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Research Article

Proteomic analysis of *Lycium barbarum* (Goji) fruit via combinatorial peptide ligand libraries

The proteome of Goji berries (*Lycium barbarum*) has been investigated in depth by using the combinatorial peptide ligand libraries (CPLL) technology. Since, in general, when plant samples are solubilized under native conditions, a limited number of identifications ensues, we have tried here solubilization under both native and denaturing conditions. In the latter case, since up to 3% SDS is used, the amount of surfactant is diluted to 0.1% or else proteins are precipitated with acetone/methanol, followed by CPLL capture. Under these conditions, a total amount of 350 proteins has been univocally identified, which represents by far the deepest proteomic investigation of Goji berries.

Keywords:

Combinatorial peptide ligand libraries / Goji berries / *Lycium barbarum* / MS

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1 Introduction

Wolfberries (*Lycium barbarum* (Goji berry) and *L. Chinese*, Solanaceae) grow in China, Tibet and other parts of Asia and have been used in the traditional medicine as ripe fruits, concentrate extracts, or infusions for their benefits to anti-aging, vision, kidney, and liver functions [1–4]. Since the beginning of the 21st century, Goji products have been introduced in Europe and North America and their consumption has increased rapidly due to their beneficial properties for well-being and longevity.

Recent studies suggest that the *Lycium barbarum* polysaccharide (LBP) extracts from wolfberries possess a range of biological activities, including effects on ageing, neuroprotection, antifatigue/endurance, increased metabolism, glucose control in diabetics, glaucoma, anti-oxidant properties, immunomodulation, antitumor activity, and cytoprotection [4]. LBPs consist of a complex mixture of highly branched and only partly characterized LBPs and proteoglycans. LBPs have been shown in several studies to promote splenocyte proliferation in vitro [1]. Although the LBPs contained in the

LBP extracts have been widely studied [5–9], little is known about the identity of the protein part of these proteoglycans, encouraging thus a deep profiling of the goji berry proteome.

On the other hand, the introduction of new foods into the diet could induce new allergic sensitizations. Goji berries have been recently introduced in Western diets, resulting in some cases of allergic reactions, including anaphylactic reactions [10, 22]. Cross-reactivity with tomato, peach, different nuts, tobacco, and *Artemis vulgaris* has been demonstrated [10, 22, 23]. The clinical profile of the individuals and the analysis by LC-MS/MS demonstrated that the most allergenic protein is lipid transfer protein (LTP) [23]. Nevertheless, most patients also recognized other bands, suggesting the presence of different allergens, such as high-molecular-mass species, that may also play a role in Goji berry allergy [22, 23]. Consequently, further studies are necessary to describe and identify the possible low-abundance allergenic proteins involved and the allergenic profile of sensitized individuals.

The wolfberry composition has been investigated in depth, producing an ample knowledge about LBPs, carotenoids, vitamins, fatty acids, and free amino acids constituents [1]. However, and despite their relevance, no study has focused on identifying the proteome of wolfberries. Presumably extraction of proteins from wolfberry might not be an easy task, due to the high amount of LBPs, representing 23–68% of the dried fruit [10, 11], and a wide variety of small metabolites such as carotenoids, vitamins, or flavonoids [1]. When using conventional extraction methods based on

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Abbreviations: CPLL, combinatorial peptide ligand libraries; GO, gene ontology; LBP, *Lycium barbarum* polysaccharide; LTP, lipid transfer protein

Colour Online: See the article online to view Figs. 2 and 3 in colour.

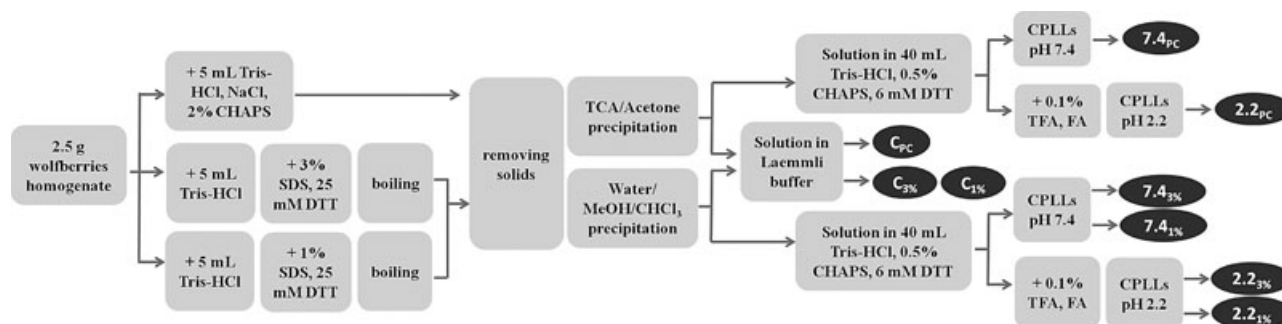


Figure 1. Simplified scheme of the designed methods for the extraction and capturing of proteins from Goji berries.

organic solvent precipitation, those metabolites can be isolated from proteins, which would have interfered in the subsequent analysis. Consequently, more advanced protein extraction methodologies are required. In this field, the use of combinatorial peptide ligand libraries (CPLLs) has demonstrated to be a very powerful tool. The efficiency of CPLLs in the capture of low to very-low abundance proteins has been widely demonstrated in the treatment of a number of samples [12–15], but it was just in the last few years when this technology has been applied to the study of vegetable samples such as maize kernels [16], spinach leaves [17], olive [18], avocado [19], and banana [20]. Generally, the increment of identified species has been between 200 to 500% as compared with the nontreated control samples.

