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Proteomic fingerprinting of mistletoe (*Viscum album* L.) via combinatorial peptide ligand libraries and mass spectrometry analysis

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ABSTRACT

Combinatorial peptide ligand libraries (CPLLs), coupled to mass spectrometry (MS) analysis, have been used to investigate in depth the proteome of *Viscum album* L. (VA), commonly named European mistletoe, in order to provide a first proteomic fingerprinting. For this purpose, the proteins were captured via CPLLs at two different pH values (acidic and neutral). A total of 648 non-redundant proteins were identified by using two different databases. The two pH values, chosen for bead incubations, have contributed to increment the capture ability: 56% and 31% of CPLLs species were respectively recognized at pH 7.2 and at pH 2.2. Finally the biological function of identified proteins was evaluated in order to understand their role on human health and the potential benefits of mistletoe extracts in medicine.

Significance: Viscum album L. (VA) extracts are recently used as supporting medicine for cancer therapy, improving patients' survival and increasing their quality of life in medicine. These anticancer effects are investigated and they are probably due to mistletoe's capability to favor tumor cell's death and to modulate the immune system. Although the increasing interest in VA medical benefits, the role of its components in human health remains unclear. In order to exploit this aspect, it is important to comprehensively study proteins present in *Viscum album* L. (VA) extracts. Nevertheless, since plant proteomics analysis is in most cases handicapped by the presence of high-abundance proteins masking the detection of the low-abundance ones, it is important to overcome this challenge. In this sense, combinatorial peptide ligand libraries (CPLLs) have been used to reduce the dynamic protein concentration range to enable the identification of a higher amount of proteins than employing conventional methods. In this work, a total of 648 non-redundant proteins were identified: 56% and 31% of CPLLs species were respectively recognized at pH 7.2 and at pH 2.2. This deep proteome identification was useful to investigate the biological functions of proteins in order to evaluate their potential role in human health.

1. Introduction

Mistletoe is defined as a "hemiparasite" because as a parasitic plant, it grows on the branches or trunk of a tree but it is also capable of growing on its own. The name mistletoe originally referred to the species *Viscum album* L. (VA), an European mistletoe belonging to *Santalaceae* family in the order *Santalales* native to Great Britain and much of Europe. However, a different species, *Viscum cruciatum*, occurs in Southwest Spain and Southern Portugal, as well as North Africa, Australia and Asia. Extracts of VA have been used in traditional medicine for treatment of jaundice, indigestion, common fever and asthma [1]. Recently, some biological activities of mistletoe preparations were investigated, such as anticancer function [2], probably due to induction of tumor cell death [3] and to exertion of direct necrotic effects or apoptosis [4]. For this reason, VA extracts have been tested in clinical trials [3] as supporting medicine for cancer therapy, improving patients' survival [5,6] and increasing their quality of life [7,8]. Despite the development of individualized medicine, chemotherapy and surgery remain the preferred choice especially for advanced malignancies [9]. For this reason, the possible additive antitumor activity of VA extracts with a chemotherapy drug, doxorubicin, was recently investigated, demonstrating an increase of drug antileukemic effectiveness [10]. Furthermore, recent literature has showed that VA modulates immune system and exerts immune-adjuvant activities, influencing tumor regression [11].

Although the knowledge of VA medical effects is increasing, the role of its components is still unclear. The mistletoe plant contains many kinds of metabolites like several pentacyclic triterpenes, among them oleanolic acid, betulinic acid, ursolic acid and lupeol [12,13], flavonoids,

Abbreviations: CPLLs, combinatorial peptide ligand libraries; MS, mass spectrometry; VA, *Viscum album* L.; AmBic, ammonium bicarbonate; LAPs, low abundance proteins; ML, mistletoe lectin.

