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Biochemical and mass spectrometry recognition of phospholipid–peptide complexes in wheat sprouts extract

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Total hydroalcoholic extract of wheat sprouts was treated with 90% cold acetone as a preliminary step directed to separate antioxidant peptides from antioxidant polyphenols. Surprisingly, the addition of acetone causes the formation of a yellow buoyant gelatinous drop that prevailingly contains peptides and phospholipids. In this context, evidences have been presented that support the hypothesis that peptides (and perhaps other active molecules) are complexed with phospholipids. In fact, the MS/MS analysis of some main ions, present in RP HPLC fractions of wheat sprout extract, generates several ions that correspond to molecular weight of phospholipids or phospholipid fragments. Moreover, several ions were detected that correspond to lysophosphatidylcholine or phosphatidylcholine–peptide complexes. The possibility that phospholipids can be complexed with peptides has been discussed in the light of potential involvement in the peptide bioavailability. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bioactive peptides; peptide-phospholipid complex; phospholipids; wheat sprout

Introduction

Attempts of increasing the bioavailability of biologically active hydrophilic molecules are a main research goal for many authors. In this context, designing and formulating a polypeptide drug has been a persistent challenge because of their unfavorable physicochemical properties, which include enzymatic degradation, poor membrane permeability and large molecular size [1,2]. A main challenge is to improve peptide oral bioavailability from less than 1% to at least 30–50%. Consequently, efforts have intensified over the past few decades, where every oral dosage form used for the conventional small molecule drugs, has been used to explore oral protein and peptide delivery. Various strategies currently under investigation include chemical modification, formulation vehicles and use of enzyme inhibitors, absorption enhancers and mucoadhesive polymers. Porcine insulin was used as a model drug due to its water solubility, ease of analysis and ready availability. To improve oral delivery insulin was complexed with phospholipid by an anhydrous co-solvent lyophilization procedure [3,4]. The authors have shown that a hydrophilic peptide, such as insulin, can be successfully formulated into biodegradable nanoparticles by formation of a phospholipid complex. Results of an *in vivo* evaluation allow to conclude that insulin–phosphatidylcholine (PC) complex loaded nanoparticles are able to markedly improve the intestinal absorption of insulin. Another model of peptide–phospholipids complex designed to increase peptide bioavailability is represented by 5A apolipoprotein mimetic peptide [5,6]. 5A/PLPC [apolipoprotein (apo)A-I mimetic peptide 5A complexed with phospholipids 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC)], reduced: the increase in (i) endothelial expression of cell adhesion molecules, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1; (ii) O_2^- production, as well as the expression of the Nox4 catalytic subunits of the NADPH oxidase and (iii) infiltration of circulating neutrophils into the carotid intima-media. In human coronary artery endothelial cells, 5A/PLPC inhibited tumor necrosis factor-induced, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression, as well as the nuclear factor B signaling cascade and O_2^- [7]. The results from these and other authors show that the strategy of performing protein/peptide complexes with phospholipids in order to attempt a substantial increase of their bioavailability is evolved in the last decades. Following the use of liposomes, complexes were prepared by incubation of peptides with small unilamellar

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vesicles of dimyristoylphosphatidilcholine [5–7]. Recently, peptide/protein was complexed with phospholipids by an anhydrous co-solvent lyophilization procedure. IR analysis of the complex obtained by means of this very innovative method shows that some weak physical interactions between insulin and soybean phosphatidylcholine (SPC) took place during the formation of the complex [3]. From a more general point of view, phospholipids complexes of natural polyphenol compounds such as curcumin [8], silybin [9], saponin [10], etc., have been studied in order to improve its bioavailability, even if the conditions for the formation of these complexes called phytosomes, are much more stringent (for example acetone under heating and stirring) [8]. We reported the isolation and characterization of chromatin binding peptides active in the control of DNA transcription [11–13]. However, the isolated and synthesized molecules are almost completely inactive at cellular level. Following our experimental experience the lost of activity in cellular systems is progressive with the purification process. Our hypothesis is that in crude extract some molecules complexed with the active peptide by weak interaction are present, thus acting as carrier for cell internalization. The progress in the purification process could destroy these complexes. The results reported in these articles show evidences that phospholipids are complexed with peptides in extracts from wheat sprout.