The main target of this work is to apply a highly improved CPLL technology to delve in depth into the Goji berry proteome in order to obtain new information about the proteins contained in this fruit and new clues for the characterization of not-yet identified allergenic proteins.

2 Materials and methods

2.1 Chemicals and samples

ProteoMiner™ (combinatorial hexapeptide ligand library beads), Laemmli buffer, 40% acrylamide/Bis solution, TEMED, molecular mass standards, and electrophoresis apparatus for 1D electrophoresis were from Bio-Rad Laboratories (Hercules, CA, USA). β -Mercaptoethanol, DTT, ammonium persulphate, urea, thiourea, CHAPS, isopropanol, ACN, TFA, TCA, SDS, and all other chemical used all along the experimental work were current pure analytical grade products and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Complete protease inhibitor cocktail tablets and sequencing grade trypsin were from Roche Diagnostics (Basel, Switzerland). Dried Goji berries (imported from China) were purchased in a local shop in Milan.

2.2 Extraction of proteins and treatment with peptide libraries

The protocols are summarized in Fig. 1, as based on previously published works [18–20]. In a first extraction method,

performed by using physiological conditions, 5 g of Goji berries were ground and homogenized with 5 mL of a buffer containing 0.125 M Tris-HCl (pH 7.4) and protease inhibitor cocktail. To 2.5 g of homogenate 5 mL of a buffer containing 0.125 M Tris-HCl (pH 7.4), 50 mM NaCl, and 2% w/v CHAPS were added. Solids were removed by centrifugation at 9000 rpm during 10 min and proteins precipitated with 10% w/v TCA/acetone in order to eliminate interfering compounds. The resulting pellet was used as control by directly dissolving in Laemmli buffer or by dissolving in 5 mL of 0.125 M Tris-HCl (pH 7.4), 50 mM NaCl, and 2% w/v CHAPS for performing the subsequent CPLL capture. A second extraction method, under denaturing conditions, started with the addition of 5 mL of 0.125 M Tris-HCl (pH 7.4) to 2.5 g of goji berries homogenate. Next, SDS and DTT were added until reaching concentrations of 1 or 3% w/v and 25 mM, respectively. The mixture was boiled during 10 min and the remaining solids were removed by centrifugation. The proteins of the supernatant were isolated by precipitation with a water/MeOH/chloroform precipitation. The resulting pellet was used as control by directly dissolving in Laemmli buffer or dissolving in 10 mL of 0.125 M Tris-HCl (pH 7.4), 2% w/v CHAPS, and 25 mM DTT. Finally, 30 mL of 0.125 M Tris-HCl (pH 7.4) were added in order to decrease the concentration of CHAPS for a most effective protein capture.

Next, we used the CPLLs technology for capturing the extracted proteins and enhancing the signal of low-abundance species. A first capture was performed at pH 7.4 by adding 100 μ L of ProteoMiner, overnight at room temperature under gentle shaking. In a second capture, CPLLs were also employed for the capture mimicking reverse-phase conditions, that is, in the presence of 0.1% v/v TFA as ion-pairing reagent and at pH 2.2 by adjusting with formic acid. Beads of both captures were recovered by filtering with Micro Bio-Spin chromatographic columns (Bio-Rad) under vacuum. The latter were then washed twice with their respective buffer to eliminate the unbound protein excess. The captured proteins from each sample were desorbed (twice, with 40 μ L) with an extraction buffer containing 4% w/v SDS, 12.5% v/v glycerol, 2.5% v/v 2-mercaptoethanol, 0.005% w/v bromophenol blue, and 62.5 mM Tris-HCl (pH 6.8), for 10 min, under boiling conditions.

SDS-PAGE, nLC-MS/MS analysis were performed according to standard protocols [18, 19]. The raw data of lanes

