steps (two ion exchange chromatographies, one size exclusion and one hydrophobic interaction chromatography (Bérot et al., 2005)). The first part of this work proposes a simple extraction/purification process of napins from a rapeseed meal obtained at industrial scale under denaturing conditions (i.e., seed trituration at around 55 °C and oil/*n*-hexane extraction). The second part is a structural study of the purified napins aimed at checking the impact of the napins purification process (from meal production to napins purification). The last part is a study of the antimicrobial activity of these rapeseed meal napins on *F. langsethiae*, which is particularly involved in cereal contamination by mycotoxins.

2. Material and methods

2.1. Materials

The industrial defatted rapeseed meal, obtained by the prepress solvent extraction system was supplied by Robbe-Novance (Compiègne, France). The meal was ground to pass through a 1-mm screen. This rapeseed contained 8% (w/w) water, 3% (w/w) residual oil and 38% (w/w) proteins (estimated from Kjeldahl assay). Common reagents for extraction step (NaOH and HCl) were from Carlo Erba (Milano, Italy). Reagents for SDS–PAGE (acrylamide, tris, glycine, sodium dodecyl sulfate) were from Bio-Rad (Hercules, CA). Ammonium sulfate for precipitation, Folin–Ciocalteu and gallic acid for phenols assay were from Sigma–Aldrich (St. Louis, MO). Glucose solution for carbohydrates assay was from Thermo Fisher (Waltham, MA).

2.2. Solid–liquid extraction steps

Solid–liquid extraction with an amount of solid and liquid phase of 100 g in an Erlenmeyer flask was carried out, in order to assess the effects of extraction pH, meal concentration and extraction duration on the protein extraction. For the study of the pH effect, extractions at 5% (w/w) of wet meal in water were performed at pH ranging from 2 to 11, at room temperature, for 30 min under magnetic agitation. HCl (5 N) and NaOH (1 N) were used as acid and alkali for the pH adjustment. After this step, the solid and liquid phases were separated by centrifugation at 13,200*g*, at room temperature for 30 min. Two samples of the aqueous phase were taken for protein quantification (Kjeldahl assay) and SDS–PAGE analysis. Considering the effect of meal concentration, extractions were carried out at pH 2, room temperature for 30 min at meal concentrations ranging from 3% to 18% (w/w). Samples of the aqueous phase were taken after 30 min and centrifuged as mentioned above. Liquid phases were submitted to a Kjeldahl assay for protein quantification. Finally, an extraction at pH 2 and 12% (w/w) of meal was carried out for 60 min; samples at 5, 10, 15, 20, 30, 40, 50 and 60 min were taken and centrifuged and submitted to the protein quantification in the aqueous phase (by Kjeldahl analysis).

2.3. Napins purification

The first step was a salting out precipitation by ammonium sulfate. The appropriate saturation for the napins precipitation was established by adding ammonium sulfate in 20 mL of aqueous phase obtained by extraction at pH 2, 12% (w/w) meal after 15 min. Ammonium sulfate saturations ranging from 35% to 65% were assayed for 30 min at room temperature. Saturated solutions were then centrifuged at room temperature at 13,200*g*. Pellets were solubilised in water (added in order to obtain a final volume of 20 mL). Supernatant and solubilised pellet were then analysed by SDS–PAGE, in order to choose the minimum saturation that leads to a full napins precipitation.

The second step was a desalting size-exclusion chromatography on a G-25 column (1.6 × 10 cm). The column was loaded with a solution obtained after precipitated napins were solubilised (at 65% ammonium sulfate in aqueous extract) with deionised water. The volume sample represented 12% of the column bed volume (corresponding to 30 mg of proteins). The elution was achieved using an AKTA (GE Healthcare Life-Science, Orsay France) purifier system at 40 mL/min with deionised water. Absorbance at 280 nm and 325 nm and conductivity were monitored on-line.

2.4. Analysis

2.4.1. Proteins analysis

2.4.1.1. Quantification. Quantification of protein matter was achieved according to Kjeldahl procedure. To estimate total nitrogen in selective extraction of napins, a value of 5.70, calculated from napins sequence (Rico, Bruix, Gonzalez, Monsalve, & Rodriguez, 1996) was used for improved accuracy.