Materials and Methods

Preparation of Wheat Sprout Extract

Wheat seeds sprouted for 3–5 days on soft agar (0.8–1%) are dehydrated, the sprouts are mechanically separated from the seeds and grinded. Extract from wheat sprout powder was prepared as previously reported [14]: 20 g of powder were suspended and homogenized (by means of a Waring Blendor) with 400 ml of water/ethanol (30 : 70, v/v) and centrifuged at 10 000 *g* for 30 min at 4 °C. After storage at −20 °C overnight, the extract was again centrifuged at 10 000 *g* for 30 min at 4 °C. The ethanol was then removed by evaporation and the aqueous residue lyophilized.

Treatment of Wheat Sprout Extract with Acetone

Forty milligram of lyophilized extract were solubilized in 2 ml of 0.2 M ammonium acetate (extract final concentration 20 mg/ml) and treated inside a centrifuge tube with 18 ml of cold acetone. The mixture was shaken and left at −20 °C overnight. Subsequently, the sample was vortexed and centrifuged at 7900 *g* for 30 min. Following centrifugation, a small white pellet was obtained while near the bottom of the tube a yellow buoyant gelatinous drop was visible. The drop of about 50-µl volume was gently withdrawn with a pipette-tip and put into a 1.5 ml tube to remove the acetone by a vacuum centrifuge. The super was removed and subjected to rotary evaporation; the pellet was dried by air flow. The three samples (super, yellow drop and pellet) were solubilized in 1 ml of water. Fraction 1: 'super'; Fraction 2: 'yellow drop'; Fraction 3: 'pellet'. In control experiments, 4 mg of reduced glutathione were solubilized in 2 ml of 0.2 M ammonium acetate and mixed with 18 ml of cold acetone. After overnight at −20 °C, the sample was centrifuged at 7900 *g* for 30 min. The super was removed and subjected to rotary evaporation and the pellet dried by air flow. Super and pellet were solubilized by 1 ml of water. The ratio of glutathione concentration in the super and in the pellet was measured by spectrophotometric absorbance at 220 nm in water.

UV Spectrophotometry

The fractions obtained from wheat sprout extract, following suitable dilution in water, were analyzed by UV spectrophotometry from 200 to 400 nm, using a Varian Cary 100 spectrophotometer.

Thin-layer Chromatography

TLC was performed on silica gel plates using propanol/water 70 : 30 (v/v) as solvent system. To determine the reducing groups, the plates were sprayed with 10% phosphomolybdic acid solution in ethanol (w/v) and heated at 120 °C until spot formation was obtained. In fact, phosphomolybdic acid, in the presence of reducing substances, is transformed into molybdenum blue, which is visible on the TLC sheet as a blue spot. To detect free amino groups, the TLC sheets were sprayed with ninhydrin reagent. In the presence of free NH_2 red-violet spots appear.

RP HPLC Analysis of Wheat Sprouts Extract

Hydroalcoholic extracts from wheat sprout were fractionated by HPLC utilizing a semi preparative C18 150 mm × 10 mm (Phenomenex, Torrance, CA, USA), RP column. The column was equilibrated with 0.1% trifluoracetic acid/acetonitrile (97 : 3, v/v). After 6 min of isocratic elution, a gradient was applied: from 3 to 20% acetonitrile in 50 min. Flow was set at 2.5 ml/min.

MS Analysis of RP HPLC Fractions from Wheat Sprouts Extract

Lyophilized HPLC fractions were solubilized in methanol and injected for electrospray MS analysis: mass spectrometer, LCQ-MS THERMOQUEST/ESI-ION TRAP; capillary temperature, 220 °C; capillary voltage, 10 V; spray voltage, 4 kV; collision energy, from 17 to 23 keV. The ions shown by MS analysis of total extract HPLC fractions were scanned to cross over ions corresponding to complex between peptide (M_1) and lysophosphatidylcholine (LPC) (M_2) or PC (M_3). We recognized monocharged (I) or doublecharged (II) ions that fulfill the following relations:

(I) $[M_{complex} + H]^{+} - [(M_1 + M_2) + H]^{+} < 0.5$
(II) $[M_{complex} + 2H]^{2+} - [(M_1 + M_3)/2 + 2H]^{2+} < 0.5$

For what concerns the study of peptide structure, the mass spectra analysis was performed with a recently reported [15] automatic combinatorial method that carries out the computation of all amino acid sequences compatible with a given molecular ion. The possible sequences of these compounds are automatically obtained by considering the mass of ions that are potential breakdown products.