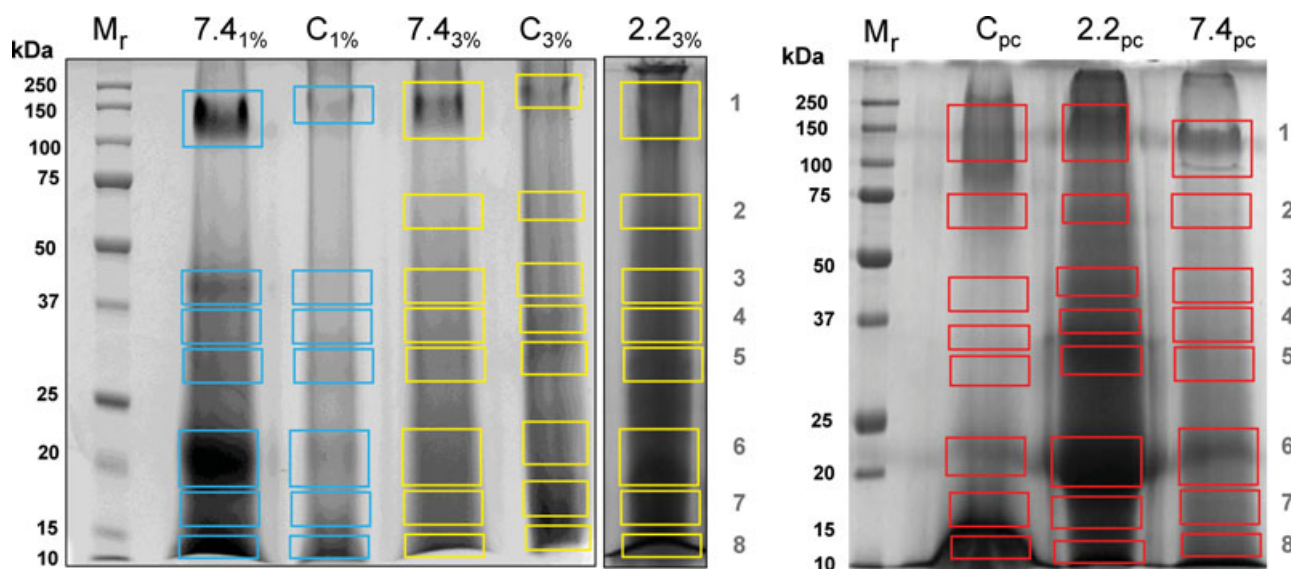


Figure 2. Coomassie-stained SDS-PAGE gels corresponding to the extracts obtained from Goji berries. Comparison of the protein extracts used as control and eluates at pH 2.2 and pH 7.4, coming from lysates containing 1% or 3% SDS. The zones marked by square brackets represent gel segments cut out for digestion and protein identification via nLC-MS/MS (for the various symbols see the black ovals of Fig. 1).

of controls and eluates were analyzed separately by MASCOT search engine (version 2.3.01), using Proteome Discover software (v. 1.2.0 Thermo), and consulting first of all contaminants database (version 13/05/2010, downloaded from <http://maxquant.org/downloaded.html>) to eliminate protein interference problems. Then the unassigned signals were searched by consulting the Uniprot_viridiplantae (30 264 sequences, 184 678 199 residues). Peptide mass tolerance was set to 1 Da, fragment mass tolerance to 0.8 Da, ion score cut off >30 and *p* value <0.01. The false discovery rate was less than 1%. The Gene Ontology (GO) analysis was performed using the web available software QuickGO (www.ebi.ac.uk/QuickGO).

3 Results

Figure 2 shows the SDS-PAGE profiling of Goji proteins of both controls, using 1 and 3% SDS as compared to the eluates from the CPLL beads, at pH 2.2 and pH 7.4. Control samples showed a modest number of very fine bands, distributed between 15 and 150 kDa. The eluates from the CPLL capture showed a spectrum of polypeptide chains, extending from 15 to 150 kDa too. Moreover, among all eluates, the pH 2.2 capture appears to be the most intense one, with a density higher than the SDS-PAGE profiling of the controls and the captures at pH 7.4. All lanes of the SDS-PAGE were segmented into eight zones, the protein therein digested with trypsin and analyzed via nLC-MS/MS as discussed in the previous section. Uniprot_viridiplantae database allowed the identification of 123 proteins (see Table 1). These results constitute the largest description so far of the Goji berries proteome. The CPLL beads permitted the identification, with at least two peptides,

of 75 proteins, of which 47 in common with controls. The total identified proteins, considering the identifications with one peptide too, were 350 and they are shown in Supporting Information Table 1. As it would be expected, the various extractions employing both native and denaturing conditions, followed by CPLLs treatment, permitted an increase of number of protein identifications. Moreover the use of different protein extraction methods (Fig. 1), using different chemical conditions (physiological and denaturing) could be a useful procedure to increase the amount of identified species. In addition Gene Ontology analysis was performed by filtering the found accession numbers against the QuickGO protein database. The resulting statistics, consisting in the percentage of accession numbers related to each GO category was plotted on a pie chart (Fig. 3). A high number of identified proteins is localized into membrane and implicated in nucleotide binding. In fact the main GO categories enriched are related to membrane, nucleotide binding, oxido-reduction process, ATP binding, and metabolic processes.

4 Discussion

An unexpected aspect of this study, quite annoying, was the fact that we could only identify a grand total of 350 proteins (see Supporting Information Table 1). This is precisely what happened with the combined data on olive pulp and stone proteomics [18]. However, since we adopted the recent scheme of double extraction and double CPLL capture, under native and denaturing conditions, we could easily identify >1000 species in both avocado [19] and banana [20]. Also in the case of artichoke, in an investigation about its proteome and the possible proteinaceous content of Cynar, a liquor

Table 1. Identified proteins in Goji lysate controls and after CPLs binding at pH 2.2 and 7.4