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long-chain fatty acids and hydrocarbons as well as trace amounts of volatile components including loliolide and vomifoliol [14]. Due to their insolubility in water, these compounds are not present in significant amounts in aqueous mistletoe extracts [15]; however, they have exhibited anti-angiogenic activities [16–19] and pro-inflammatory and anti-apoptotic effects [20]. Moreover VA extracts, often used as adjuvant in cancer therapy, contain several hydrophilic bioactive compounds like lectins [21,22], viscotoxins, oligosaccharides and polysaccharides [23, 24].

Although mistletoes have been shown to have other pharmacological activities, including nervine, hypertensive [25], cardiac depressant, hypolipidemic action [26], vasodilator and relaxant effects, as yet no studies have explored the entire proteome of *VA*. In the present research, a deep investigation on proteins, extracted from *Viscum album* L. leaves, has been performed by the application of combinatorial peptide ligand libraries (CPLLs) technology, coupled to mass spectrometry (MS) analysis, in order to enlarge the identification of the VA proteome. The related biological functions, in order to understand the biomedical role of proteomic profiling on human health, have been also evaluated.

2. Materials and methods

2.1. Chemicals and biologicals

ProteoMiner™ (combinatorial hexapeptide ligand library beads, PM), Laemmli buffer, 40% acrylamide/bis solution, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), molecular mass standards (Precision Plus Protein Standard) and electrophoresis apparatus for one-dimensional electrophoresis were from Bio-Rad Laboratories (Hercules CA). β-Mercaptoethanol, ammonium persulphate, acetonitrile (ACN), trifluoroacetic acid (TFA), sodium dodecyl sulphate (SDS), dithiothreitol (DTT), ammonium persulphate, methanol (MeOH), ammonium bicarbonate (AmBic), formic acid (FA) and all other chemicals used all along the experimental work were pure analytical grade products and purchased from Sigma-Aldrich (St Louis, MO). Complete protease inhibitor cocktail tablets and sequencing grade trypsin were from Roche Diagnostics, (Basel, CH).

2.2. Extraction of mistletoe proteins

Mistletoe (*Viscum album* L.) growing on host tree, called *Pinus nigra*, located in El Toro (Castellòn de la Plana, Spain, UTM coordinates: X = 692600 and Y = 4422700) was collected in January 2015. The green parts (leaves) were selected and washed with water to eliminate bacterial and surface contamination from human hands. The age of mistletoe was estimated by counting the total number of nodes (or branch nodes) on the longest stem of mistletoe. In our case, a large number of node branches were found, being 10-year-old mistletoe. Then, the green parts were sliced and transferred to a mortar and frozen in liquid nitrogen. The frozen material was ground to a fine powder in a pre-cooled mortar and pestle and then this powder was homogenized. The protein extraction protocol was adapted from Olsner et *al.* [27].

Briefly, 10 g of powdered mistletoe were solubilized in 10 volumes of 10 mM Tris-HCl at pH 8.3, containing 100 mM lactose in order to avoid the possible binding of lectins present in the extract (which are the most abundant proteins) to carbohydrate components in the homogenate. The addition of lactose was considered as the best compromise considering the diversity of sugar specificity of lectins (galactose and *N*-acetyl-D-galactosamine) [27]. Next, the suspension was mildly shaken at 4 °C overnight. To study the whole proteome of mistletoe, 5 mL of this protein extract were diluted with 45 mL of a buffer containing 50 mM Tris-HCl at pH 7.2 and 50 mM NaCl.

Prior to capture with CPLLs, the extract was centrifuged at 18,000 rpm for 10 min to isolate the clarified protein solution from the insoluble residue, *ca.* 40 mL of mistletoe solution being thus recovered. Two biological replicas were performed. To determine the protein concentration, a Bio-Rad DC Protein Assay was performed in mistletoe extract. It was a colorimetric assay based on Lowry assay where proteins create complexes with copper in alkaline medium able to reduce the Folin reagent, producing a blue colour, proportional to protein concentration, with maximum absorbance at 750 nm. This extract was divided into two fractions (20 mL each containing 28.8 mg of proteins), one of which was maintained at pH 7.2, whereas the other was adjusted at pH 2.2 by adding FA and 0.1% TFA. To all mentioned aliquots, 30 μL of ProteoMiner (CPLL) bead volume were added and protein capture was carried out via gentle shaking overnight at room temperature; then the beads were collected by filtration and washed with the appropriate extraction buffer.