To estimate the non-protein part of nitrogen, 20% TCA were mixed with protein extract (v/v) under stirred agitation for 10 min, and then centrifuged at 4500*g* for 15 min. Non-protein nitrogen contained in the supernatant was analysed by the Kjeldahl method. Protein nitrogen was calculated from the difference between total nitrogen and non-protein nitrogen.

2.4.1.2. SDS–PAGE. Electrophoresis gels (sodium dodecyl sulfate polyacrylamide) based on the Leammli method (Laemmli, 1970) were performed using 15% (w/v) concentration of polyacrylamide in presence of 10% SDS. Each sample was prepared at around 1 g L^{-1} of proteins in a solution of bromophenol blue, with or without β-mercaptoethanol. Then, the mixture was heated at 100 °C for 5 min. After migration of samples at 20 mA for 50 min, the gel was coloured with a solution of Coomassie R 250 Brilliant Blue. A standard molecular weight kit (Bio-Rad Precision Plus Protein Standards) from 250 to 10 kDa was used to determine the molecular weights of rapeseed meal protein extracts.

2.4.2. Carbohydrate assay

Sugars were determined by the Nelson–Somogyi method (Nelson, 1944). A volume of 0.4 mL of sample, mixed with copper reagent (v/v), was incubated at 100 °C for 10 min. Cuprous oxide was formed and treated with 0.4 mL of arsenomolybdic acid. The blue colour developed was read at 500 nm with a Cary 50 UV–Vis spectrophotometer (Varian, Massy, France). The absorbance of sample was compared with a standard curve, established with 1.67 mM glucose solution.

2.4.3. Polyphenolic compounds assay

The total polyphenol part was extracted by an acid hydrolysis and then quantified with Folin–Ciocalteu reagent and compared with a calibration curve established with gallic acid according to the method described by the International Organization for Standardization (ISO 14502-1, 2005). The detailed procedure for preparation of gallic acid solutions was as follows. A solution of 40 g L^{-1} of gallic acid was prepared and then diluted to obtain various concentrations at 5, 10, 20 and 30 g L^{-1}, to construct a calibration curve.

Calibration solutions, properly diluted samples, and blank (0.5 mL) were in a first step submitted to a hydrolysis with hydrochloric acid (4 N) and 10 mL of ethanol at 90 °C for 2 h. Phenols extracted with ethyl acetate were thus dosed as follows: 1 mL of sample was mixed with sodium carbonate, Folin–Ciocalteu reagent (v/v) and incubated for 10 min at 38 °C. The colour generated was read at 660 nm.

The absorbance was measured by a Cary 50 UV–Vis spectrophotometer (Varian).

2.4.4. Ash content assay

The ash content was determined according to AOAC method (1990). The sample (0.5 g) was placed in a muffle furnace capable of maintaining temperatures between 500 and 600 °C for 12 h. Ash content was expressed as the ratio of weight sample after combustion over its dry weight.

2.5. Protein structure assessment

2.5.1. Circular dichroism

The secondary structure of napins was recorded by using a CD6 dichrograph (Jobin Yvon, Longjumeau, France). Far-UV spectra were recorded between 185 and 250 nm, at 20 °C with a 1-mm path length quartz cell and 2-nm bandwidth. The protein concentration was 10 mM and samples were dissolved in 20 mM phosphate buffer, pH 7. The results are reported as a mean residual ellipticity in degrees of square centimetres per decimole. All experiments were conducted in triplicate. The percentage of secondary structure was determined by the Dicroprot method (Deléage & Geourjon, 1993).

2.5.2. Fluorescence measurement

Fluorescence spectra were recorded using a SPEX Fluorolog-3 spectrofluorometer (Jobin Yvon) equipped with a thermostatted cell compartment (25 °C) having a 450 W Xenon lamp. Measurements were performed with napins under native conditions and after incubating napins with different concentrations of chemical denaturant (GdnHCl from 1 to 8 M) overnight before the measurements were performed. The excitation wavelength was 280 nm and the emission intensity was monitored at wavelengths in the range of 320–450 nm. Protein concentration was 0.4 g L^{-1}.

2.5.3. Differential scanning calorimetric measurement

Differential scanning calorimetry (DSC) was performed using a VP-DSC (MicroCal, Northampton, MA); Data were analysed by a Windows-based software package. All DSC experiments were achieved with a protein concentration of 7 g L^{-1} in phosphate buffer (20 mM, pH 7). Samples were degassed prior to loading the DSC cell. The DSC scan rate was set to 10 °C min^{-1} for all experiments.