Accession number	Protein name	Mascot score	Mr	N peptide	E	Ctrl
tr A5AKB1 A5AKB1_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	452	30500	2	X	X
sp P80471 LIPC_SOLTU	Light-induced protein, chloroplastic OS = <i>Solanum tuberosum</i>	1140	35671	9	X	X
tr Q24141 Q24141_TOBAC	Plastid-lipid-Associated Protein (Fragment) OS = <i>Nicotiana tabacum</i>	672	29385	5	X	X
tr B7FM02 B7FM02_MEDTR	Putative uncharacterized protein OS = <i>Medicago truncatula</i>	621	62020	3	X	X
sp P36401 E13H_TOBAC	Glucan endo-1,3-beta-glucosidase, acidic isoform PR-Q~ OS = <i>Nicotiana tabacum</i>	555	37086	3	X	X
sp P17614 ATPBM_NICPL	ATP synthase subunit beta, mitochondrial OS = <i>Nicotiana plumbaginifolia</i>	531	59933	10	X	X
tr Q38HV4 Q38HV4_SOLTU	Fructose-bisphosphate aldolase OS = <i>Solanum tuberosum</i>	454	38812	6	X	X
tr Q9XG67 Q9XG67_TOBAC	Glyceraldehyde-3-phosphate dehydrogenase	453	36816	6		X
tr F2D4W3 F2D4W3_HORVD	Enolase OS = <i>Hordeum vulgare</i> var. <i>distichum</i>	380	48601	2	X	X
tr F2DYT5 F2DYT5_HORVD	Predicted protein OS = <i>Hordeum vulgare</i> var. <i>distichum</i>	367	71371	6	X	X
tr Q43778 Q43778_SOLLC	Glucan endo-1,3-beta-D-glucosidase OS = <i>Solanum lycopersicum</i>	367	37957	3		X
tr Q8L5C8 Q8L5C8_SOLTU	Malate dehydrogenase OS = <i>Solanum tuberosum</i>	364	36429	5	X	X
tr C5YZAI C5YZAI_SORBI	Putative uncharacterized protein Sb09g022580 OS = <i>Sorghum bicolor</i>	362	71357	6		X
tr E4MWE3 E4MWE3_THEHA	mRNA, clone: RTFL01-11-D08 OS = <i>Thellungiella halophila</i>	347	36888	6		X
tr D0R8X7 D0R8X7_9ROSI	Glyceraldehyde-3-phosphate dehydrogenase (Fragment) OS = <i>Manihot cecropiifolia</i>	345	14536	6	X	X
tr Q8H135 Q8H135_ARATH	ATP synthase subunit beta (Fragment) OS = <i>Arabidopsis thaliana</i>	292	48284	8	X	X
tr A9XHZ7 A9XHZ7_CHLVU	Heat shock protein 70 OS = <i>Chlorella vulgaris</i>	241	71149	3	X	X
tr B6U5H4 B6U5H4_MAIZE	Putative uncharacterized protein OS = <i>Zea mays</i>	237	8273	3	X	X
tr A9T619 A9T619_PHYPA	Predicted protein OS = <i>Physcomitrella patens</i> subsp. <i>patens</i>	205	42614	3		X
sp Q42699 METE_CATRO	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase OS = <i>Catharanthus roseus</i>	204	85089	5	X	X
tr B9VUV0 B9VUV0_PETHY	Xyloglucanase-specific endoglucanase inhibitor protein OS = <i>Petunia hybrida</i>	204	47069	2	X	
tr A5AH72 A5AH72_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	203	110823	6	X	
sp P29196 CAPP_SOLTU	Phosphoenolpyruvate carboxylase OS = <i>Solanum tuberosum</i>	200	110871	6	X	X
sp P12437 PERX_SOLTU	Suberization-associated anionic peroxidase OS = <i>Solanum tuberosum</i>	196	39220	3	X	X
tr Q5CCU4 Q5CCU4_PYRCO	Vacuolar H ⁺ -ATPase catalytic subunit OS = <i>Pyrus communis</i>	188	69014	3	X	X