The captured proteins from the peptide library were desorbed twice (each time with 30 μL) by washing with a boiling solution composed of 4% SDS and 20 mM DTT for 15 min [28]. The entire eluates (60 μL each) and the 50 μL of mistletoe extract (untreated by CPLLs), were precipitated with MeOH/chloroform as follows: to one volume of protein solution, 4 volumes of cold MeOH were added, mixed and kept at 4 °C for 5 min. One volume of chloroform and 3 volumes of water were added, mixed and centrifuged at 14,000 rpm for 15 min. The supernatant was rejected and the pellet was washed twice with cold MeOH and finally centrifuged at 14,000 rpm for 5 min. The obtained pellet was dissolved in 20 μL of Laemmli buffer for SDS-PAGE (which was performed according to Candiano et al. [28]).

2.3. SDS-PAGE analysis

The three pellets obtained in the previous step (one for control and the others for eluates at pH 2.2 and 7.2), which were dissolved in 20 μL of Laemmli buffer, were loaded onto an SDS-PAGE gel. The gel was composed by a 4% polyacrylamide stacking gel (125 mM Tris–HCl, pH 6.8, 0.1%, m/v, SDS) and a 12% resolving polyacrylamide gel (in 375 mM Tris–HCl, pH 8.8, 0.1%, m/v, SDS buffer). A Tris-glycine buffer at pH 8.3 (with 0.1% SDS, m/v) was employed to fill the cathode, whereas a Tris buffer at pH 8.8 was used in the anode. Electrophoresis was set at 100 V until the dye front reached the bottom of the gel. Staining and destaining were performed with Colloidal Coomassie Blue and 7% (v/v) acetic acid in water, respectively [28]. Finally, the SDS-PAGE gels were scanned with a VersaDoc imaging system (Bio-Rad).

2.4. Mass spectrometry and data analysis

All sample bands obtained by SDS-PAGE were cut out and destained by washing with ACN and 50 mM ammonium bicarbonate (AmBic) at 56 °C [29]. Afterwards, the gel pieces were reduced and alkylated with 1.5 mg/mL DTT (in 50 mM AmBic) at 56 °C and 10 mg/mL iodoacetamide (in 50 mM AmBic) at room temperature, respectively. Finally, proteins were digested with 0.02 μg/μL trypsin (in 25 mM AmBic) at 37 °C overnight. The tryptic mixtures were acidified with FA up to a final concentration of 10%. Eight microliters of trypsin digested sample was injected on a reversed phase trap column (Acclaim PepMap100, C18, 100 Å, 5 μm, 100 μm ID × 2 cm length, Thermo Scientific) for peptide clean-up and preconcentration. After clean-up, the trap column was placed in series with a fused silica reverse-phase column (pico Frit column, C18 HALO, 90 Å, 75 μm ID, 2.7 μm, 10.5 cm length, New Objective). Separations were performed using a nano chromatographic system (UltiMate 3000 RSLCnano System, Thermo Scientific) at a constant flow rate of 300 nL/min with a gradient from 4% buffer A (2% ACN and 0.1% FA in water) to 96% buffer B (2% water and 0.1% FA in ACN) in 60 min.

The eluting peptides were on-line sprayed in a LTQ XL mass spectrometer (Thermo Scientific). Full scan mass spectra were collected in the linear ion trap in the mass range *m*/*z* 350 to *m*/*z* 1800 Da and the 5 most intense ions were selected and fragmented in the ion trap. Target ions already selected for fragmentation were dynamically excluded for 30 s.

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