Normal-phase HPLC–Tandem MS

Fraction 2 (see Materials and Methods Section) was analyzed by HPLC–MS, which was carried out using a pump module (Jasco PU-980) and a ternary gradient module (Jasco LG-980-02, Tokyo, Japan). The column was a Polaris Si-A 3_150 mm × 4.6 mm (Varian, Middelburg, The Netherlands) protected with a silica precolumn (4 mm × 3.0 mm i.d.) from Phenomenex. The phospholipids present in Fraction 2 were separated according to Malavolta *et al*. [16], with a modified flow rate, as reported below. Briefly, the mobile phase was a gradient of solvent A [$CHCl_3$/MeOH/NH_4OH(30%) 80 : 19.5 : 0.5, v/v], and solvent B [$CHCl_3$/MeOH/H_2O/NH_4OH(30%) 60 : 34 : 5.5 : 0.5, v/v]. The gradient started at 100% of A, decreased to 0% in 10 min, then was held for 15 min and then reached back

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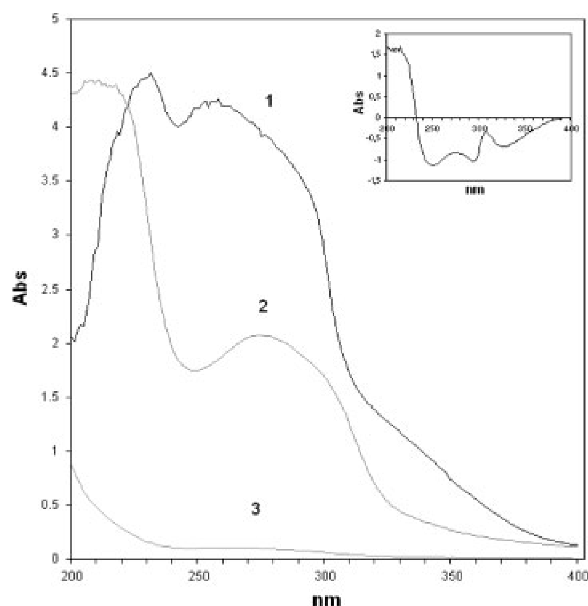

Figure 1. Absorption spectrum from 200 to 400 nm of the samples from wheat sprout extract, following treatment with acetone. Fractions 1, 2 and 3 (see Materials and Methods Section). Before the spectrophotometric analysis, the samples were diluted 1 : 5 with water. Inset: the differential absorption spectrum from 200 to 400 nm of the Fraction 2 (sample cuvette) against the Fraction 1 (blank cuvette) is reported.

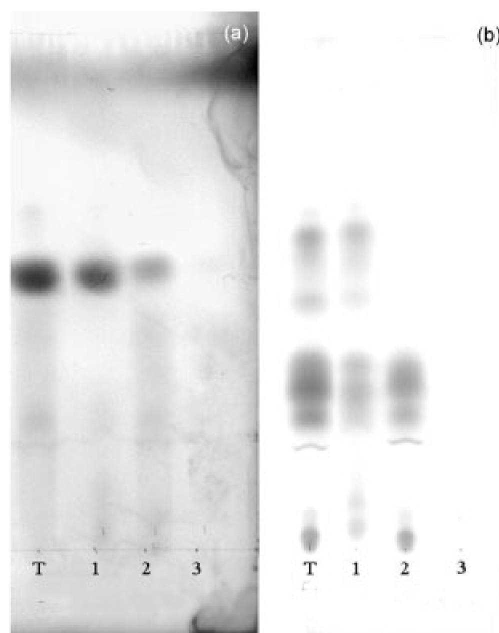

Figure 2. Ascendent TLC on silica gel plates (10 × 20 cm). Solvent system: propanol/water 70 : 30 (v/v). The plates were stained with phosphomolybdic acid (panel a) or ninhydrin (b). Fractions 1, 2 and 3 (3 μl) (see Materials and Methods Section). T represents TLC pattern of total extract.

100% A in 5 min. The flow rate was 1.0 ml/min and the injection loop was 5 μl. The HPLC system was coupled on-line to an LCQ ion-trap mass spectrometer (Finnigan, San Jose, CA, USA) equipped with an ESI source. The HPLC effluent was splitted and 0.4 ml min^{-1} entered the MS through a steel ionization needle set at 5.0 kV and a heated capillary set to 200 °C. The sheath gas flow was approximately 90 arbitrary units. The ion source and the ion optic parameters were optimized with respect to the positive molecular related ions of the glycolipids and phospholipids standards. The molecular mass peaks from the HPLC effluent were detected using positive ion full-scan ESI-MS analysis. Mass resolution was 0.1 Da. Tandem mass (MS_2) experiments were carried out with relative collision energy of 45%. The integration was performed with the interactive chemical information system peak detection algorithm software provided by Finnigan, after correction for the contribution from the ^{13}C isotope effect [17].