tr Q40151 Q40151_SOLLC	Hsc70 OS = <i>Solanum lycopersicum</i>	187	71869	6	X	
tr Q93WZ9 Q93WZ9_PHAVU	Phosphoenolpyruvate carboxylase housekeeping isozyme pepc2 (Fragment) OS = <i>Phaseolus vulgaris</i>	183	44586	2	X	
tr P93570 P93570_SOLTU	Chaperonin-60 beta subunit OS = <i>Solanum tuberosum</i>	182	63266	5	X	X
tr Q9M5Z8 Q9M5Z8_TOBAC	Vacuolar H ⁺ -ATPase B subunit OS = <i>Nicotiana tabacum</i>	169	53975	5	X	X
tr Q5M9V4 Q5M9V4_TOBAC	ATP synthase subunit alpha OS = <i>Nicotiana tabacum</i>	165	55574	6	X	X
tr O81536 O81536_SOLLC	Annexin p34 OS = <i>Solanum lycopersicum</i>	161	35894	6	X	X
tr A8QID6 A8QID6_CAPAN	Catalase OS = <i>Capsicum annuum</i>	158	56960	3	X	X
tr D6QY28 D6QY28_SOLME	Chloroplast polyphenol oxidase OS = <i>Solanum melongena</i>	149	68227	3	X	X
tr Q8HFC9 Q8HFC9_9POAL	ATP synthase subunit alpha (Fragment) OS = <i>Ecdeiocolea monostachya</i>	143	44698	2	X	X
tr Q9XQP4 Q9XQP4_TOBAC	NAD-malate dehydrogenase OS = <i>Nicotiana tabacum</i>	140	43679	4		X
tr A5AXH5 A5AXH5_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	140	93154	2		X
tr Q2XTE7 Q2XTE7_SOLTU	Annexin p34-like protein-like OS = <i>Solanum tuberosum</i>	136	36023	5	X	X
tr Q9LDS8 Q9LDS8_SOLPN	Beta fructosidase OS = <i>Solanum pennellii</i>	136	67385	3	X	X
tr A9P828 A9P828_POPTR	Phosphoglycerate kinase OS = <i>Populus trichocarpa</i>	136	42630	2		X
tr C0K2V3 C0K2V3_RIBNI	GDP-D-mannose-3~,5~-epimerase OS = <i>Ribes nigrum</i>	135	42940	4	X	X
sp Q1S053 H2A3_MEDTR	Probable histone H2A.3 OS = <i>Medicago truncatula</i>	135	16132	2	X	
tr D8SQC5 D8SQC5_SELML	Putative uncharacterized protein OS = <i>Selaginella moellendorffii</i>	131	53696	4		X
sp Q03684 BIP4_TOBAC	Luminal-binding protein 4 OS = <i>Nicotiana tabacum</i>	129	73705	4		X
tr E0ZQA2 E0ZQA2_VIGMU	Annexin 1 (Fragment) OS = <i>Vigna mungo</i>	127	35882	2	X	X
tr F2ZAM1 F2ZAM1_TOBAC	Non-specific lipid-transfer protein OS = <i>Nicotiana tabacum</i>	126	12263	2	X	X
tr E0YCS8 E0YCS8_SOLLC	Methylketone synthase IIa OS = <i>Solanum lycopersicum</i>	125	23515	2	X	X
tr C5YC13 C5YC13_SORBI	Putative uncharacterized protein Sb06g022410 OS = <i>Sorghum bicolor</i>	124	58873	2	X	X
tr Q4LDR0 Q4LDR0_SOLLC	Heat shock protein OS = <i>Solanum lycopersicum</i>	124	110564	3		X
tr Q9M619 Q9M619_COFAR	Methionine synthase (Fragment) OS = <i>Coffea arabica</i>	124	24659	2	X	
tr E2JEI1 E2JEI1_SOLTU	Invertase OS = <i>Solanum tuberosum</i>	117	67422	3	X	X
tr B9IAB4 B9IAB4_POPTR	Predicted protein OS = <i>Populus trichocarpa</i>	113	64759	4		X
sp P48495 TPIS_PETHY	Triosephosphate isomerase, cytosolic OS = <i>Petunia hybrida</i>	113	27343	4		X