2.6. Antifungal assay

Antifungal activity assays were performed in Petri dishes (∅ = 100 mm), containing 10 mL of yeast malt agar (YM) medium at napins concentrations ranging from 0 to 5.25 g L^{-1}. The Petri dishes were inoculated centrally by a highly toxigenic *F. langsethiae* strain isolated from the 2008 barley harvest. Cultures were incubated for five days at 25 °C under alternating light and darkness periods of 12 h. Two colony diameter measurements were performed each day, taking into account minimal and maximal diameter values. Each experimental condition was performed in triplicate and colony growth rates were determined for each initial condition. IC_{50} of napins for *F. langsethiae* was the concentration of napins leading to a 50% decrease of the colony growth rate, compared with the standard culture without napins.

3. Results and discussion

3.1. Napins solid–liquid extraction from an industrial rapeseed meal

3.1.1. Effect of pH extraction on protein content

Proteins from rapeseed or rapeseed meal are classically extracted by a solid/liquid extraction step using an aqueous phase. The influence of the pH, well known to be the main parameter impacting the protein extraction, was first investigated. Fig. 1A displays the concentration of proteins assessed from nitrogen assayed by Kjeldahl analysis in the liquid phase after 30 min of extraction at pH ranging from 2 to 10 and at 5% (w/w) of meals. The limits of this range were set to avoid any chemical proteolysis and to use a reasonable amount of alkali or acid. It can be observed that the highest protein concentration in the liquid phase is obtained at the highest pH (2.15 g L^{-1} at pH 10). From pH 10 down to 6, the protein extraction goes down (1.2 g L^{-1}) and rises again by decreasing the pH to 2 (2.05 g L^{-1}). Thus, the efficiency of protein extraction is clearly improved at 'extreme' pHs. This has been widely observed and might be explained by electrostatic repulsions. Indeed, the major protein fraction (i.e., cruciferins) has an IP near neutrality (Schwenke, 1994). As a consequence, electrostatic repulsion between cruciferins should reach a maximum at acid and basic pH values with improved extraction from solid meal under these conditions.

Napins IP is reported to be particularly high (IP = 11 according to Schwenke, 1994). So a selective extraction of napins under acidic conditions may be expected. To check that out, extracted proteins from pH 2 to 10 were analysed by SDS–PAGE (Fig. 1B). In Fig. 1B, lanes 10 down to 5 (corresponding to extraction pH from 10 to 5 respectively) show bands at around 50, 25, 20 and 15 kDa, whose were identified from molar masses as cruciferin β-subunits dimers, cruciferin β-subunits, cruciferin α-subunit and napins, respectively. Clearly, each band intensity fades from lanes 10 to 4. Lanes 4 down to 2 (corresponding to extraction pH from 4 to 2) only show the band of napins along with a slight band just below 30 kDa (that might be a dimer of napins). The more acid the extraction is, the more intense the napins band looks. This clearly confirms that a selective extraction of napins takes place under acidic conditions and indicates that the best extraction pH is 2. However, two points are surprising: (i) cruciferins band intensities decrease from basic pH to pH 4 and do not rise under acidic conditions and (ii) napins band is intense under basic condition.

Interestingly, other authors reported a selective extraction of alfalfa RuBisCO small subunit (IP around 6) at pH 2 from a solid concentrate containing other proteins (having IP close to neutrality). Obviously, this indicates that electrostatic repulsions probably act with other factors on protein extraction (such as protein solvation difficulties at solid/liquid interface or strong protein/protein interactions in the solid) (Trovaslet et al., 2007).

Napins concentration in the aqueous extract had to be improved since subsequent purifying and drying processes require to have concentrated starting material. The main parameter of the extraction step that could lead to napins extracted concentration improvement is clearly the starting meal concentration.