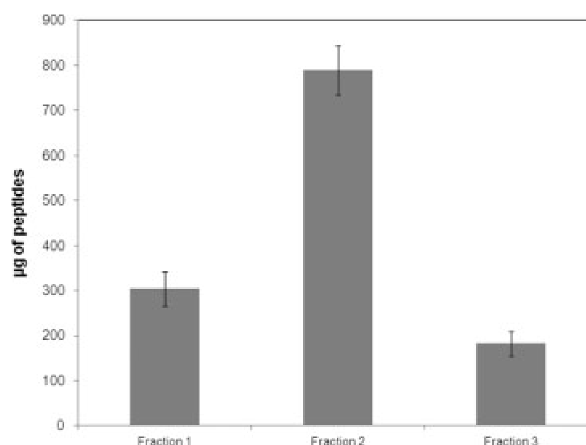

Figure 3. Measure of protein content by Biorad assay. The samples were mixed with equal volume of Biorad reactive and after 15 min at room temperature the absorption read at 595 nm. The absorption value was transformed in protein amount by a titration curve obtained with bovine albumin. For Fractions 1, 2 and 3, see Materials and Methods Section. The values are the mean of five independent measures ± standard deviation.

Results

Total hydroalcoholic extract of wheat sprout contains a powerful cocktail of antioxidant compounds [18–21]. Gel filtration, ion exchange chromatography and HPLC, coupled to analytical methods, demonstrated that this cocktail is composed both of hydrophilic molecules such as peptides and more hydrophobic compounds such as polyphenols. To enrich the extract of the polyphenol component, 90% acetone was utilized to remove the main portion of peptides. We observed that 90% acetone causes the precipitation of 70% glutathione, a hydrophilic peptide (see Materials and Methods Section) (data not shown). The treatment of the extract with acetone interestingly causes the formation of a yellow buoyant gelatinous drop. Following sample centrifugation, the drop was withdrawn (Fraction 2); moreover super (Fraction 1) and pellet (Fraction 3) were separately collected. It is noteworthy that all the three fractions, following acetone removal, were easily solubilized by 1 ml of water. The absorption spectra of the samples are reported in Figure 1. The spectra demonstrate that the 65% of absorption in the wavelength range from 250 to 350 nm, that may be ascribed to aromatic structure, is shown in the Fraction 1 and the 33% in the Fraction 2. To better identify the absorption distribution between these two samples, a differential absorption spectrum of the Fraction 2 was performed against the Fraction 1 (insert of Figure 1). The differential spectrum shows a positive difference from 200 to 215 nm that could be due to peptidic bonds, while a strong negative difference may be observed from 230 to 300 nm.

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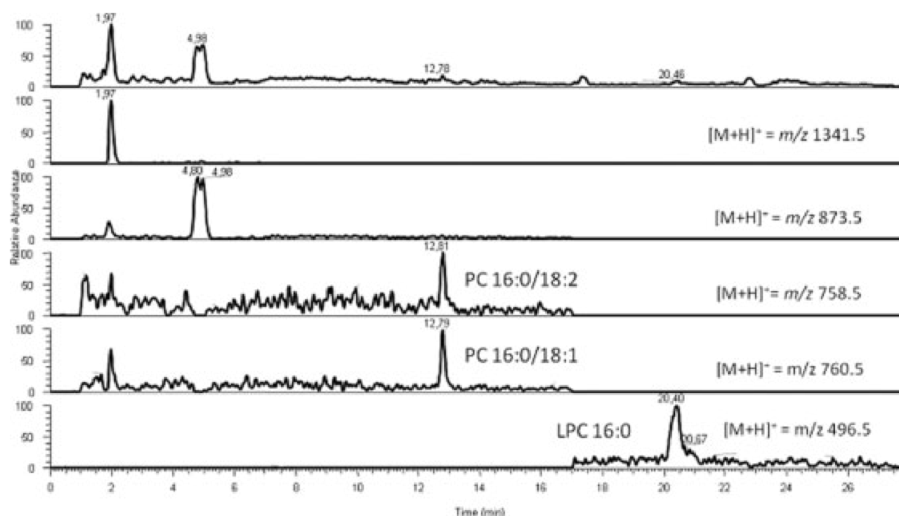

Figure 4. Reconstructed positive ion HPLC-ESI-MS chromatogram of phospholipids from Fraction 2 with the MS operating in scan mode. PC, phosphatidylcholine; LPC, lysophosphatidylcholine. $C_{n:m}$ = fatty acid (n = carbon number; m = number of double bonds). The conditions of HPLC chromatography and MS analysis are described in Materials and Methods Section.