Table 1. Continued

Accession number	Protein name	Mascot score	Mr	N peptide	E	Ctrl
tr Q0ZPA3 Q0ZPA3_SOLLC	Plastid lipid associated protein CHRC OS = <i>Solanum lycopersicum</i>	113	35685	3	X	
tr B7FL18 B7FL18_MEDTR	Putative uncharacterized protein OS = <i>Medicago truncatula</i>	112	22646	3	X	
tr Q8W283 Q8W283_9GENT	Phosphoenolpyruvate carboxylase (Fragment) OS = <i>Gaertnera cuneifolia</i>	111	9286	3	X	X
sp Q9FV65 CAPPC_FLATR	Phosphoenolpyruvate carboxylase 2 OS = <i>Flaveria trinervia</i>	111	111260	4	X	X
tr D0V3Y6 D0V3Y6_SOLTU	Calmodulin isoform 1 OS = <i>Solanum tuberosum</i>	109	16960	2	X	
tr C5YC13 C5YC13_SORBI	Putative uncharacterized protein Sb06g022410 OS = <i>Sorghum bicolor</i>	108	58873	2	X	
tr Q8L4N2 Q8L4N2_SOLLC	Cell-wall invertase OS = <i>Solanum lycopersicum</i>	107	66435	2	X	
sp P21568 CYPH_SOLLC	Peptidyl-prolyl cis-trans isomerase OS = <i>Solanum lycopersicum</i>	107	18184	2	X	
tr B2CZJ9 B2CZJ9_CAPAN	PR5-like protein OS = <i>Capsicum annuum</i>	107	28049	3	X	
tr A0ZSE5 A0ZSE5_ZOSMR	Vacuolar H ⁺ -ATPase subunit B OS = <i>Zostera marina</i>	106	54451	2		X
tr COLL39 COLL39_9CHLO	Translation elongation factor-1 alpha (Fragment) OS = <i>Blastophrys rhizopus</i>	105	28875	2		X
tr D8L9J1 D8L9J1_WHEAT	Retinal dehydrogenase, putative, expressed OS = <i>Triticum aestivum</i>	104	54325	2	X	X
tr B2ZBG8 B2ZBG8_MALDO	Heat shock protein 17.5 OS = <i>Malus domestica</i>	102	17544	2		X
tr Q45TN2 Q45TN2_PAPSO	Phytoene desaturase (Fragment) OS = <i>Papaver somniferum</i>	102	33908	4		X
tr Q8L5D5 Q8L5D5_PSEMZ	Small heat-shock protein OS = <i>Pseudotsuga menziesii</i>	101	18160	2		X
tr B5M6X8 B5M6X8_TOBAC	Calreticulin OS = <i>Nicotiana tabacum</i>	100	47641	2	X	
tr C4PG28 C4PG28_9SOLN	Polygalacturonase inhibiting protein OS = <i>Solanum torvum</i>	100	37361	2	X	
tr Q3SC86 Q3SC86_SOLLC	Xyloglucan-specific fungal endoglucanase inhibitor protein OS = <i>Solanum lycopersicum</i>	100	47160	2	X	
tr D8QX04 D8QX04_SELML	Putative uncharacterized protein OS = <i>Selaginella moellendorffii</i>	99	17934	2	X	X
tr Q0WS75 Q0WS75_ARATH	Histone H2B OS = <i>Arabidopsis thaliana</i>	97	15651	2	X	X
tr B6CIL7 B6CIL7_9LAMI	Cytosolic glyceraldehyde-3-phosphate dehydrogenase (Fragment) OS = <i>Conradina sp. Edwards 133</i>	96	10150	5	X	X
tr B0M1A9 B0M1A9_SOYBN	Peroxisomal 3-ketoacyl-CoA thiolase OS = <i>Glycine max</i>	95	49213	2		X
tr Q5QHT2 Q5QHT2_VIGRA	70 kDa heat shock cognate protein 3 OS = <i>Vigna radiata</i>	94	71870	4	X	X
tr Q09224 Q09224_9SOLA	Water channel protein OS = <i>Nicotiana excelsior</i>	94	31037	2		X
sp Q9ZWQ8 PAP_CITUN	Plastid-lipid-associated protein, chloroplastic OS = <i>Citrus unshiu</i>	93	35252	3		X
tr Q24379 Q24379_SOLTU	Lipoxygenase OS = <i>Solanum tuberosum</i>	92	97178	2	X	
tr C0PHC0 C0PHC0_MAIZE	Putative uncharacterized protein OS = <i>Zea mays</i>	92	68689	2		X
sp Q9M7P6 NDK_CAPAN	Nucleoside diphosphate kinase OS = <i>Capsicum annuum</i>	91	16373	3		X
tr B5LAT1 B5LAT1_CAPAN	Putative ketol-acid reductoisomerase OS = <i>Capsicum annuum</i>	88	63652	2	X	X
tr F2D735 F2D735_HORVD	Predicted protein OS = <i>Hordeum vulgare var. distichum</i>	88	18248	2		X
tr A5B8T3 A5B8T3_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	88	35405	2		X
tr Q41428 Q41428_SOLTU	Polyphenol oxidase OS = <i>Solanum tuberosum</i>	86	68110	4	X	X
tr B1NYI4 B1NYI4_NICBE	Phytoene desaturase OS = <i>Nicotiana benthamiana</i>	85	65942	2	X	
tr AIBY89 AIBY89_9SOLA	Pectinesterase (Fragment) OS = <i>Nicotiana attenuata</i>	84	20266	2	X	X
tr A2X2W9 A2X2W9_ORYSI	Putative uncharacterized protein OS = <i>Oryza sativa subsp. indica</i>	84	42594	3		X
tr F6HEZ8 F6HEZ8_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	84	41488	3		X
tr C5Y9Z1 C5Y9Z1_SORBI	Putative uncharacterized protein Sb06g031910 OS = <i>Sorghum bicolor</i>	82	64709	2	X	X
tr A5B109 A5B109_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	82	42157	2	X	
tr A5AS18 A5AS18_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	81	21726	3		X
tr A5BS35 A5BS35_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	80	25425	2		X
tr F4YF87 F4YF87_CAMSI	Histone H4 OS = <i>Camellia sinensis</i>	78	14989	2	X	X
tr A9NS28 A9NS28_PICSI	Putative uncharacterized protein OS = <i>Picea sitchensis</i>	78	18210	2		X
tr D3GGK1 D3GGK1_9FABA	Putative plasma membrane intrinsic protein PIP family member 1 aquaporin (Fragment) OS = <i>Schizolobium parahyba</i>	77	16483	3		X
sp Q0JNL7 CALM3_ORYSJ	Calmodulin-3 OS = <i>Oryza sativa subsp. japonica</i>	77	16878	2	X	
tr D2CNC8 D2CNC8_TOBAC	Subtilase (Fragment) OS = <i>Nicotiana tabacum</i>	76	57309	2		X
tr D2CNC6 D2CNC6_TOBAC	Subtilase OS = <i>Nicotiana tabacum</i>	76	83248	2		X
tr Q6L460 Q6L460_SOLDE	ATP synthase D chain, mitochondrial, putative OS = <i>Solanum demissum</i>	76	19795	2	X	
tr COPQJ1 COPQJ1_PICSI	Elongation factor 1-alpha OS = <i>Picea sitchensis</i>	76	49639	2	X	
tr E3NYT1 E3NYT1_9FABA	Harpin binding protein (Fragment) OS = <i>Arachis diogeni</i>	74	18032	2	X	
tr B4G039 B4G039_MAIZE	Putative uncharacterized protein OS = <i>Zea mays</i>	73	41741	2		X
tr Q6H3X6 Q6H3X6_SOLLC	Protease inhibitor II OS = <i>Solanum lycopersicum</i>	73	17194	2	X	
tr COPHD8 COPHD8_MAIZE	Putative uncharacterized protein OS = <i>Zea mays</i>	72	55138	2		X
sp Q8H8T0 RGP1_ORYSJ	UDP-arabinopyranose mutase 1 OS = <i>Oryza sativa subsp. japonica</i>	72	41835	2		X