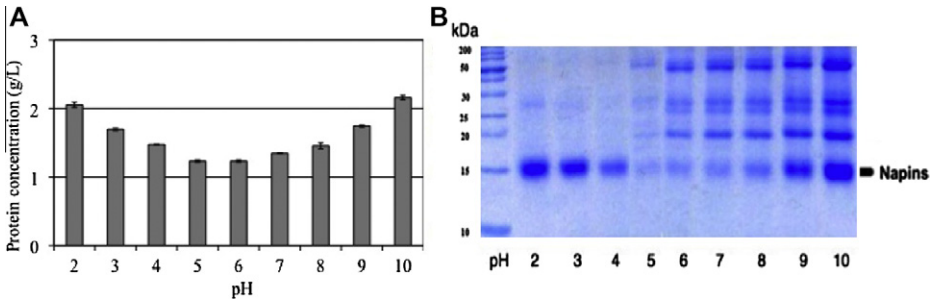

Fig. 1. (A) Protein concentration in the supernatant obtained after centrifugation of 5% (w/w) rapeseed meal in water at pH ranging 2–10, after 30 min of extraction at room temperature. (B) SDS–PAGE 15% of polypeptides extracted (lane 2 to lane 10). Molecular weight markers in the range 10–200 kDa were run along with the protein (left lane). Concentration of loaded protein was 1 g L^{-1}. The error bars are based on duplicate runs.

Therefore, the effect of this parameter on the napins extraction was investigated.

3.1.2. Effect of meal concentration on napins extraction

Extractions at pH 2 and rapeseed meal concentration ranging from 3% to 18% (w/w) in water were carried out. Meal concentration higher than 18% (w/w) was inappropriate, since above this concentration, slurries became difficult to homogenise. Protein concentration and extracted proteins over meal amount used were determined in each condition (Fig. 2A). Protein concentration was still assayed from Kjeldahl analysis but in this case, a conversion factor of 5.70 calculated from napins sequence (Rico et al., 1996) was used. Fig. 2A shows a linear evolution of the extracted protein concentration from 1.03 to 6.39 g L^{-1} for meal concentrations from 3% to 18% (w/w). The extracted proteins over meal amount ratio remains constant around 30 mg extracted proteins per gram of meal but decreases drastically at 15% and 18% meal (down to 15 mg g^{-1}). This is due to a significant lowering in the aqueous extract volume obtained after centrifugation from an initial meal concentration of 15%. From this result, the meal concentration of the extraction step was set at 12% (w/w), which seems to be a good compromise between protein concentration in the extract and extraction yield. Under these conditions, the step productivity was around 1 mg g^{-1} min^{-1} (mg of extracted proteins per gram of meal per minute of extraction). The productivity is directly linked to the extraction step duration. Therefore, the impact of the last parameter on the process productivity was considered by studying the kinetics of the protein extraction (at pH 2 and 12% w/w of meal).

3.1.3. Effect of the extraction duration on napins extraction

The evolution of the protein concentration in the aqueous phase was followed for 60 min (Fig. 2B). Fig. 2B shows that the concentration rises for 15 min then remains constant at 4.5 g L^{-1}. This indicates that, under these conditions, equilibrium is reached after 15 min. The productivity of the step using 15 min extraction duration was then doubled at around 2 mg g^{-1} min^{-1}. Other studies dealing with total proteins extraction from rapeseed meal reported extraction times around 60 min (Bérot et al., 2005; Wu et al., 2008). The effect of the temperature on protein extraction could also have been investigated but this would probably have led to a partial decomposition of the protein, potentially impairing its anti-fungal bioactivity. Thus, the extraction conditions chosen for the study were pH 2 (guarantee of a napins selective extraction) with a 12% (w/w) meal concentration for 15 min. The extract was around 88% of water and 12% dry matter mainly constituted of proteins (around 25%), free carbohydrates (4%), polyphenolic compounds (7%), and ash (10%). The rest of the dry matter (around 50%) is probably composed of salts. This high content in salt is probably due to the amount of HCl (6.82 g) added to keep the pH at 2 during the extraction step. Anyway, the extract is composed of napins (of around 15 kDa) and small molecular weight compounds. Therefore, size-exclusion chromatography (SEC) in desalting mode was chosen to purify napins, since this process shows good productivity and is easy to scale up.

3.2. Napins purification from the liquid extract by desalting SEC

Polyphenolic compounds are known to adsorb to stationary phases. This leads to washing difficulties and increases elution time. Thus, a pre-purification step allowing protein concentration and a partial elimination of polyphenols was required. A salting out precipitation using ammonium sulfate seemed adequate, since this kind of precipitation preserves protein structures. The large amount of salts used for the precipitation will be eliminated by the subsequent SEC step.