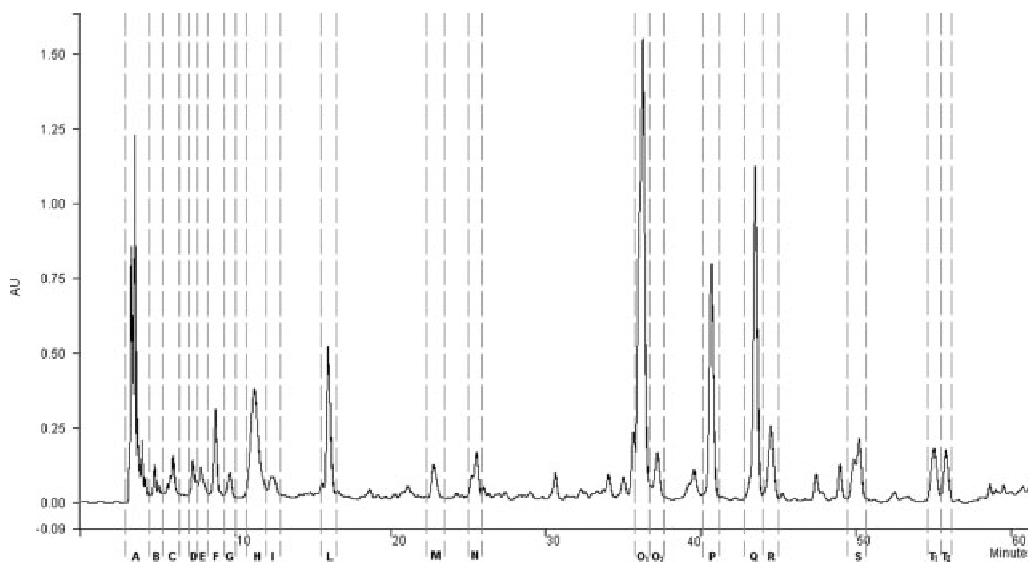

Figure 5. RP analytical HPLC chromatography of the wheat sprout total hydroalcoholic extract. The HPLC conditions and the RP column are described in Materials and Methods Section.

This absorption range is characteristic of aromatic structures. However, it is also possible to observe that the absorption kinetic of the Fraction 2 shows a sharp peak at about 275 (280) nm that is characteristic of the aromatic amino acids, while the Fraction 1 shows a large shoulder between 260 and 300 nm. TLC (Figure 2) and peptide titration by Biorad assay (Figure 3) confirms the absorption data. TLC staining with phosphomolybdic acid shows that the antioxidant molecules are almost completely contained in the Fraction 1, while the Fraction 2 contain the main peptide fraction. The amounts of protein/peptide measured by Biorad assay are 304 ± 38 µg in Fraction 1, 790 ± 54 µg in Fraction 2 and 183 ± 27 µg in Fraction 3. Phosphomolybdic acid or ninhydrin positive spots appear not detectable in the TLC of the Fraction 3. Interestingly, the normal-phase HPLC of Fraction 2 coupled to MS analysis shows three sharp peaks that correspond to PC (*m/z* 758.5 and *m/z* 760.5) and LPC (*m/z* 496.5) (Figure 4). Moreover, the HPLC/MS spectra show a lot of molecules that are not referable to phospholipids. In Figure 5, the fractionation pattern of total wheat sprout extract by RP HPLC is reported. On the fractions obtained from RP HPLC of the total hydroalcoholic extract and from normal-phase HPLC of Fraction 2, the MS analysis has been performed. Some molecular ions, from 700 to 1000 *m/z*, shown by the MS analysis of Fraction 2 (Figure 6) are also present in the MS spectra of total extract RP HPLC fractions (Fraction G, *m/z* 930.8, 939.6; Fr. H 785.6, 799.7, 881.6; Fr. L 817.1; Fr. M 881.1, 905.9; Fr. N 870.9; Fr. P 785.7; Fr. S 791.2; Fr. T_2 905.5). This would confirm that the non lipid ions observed in the Fraction 2 correspond to molecules present in the total extract. The MS/MS analysis of these ions allows us to elucidate some features of peptidic structures. For example, a molecular model may been obtained from the MS/MS spectrum of the ion with *m/z* 905.5 (Figure 7); the series of ions at *m/z* 906.6 (molecular ion MH^+), 311.3, 414.5, 511.3, 568.1, 625.3 (*N*-terminal fragments) and 596.1, (*C*-terminal fragment) is compatible with the sequence pyroGlu-Ala-Gln (or