Table 1. Continued

Accession number	Protein name	Mascot score	Mr	N peptide	E	Ctrl
tr C0P2V1 C0P2V1_MAIZE	Putative uncharacterized protein OS = <i>Zea mays</i>	70	28634	2		X
tr A2YUJ2 A2YUJ2_ORYSI	Putative uncharacterized protein OS = <i>Oryza sativa subsp. indica</i>	68	110728	5		X
tr D2DEK8 D2DEK8_LYCBA	Betaine aldehyde dehydrogenase OS = <i>Lycium barbarum</i>	67	55654	2		X
tr Q0J5H3 Q0J5H3_ORYSJ	Malate dehydrogenase (Fragment) OS = <i>Oryza sativa subsp. japonica</i>	67	37641	2		X
tr Q41028 Q41028_PEA	Pisum sativum 17.9 kDa heat shock protein (hsp17.9) (Fragment)	67	17633	2		X
tr D8QN83 D8QN83_SELML	Putative uncharacterized protein OS = <i>Selaginella moellendorffii</i>	67	55277	2		X
tr B9S392 B9S392_RICCO	Heat-shock protein, putative OS = <i>Ricinus communis</i>	65	17505	2		X
tr Q1W388 Q1W388_STRAF	Quinone-oxidoreductase QR2 OS = <i>Striga asiatica</i>	65	21848	2	X	
tr A5BUA5 A5BUA5_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	64	13350	2	X	X
tr A6YS00 A6YS00_SOLLC	Zeta-carotene desaturase OS = <i>Solanum lycopersicum</i>	63	65042	2	X	
tr Q8VXH8 Q8VXH8_KALFE	Phosphoenolpyruvate carboxylase, isoform 1 (Fragment) OS = <i>Kalanchoe fedtschenkoi</i>	62	41119	2		X
tr A9YYG9 A9YYG9_CAPAN	Small heat shock protein class I (Fragment) OS = <i>Capsicum annuum</i>	60	9291	2	X	X
tr F6HNM7 F6HNM7_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	60	18008	2	X	
tr B9HT33 B9HT33_POPTR	Predicted protein OS = <i>Populus trichocarpa</i>	59	18552	2		X
tr B8QHE4 B8QHE4_9MONI	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (Fragment) OS = <i>Dennstaedtia dissecta</i>	52	10429	2		X
tr B2YKT9 B2YKT9_TOBAC	Glycine-rich RNA-binding protein OS = <i>Nicotiana tabacum</i>	52	15673	2	X	
tr D8RF84 D8RF84_SELML	Putative uncharacterized protein OS = <i>Selaginella moellendorffii</i>	43	73055	2		X

made from an artichoke infusion, we could identify close to 1000 proteins [21]. So, the present data in a way are quite disappointing to us, considering also that we made two different extractions procedures under denaturing conditions. What has happened? We can only offer two possible explanations. To start with, all the fruits we have analyzed so far [18–20] were fresh fruits, harvested just a few days before consumption. On the contrary, Goji berries, imported from China, can only be found in Europe as dried berries, which can be obtained only in specialized shops selling herbal medications (herbalist's stores). We do not know which kinds of industrial processing the berries have been subjected to, such as drying or thermal treatments and the like, or how long the berries have been stored for and under which conditions. In one scenario, proteases activated during the treatment might have destroyed a substantial population of cytoplasmic proteins, especially those of low-abundance, which would thus be subtracted to analysis. In another scenario, many proteins might have been irreversibly cross-linked, to the point of resisting solubilization even under denaturing conditions. It is a fact that, when analyzing the GO pie chart of Fig. 3, one can notice that the first two high-abundance categories are membrane proteins and integral to membrane proteins, whereas, normally, the categories most represented are cytoplasmic species, as observed for olive [18], avocado [19], and banana [20]. Notwithstanding these shortcomings with dried fruits, the exploration of the Goji berries proteome has given substantial results, which hopefully might be expanded upon analysis of fresh berries, as might be available in the future. Additionally, the present data have shown the utility of the CPLL methodology in in-depth exploration of proteomes, as amply reported in previous work [18–20].

In general, when trying to identify proteins in plant systems and in particular in fruits, the main concern of investigators is to assess the presence of allergens. Thus, even if the proteomic asset of edible fruits is not known, certainly their allergens (if any) are well catalogued. In recent investigations on fresh fruits, such as avocado and banana, while we were able to explore their proteomes to a very deep extent (>1000 unique gene products listed and identified for the first time) we could also recognize a number of allergens, most of them well known, some new. In particular, in avocado: *Pers a 1* (class I endochitinase), *Pers a 4* (profilin), a 1,3- β -glucanase, a thaumatin-like protein, and a polygalacturonase were identified [19]. In the banana fruit, we could assess the presence of the following allergens: *Mus a 1* (profilin), *Mus a 2* (class I chitinase), *Mus a 4* (thaumatin-like protein), and *Mus a 5* (β -1,3-glucanase), a pectinesterase and a superoxide dismutase [20]. Also in the olive fruit pulp we could identify at least two allergens: a thaumatin like protein (*Ole e 13*) and a Cu/Zn superoxide dismutase, described in the olive pollen as the allergen *Ole e 5* [18].