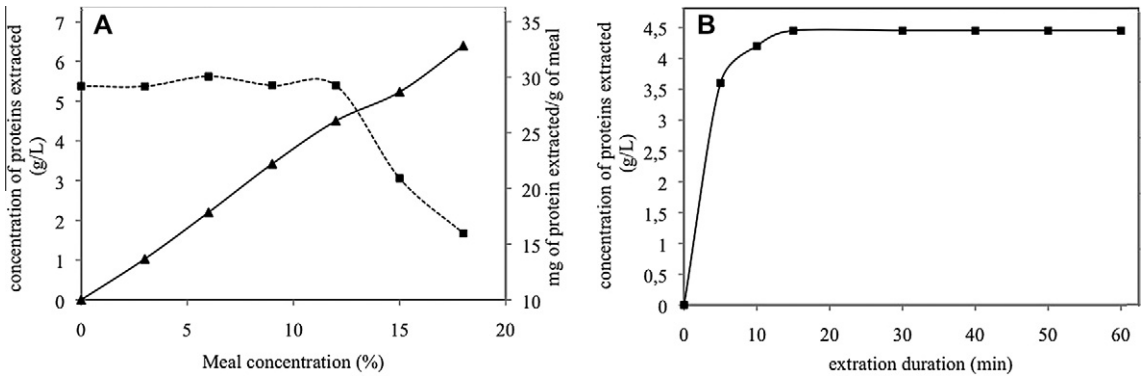

Fig. 2. (A) Protein concentration in supernatant after 30 min of pH 2 extraction from rapeseed meal at 3% to 18% (w/w). Protein concentration (black triangle) and quantity of protein extracted as a function of meal concentration (black square). Each sample was analysed three times. Estimate error was ±1.14%. (B) Protein concentration after extraction of 12% (w/w) rapeseed meal in water at pH 2 for 5–60 min.

Napins were precipitated at 65% of ammonium sulfate saturation and resuspended in water at a third of the initial volume (in order to increase napins concentration). A loss of coloration was observed after ammonium sulfate precipitation, indicating a partial elimination of polyphenols. This solution was loaded (12% of the bed volume, i.e., 30 mg of proteins) onto a laboratory-scale column (1.6 × 10) and eluted at a rate of 30 cm h^{-1} using deionised water. Absorbance at both 280 and 325 nm was recorded on-line. These two wavelengths were chosen to distinguish the elution of proteins (absorbing UV at 280 nm) and polyphenols (absorbing UV at both 280 and 325 nm). The conductivity was followed to ensure that protein fraction was separated from salts.

The chromatogram at 280 and 325 nm (Fig. 3A) shows two well-separated peaks at 10 (elution in the column void volume) and 46 min (elution over two bed volumes). The conductivity is under a major peak at around 15 min. As expected, SDS–PAGE analysis (Fig. 3B) revealed that napins are eluted in the void volume. Indeed, the exclusion molecular weight of this stationary phase is given at around 5 kDa. This indicates that napins fraction is clearly free from minerals but remains slightly contaminated by polyphenolic compounds. The rest of the polyphenols eluted after two bed volumes, denoting an adsorption onto the stationary phase. The contamination of the protein fraction can be due to a protein/polyphenol association. Xu and Diosady (2000) showed that such interactions exist between rapeseed proteins and polyphenols. These interactions may occur through weak (hydrogen bridges, hydrophobic interactions, salt bridges) or covalent bonds. Napins fraction from SEC was further analysed by RP-HPLC (data not shown). The chromatogram at 280 and 325 nm are under a single peak with the same retention time. This indicates that in spite of RP-HPLC dissociating elution conditions (acetonitrile/H_2O/trifluoroacetic acid mixture as mobile phase) polyphenols and napins remain associated. This supports the assumption of a strong association and possibly covalent bonds. The fraction was analysed by SDS–PAGE in reducing (with β-mercaptoethanol) and non-reducing (without β-mercaptoethanol) conditions. The gel obtained is presented in Fig. 3B. Under non-reducing conditions (lane 3), the profile is under a major band at 15 kDa previously identified as napins, the band at around 30 kDa (which was meant to be napins dimers, according to Tan, Mailer, Blanchard, & Agboola, 2011) and a band at 10 kDa. In reducing condition (lane 2), three bands at around 5, 10 and 15 kDa can be seen. This confirms the identification of the band at 15 kDa as napins, since this kind of protein is known to be a heterodimer of large and small subunits of around 10 and 5 kDa respectively. The hypothesis of a napins dimer held by disulfide bond for explaining the presence of the 30 kDa band in the non-reducing gel also appeared to be reinforced. Finally, protein fraction represented more than 92% of the dry matter, which suggested that the SEC step was enough for napins purification. Thus, a simple process based on a simple solid/liquid extraction, a pre-purification step by salting out protein precipitation and SEC gave a purified fraction of napins from a rapeseed meal generated at industrial scale. Interestingly, this process can be easily scaled up to mass-produce napins. A replacement of the salting out precipitation would avoid the use of large amounts of salts. This could be easily done by tangential filtration for example.