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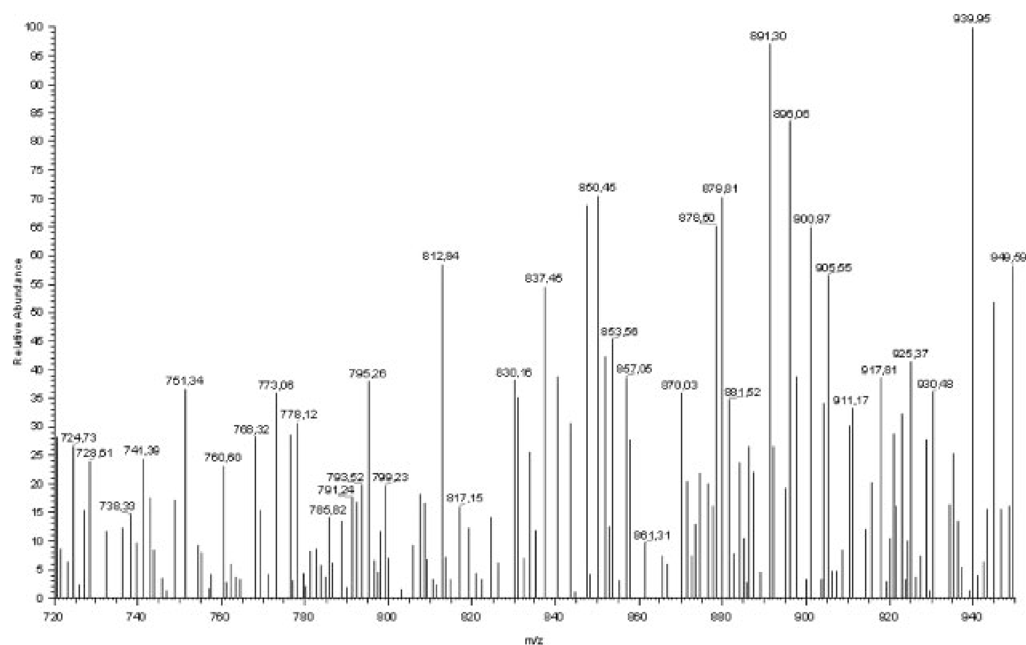

Figure 6. Mass spectrum of normal-phase HPLC eluate collected between 2 and 10 min (Figure 4). The conditions of MS analysis are described in Materials and Methods Section.

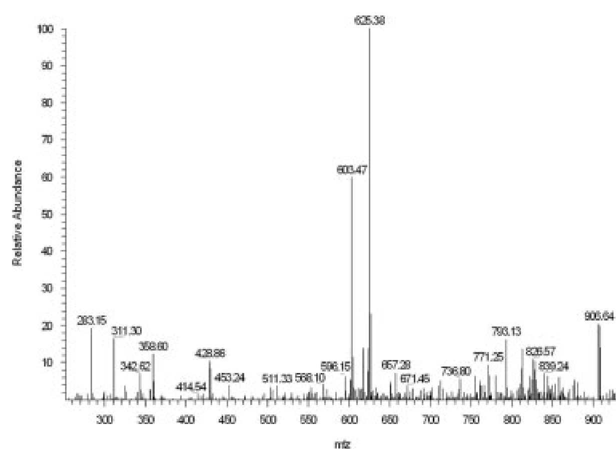

Figure 7. Mass/mass spectrum of the ion with *m/z* 906.6 (MH^+). The conditions of MS analysis are described in Materials and Methods Section.

Gln-Ala)-Cys-Pro-Gly-Gly-Phe-AsnOH. Subsequently, taking into account that using electrospray MS non covalent interaction of biomolecules could survive the transfer into the gas phase [22], we have checked if ions corresponding to complexes between non lipid ions (Figure 6) and LPC or PC, assembled by acetone in Fraction 2, are present in the HPLC fractions of wheat sprouts total extract. The results reported in Table 1 demonstrate that several ions are representative of peptide molecules complexed with LPC or PC. Examples of potential LPC complex and PC complex are reported in Figures 8 and 9. In addition, the MS/MS analysis of some main ions observed in MS spectra of total extract RP HPLC fractions allows us to identify complexes of LPC or PC with peptides not detected in direct-HPLC of Fraction 2. Two molecular models are reported here:

1. Fraction Q: ion *m/z* 1117.87: [PC + NH_2Gly-His-PheOH) + H]$^+$
2. Fraction P: ion *m/z* 1196.00: [LPC + (AcAsp-Ser-Val-Cys(or-Cys-Val)-Val-AsnNH_2) + H]$^+$

Discussion

In this article, we tried to separate polyphenols and other hydrophobic molecules from peptides by means of acetone. Acetone/water 90 : 10 (v/v) is a mixture suitable to precipitate hydrophilic peptides, while from the literature data the precipitation of lipid, phospholipids and hydrophobic polyphenols is not expected. Some authors reported precipitation of phospholipids by acetone but starting from neutral and polar lipids extracts obtained with hydrophobic organic solvents, for example petroleum ether, chloroform or ethyl acetate/ethanol (2 : 1; v/v) [23]. The results reported in this article demonstrate that the acetone apparently causes the assembly of peptides (and perhaps other molecules) selectively complexed with PC or LPC in a yellow gelatinous buoyant drop (called fraction 2). These data indicate that the wheat sprout extract contains phytocomplexes constituted of phospholipids and peptides or other active molecules such as polyphenols. The complexes are mediated by weak interactions [3], presumably characterized by hydrophobic and maybe electrostatic components. Accordingly, the biological activity of some fractions could be lost following extensive purification steps that probably causes the breaking down of the complex thus supporting the hypothesis that the complex with phospholipids may be involved in the bioavailability of peptides and other active molecules. The main goal of this work is the possibility that phospholipids can associate with peptides and other active molecules. The formation of these complexes, that may be important for active molecules bioavailability, is suggested by some observations: (i) The phospholipids present in total crude extract following any step of lyophilization during the extraction and purification procedure are quickly and completely soluble in water. This solubility in water may be explained if the phospholipids are associated with hydrophilic molecules. This observation is in agreement with the data of Li *et al*. [24] according to which phospholipids solubilized in chloroform into a vial may be slowly solubilized, after removing the chloroform by vacuum centrifuge, by an aqueous solution of binding fusion peptide; the complex with the peptide bring in

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Table 1. MS analysis

Fractions	LPC complexes	PC complexes
G	*m/z* 1335.10: [ion 812.24 + LPC($C_{18:0}$) + H]$^+$	
	m/z 1428.93: [ion 905.55 + LPC($C_{18:0}$) + H]$^+$	
	m/z 1469.27: [ion 949.59 + LPC($C_{18:2}$) + H]$^+$	
H	*m/z* 1410.57: [ion 891.30 + LPC($C_{18:2}$) + H]$^+$	*m/z* 799.71: [ion 837.45 + PC($C_{16:0}$/$C_{18:2}$) + 2H]$^{2+}$
I		*m/z* 839.50: [ion 917.81 + PC($C_{16:0}$/$C_{18:2}$) + 2H]$^{2+}$
M	*m/z* 1415.01: [ion 896.06 + LPC($C_{18:2}$) + H]$^+$	
N	*m/z* 1357.23: [ion 837.45 + LPC($C_{18:2}$) + H]$^+$	
P		*m/z* 822.02: [ion 857.05 + PC($C_{18:1}$/$C_{18:1}$) + 2H]$^{2+}$
Q	*m/z* 1374.41: [ion 850.45 + LPC($C_{18:0}$) + H]$^+$	*m/z* 843.56: [ion 925.37 + PC($C_{16:0}$/$C_{18:2}$) + 2H]$^{2+}$
	m/z 1402.91: [ion 879.81 + LPC($C_{18:0}$) + H]$^+$	
R		*m/z* 869.68: [ion 949.59 + PC($C_{18:1}$/$C_{18:1}$) + 2H]$^{2+}$
T_1	*m/z* 1349.48: [ion 830.16 + LPC($C_{18:2}$) + H]$^+$	*m/z* 815.45 = [ion 870.03 + PC($C_{16:0}$/$C_{18:2}$) + 2H]$^{2+}$
T_2		*m/z* 833.22 = [ion 905.55 + PC($C_{16:0}$/$C_{18:2}$) + 2H]$^{2+}$

Recognition in wheat sprouts extract RP HPLC fractions of potential complexes between non lipid molecules eluted in normal-phase HPLC (Figure 4) and LPC or PC.