In regard to the Goji berries, some recent reports have been focused on the study of allergens [10, 22, 23], describing as the most prevalent allergen a 7–9 kDa protein, tentatively identified as LTP, but also a 50 kDa band [22]. Effectively, and as it is shown in the Supporting Information Table 1, a nonspecific LTP was identified among the most abundant proteins. LTP is one of the most ubiquitous allergens in the plant kingdom. Plant-food allergic patients, especially in southern Europe, are frequently sensitized to this nonspecific LTP [24], which has been related with cases of cross-reactivity with other species, members of the Solanaceae family, such as tomato or tobacco, but also corresponding to different taxonomical groups, like peach [22, 23]. In addition, it has been

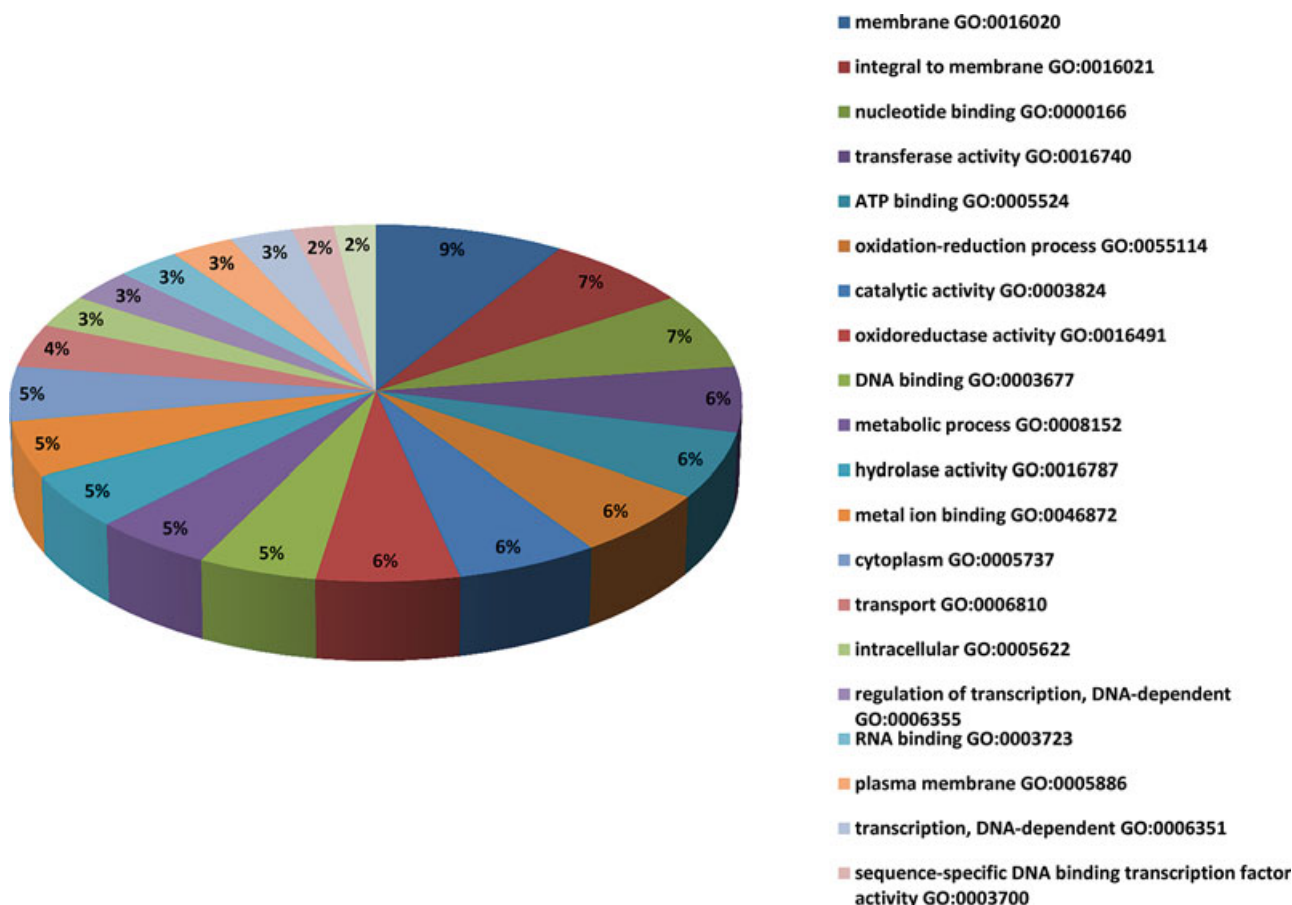


Figure 3. Gene Ontology (GO) analysis. The main GO categories enriched are related to membrane, oxido-reduction process, ATP binding, nucleotide binding, and metabolic processes.

possible to identify some other proteins suspected to be allergenic in goji berries or with confirmed allergenic activity in other fruits. We have observed the presence of a 11s globulin, also called legumin, and a peroxidase both described as tomato allergens [25, 26], and a pectinesterase, already identified by us in banana [19].

This report is part of a series of studies investigating in depth the proteome of “recalcitrant” plant tissues. Even if the number of identified species was not as high as in other fruits, probably because of the dry state of the berries, this is the first time that such a high number of proteins are identified in this just introduced food into the Western diets. This list of proteins will enable further studies on characterizing the proteoglycans of Goji berry extracts. Once again, this study offers new clues about the presence of potential allergenic proteins that have never been identified because of their low abundance. This is a new step on developing reliable detection methods for Goji berry allergens in order to ensure consumer protection and awareness.

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