The rapeseed meal used in the study was produced by an industrial rapeseed oil process extraction including a seed trituration step at around 55 °C and an *n*-hexane extraction step. These steps can be particularly denaturing for proteins and lead to a loss in the antimicrobial potential of napins.

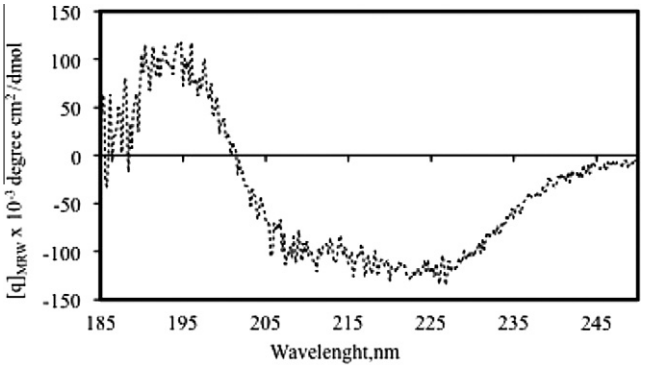

Fig. 4. CD spectrum in the far-UV region (185–250 nm) of napin purified. Scan was recorded using a cell with 1-mm path length. Napins at 10 μM were diluted in phosphate buffer 20 mM (pH 7.0). Spectrum with two minima at 208 and 222 nm indicates a predominance of the α-helix structure.

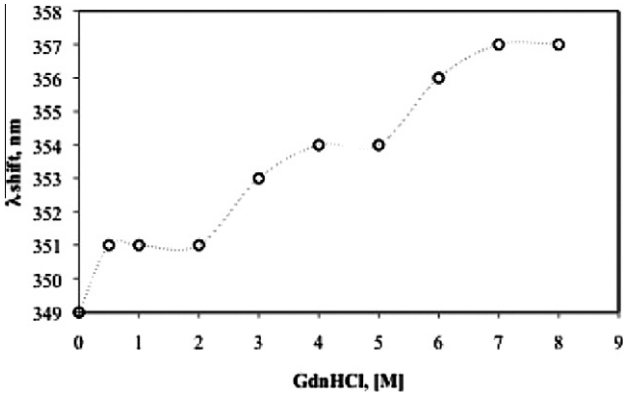

Fig. 5. Shift in the fluorescence emission wavelength maxima as a function of denaturant (GdnHCl) concentration.

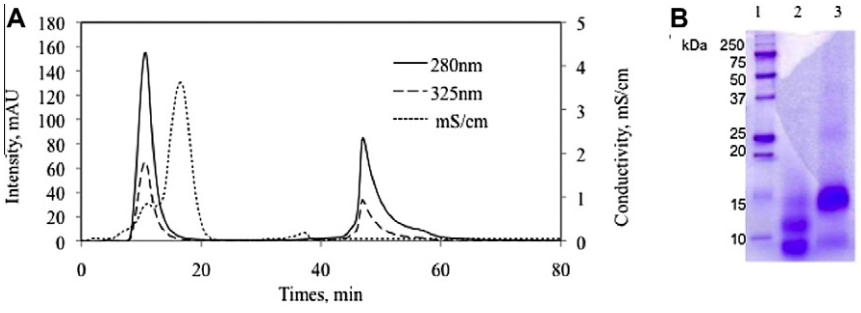

Fig. 3. (A) Size exclusion chromatography (SEC) of napins extracts (at pH 2 using 12% of rapeseed meal for 15 min) after precipitation with 65% ammonium sulfate. (B) SDS–PAGE 15% (w/v) pattern under reducing (line 2) and non-reducing conditions (line 3) for the first SEC fraction. Molecular weight markers (line 1) in the range 10–250 kDa were run along with the protein.