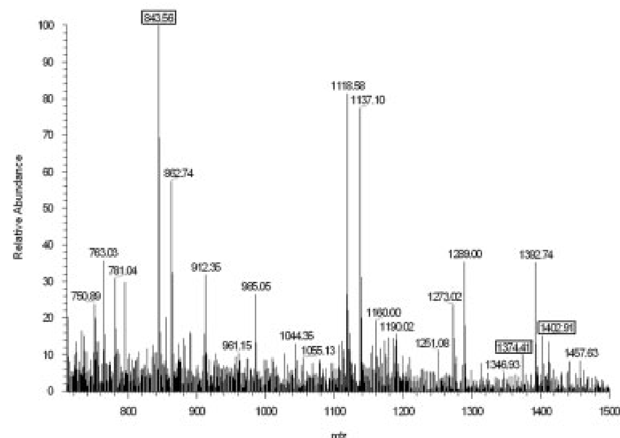

Figure 8. Mass spectrum (from 700 to 1500 *m/z*) of RP HPLC Fraction Q (Figure 5). The conditions of MS analysis are described in Materials and Methods Section. Energy collision = 17 keV. The ion with *m/z* 843.56 represents phospatidylcholine complex. The ions with *m/z* 1374.41 and 1402.91 represent lysophosphatidylcholine complexes (Table 1).

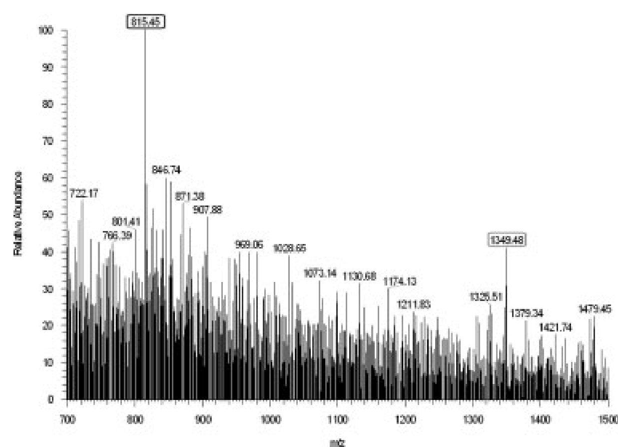

Figure 9. Mass spectrum (from 700 to 1500 *m/z*) of RP HPLC Fraction T_1 (Figure 5). The conditions of MS analysis are described in Materials and Methods Section. Energy collision = 17 keV. The ions with *m/z* 815.45 and 1349.48 represent phospatidylcholine and lysophosphatidylcholine complexes, respectively (Table 1).

solution the phospholipids. (ii) The fraction obtained by extract treatment with acetone and called Fraction 2 interestingly contains PC and LPC and a series of molecules that the MS analysis shows also in the HPLC fraction of the total extract. (iii) Some molecular ions of complexes between non lipid molecules (Figure 6) and LPC or PC have been recognized in RP HPLC fractions of wheat sprout total extract. The LPC complex ions have been recognized as monocharged ions while the PC complex as bicharged ions because the values of the monocharged ones are too near the operative limit of the MS spectrometer.

The possibility of observing molecular ions of not covalently bound molecules is depending from the MS collision energy and from the binding strength of the complex. Recently, the determination of molecular ions concerning peptide–phospholipid complexes have been observed with mass spectrometers equipped with a nanoelectrospray source [24–26].

However, it is unlikely that acetone/water 90 : 10 (v/v) causes phospholipids precipitation, while this may easily occur if they are associated with hydrophilic molecules such as peptides. In fact, we have demonstrated that, in the presence of 90% acetone, also a small peptide such as glutathione precipitate and following centrifugation go down in the pellet, so the result showing that peptides with relatively high molecular weight are concentrated in the 'yellow buoyant drop' (Fraction 2), supports once more the hypothesis of complexes with phospholipids or perhaps other amphipathic molecules. In our opinion, the treatment of wheat sprout extract with acetone does not cause the formation of the complexes but just assemble some of the possible complexes in the yellow buoyant gelatinous drop (Fraction 2). In conclusion, the hypothesis that complexes of phospholipids with other molecules are present in fractions from wheat sprouts extract is supported by several direct evidences. The possible hypothesis that these complexes are native and not produced during the extraction procedure is more speculative; however, it is interestingly in agreement with the progressive loss of bioavailability of some active molecules following the steps of purification. This has been observed by many authors in extracts not only from vegetables but also from animal cells. For this reason, the problem discussed

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in this article may represent one of the causes according to which many promising molecules active '*in vitro*' on important molecular mechanisms (replication, transcription, translation, etc.) resulted almost completely inactive in cellular and animal tests. Of course, the peptides activity *in vivo*' may be mediated also by specific mechanisms such as cell recognition through molecular receptors [27,28].

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