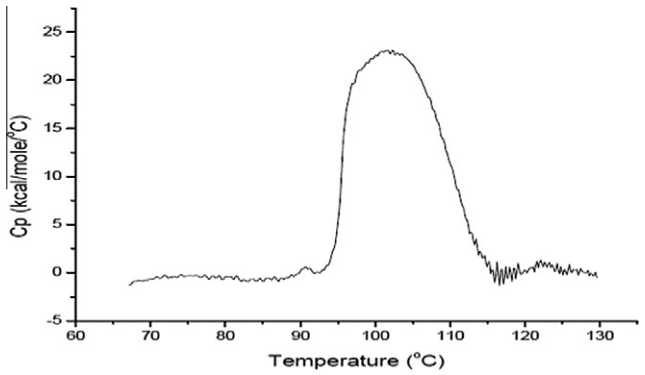

Fig. 6. Differential scanning calorimetry (DSC) of napins from rapeseed meal. DSC thermogram showing temperature for the thermal denaturation of napins (T_m 103 °C). Protein concentration was 7 g L^{-1} in 20 mM phosphate buffer (pH 7.0) and a scan rate of 1 °C min^{-1}.

3.3. Rapeseed meal napins structural analysis

First, napins were analysed by circular dichroism (CD) in the far UV (185–200 nm). CD spectra of napins were obtained at 25 °C in phosphate buffer pH 7 (Fig. 4). In the far UV region the CD spectrum gives information on secondary structures. The spectra show two minima at 222 and 208 nm. This is characteristic of high content in α-helix. From the spectrum, napins revealed to be composed of 37% α-helix and 11% β-sheet. Interestingly, such a high amount of α-helix was previously observed with native napins from different *Brassicacae* seeds (*B. napus*: Wu & Muir, 2008; *Brassica juncea*: Jyothi, Sinha, Singh, Surolia, & Appu Rao, 2007). This tends to indicate that the secondary structure of napins has not been affected drastically during the industrial process and our extraction/purification procedure.

Then, we focused on napins tertiary structure. To investigate protein tertiary structure denaturation, the intrinsic fluorescence of tryptophan is frequently used, since a shift in fluorescence occurs as it is exposed to a polar environment. In 2007, Jyothi et al. (2007) studied the impact of guanidinium chloride (GdnHCl), a strong chaotropic agent, on the tertiary structure of native *Brassicacae* napins. The same protocol was applied to our rapeseed meal napins. Fig. 5 shows a red shift (increasing of λ_{max} from 339 to 357 nm) as GdnHCl concentration increases. As expected, this denotes an aqueous solvent exposure of tryptophan side chains, thus unfolding the protein. The interesting point lies on the three states observed in the range of 0.5–2, 4–5 and 7–8 M GdnHCl. Indeed, Jyothi et al. also observed such a three state denaturing pattern (at the same GdnHCl concentration) with native napins. This tends to show that napins extracted from our industrial rapeseed meal have a tertiary structure similar to native ones. The oil extraction process seems to have not affected napins structure much.

Finally, to check that assertion out, the thermal stability of our napins was measured by DSC thermogram (Fig. 6). Fig. 6 shows a high endothermic peak at 102 °C, as in Wu et al. (2008) who investigated the thermal stability of *B. napus* napins. This high denaturing temperature is likely to be due to the compact structure of napins and its high content in stabilising secondary structures (as helix and sheets) as evoked by Jyothi et al. (2007) and Moreno, Mellon, Wickham, Bottrill and Mills (2005). This could explain why no extensive denaturation occurred during the oil extraction process or the napins extraction/purification process.

This suggested that the antimicrobial potential of our napins could be entire. Such a bioactivity was assessed on *F. langsethiae*, a fungus strain that grows on cereals and causes a food safety problem.

3.4. Antifungal bioactivity on F. langsethiae

The antifungal napins activity was tested against the highly toxigenic *F. langsethiae*. This activity was determined under the form of IC_{50} on Petri dish cultures containing 0 to 5.25 g L^{-1} of napins. Fig. 7 displays basic Petri dish aspects after 4 days and the corresponding IC_{50} curve. The antifungal effect of napins clearly appears in Fig. 7A, with an associated IC_{50} value of 70 μM (Fig. 7B). This result confirms the antifungal bioactivity of napins

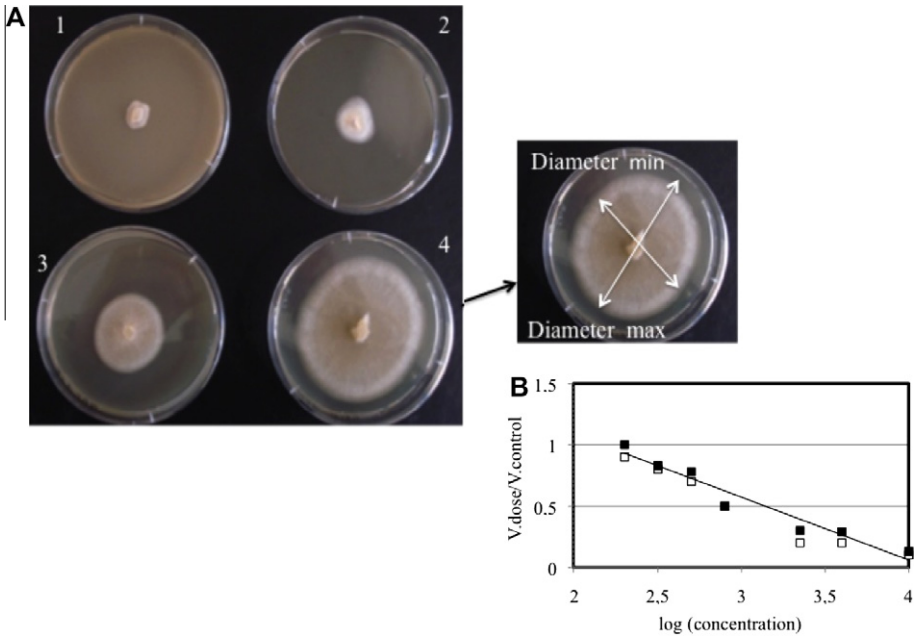

Fig. 7. (A) Evolution of diameter growth of *F. langsethiae* in presence of 5.25, 1.31, 0, 16 g $L^{-1}$of napins after 5 days (Petri dish No. 1, 2, 3, respectively) and control without napins (Petri dish No. 4). (B) Effect of various concentrations of napins on growth of *F. langsethiae*. IC_{50} value is 70 μM. Comparison is done by measuring maximum (black square) and minimum (white square) *Fusarium* growth diameters.

from *Brassica* species already observed by Terras, Torrekens et al. (1993) with rapeseed 2S albumins fraction (assumed to be napins).

Recently, it was demonstrated that a napins-like polypeptide with high thermostability often exhibits antifungal properties (Lin & Ng, 2008). Studies by Powers and Hancock (2003) have shown two structural requirements for antimicrobial activity: (i) a cationic charge and (ii) an amphipathic conformation. Rapeseed napins with their high arginine and lysine content, and amphipathicity fully comply with these two requirements. What is more, napins can also interact synergistically with thionins which produce fungal membrane damage (Terras, Schoofs et al., 1993). In another antimicrobial mechanism model, amphipathic α-helix structure of napins from *Brassicaceae* may be involved in the CaM (calmodulin) antagonist and can form pores in membranes (Neumann, Condron, Thomas et al., 1996). This is probably because CaM and two subunits of napins contain similar α-helix. Another assumption concerning the antifungal mechanism of napins could be due to a protease inhibiting capacity, which could cause an alteration of protein metabolism (Neumann, Condron, & Polya, 1996).

This work provides data confirming the antifungal potential of napins from *Brassicaceae*. However, the originality of this work lies in the fact that such activity is observed with napins from an industrial by-product of rapeseed oil extraction. Indeed, such a denaturing process (implying heat treatment steps at 55 and 105 °C and a solvent extraction) could have denatured napins and caused a loss in antifungal potential.

4. Conclusion

This study presents an original method for extracting/purifying napins from an industrial rapeseed meal. Extraction at pH 2 has been shown to allow a selective extraction of napins. The extraction is also influenced by rapeseed meal concentration and extraction duration. The best conditions observed in this work are a 12% (w/w) rapeseed meal concentration and an extraction duration of 15 min at room temperature. The extracted napins possessed similar structural shape to native napins, indicating that industrial oil extraction process did not cause a drastic napins denaturation. Our napins show a promising activity for *F. langsethiae* growth inhibition, which could be used either to complement phytosanitary molecules employed in the field, or to improve cereals storage conditions and duration. The demonstration of that bioactivity opens an interesting valorisation field for rapeseed meal in the area of food safety. However, the ammonium precipitation step should be replaced by a more productive one (i.e., diafiltration with UF membrane) and the bioactivity should be further characterised (particularly the impact of napins on *Fusarium* mycotoxins release).

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