



The peel and pulp of mango fruit: A proteomic samba

Elisa Fasoli, Pier Giorgio Righetti*

Department of Chemistry, Materials and Chemical Engineering “Giulio Natta”, Politecnico di Milano, 20131 Milan, Italy

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ABSTRACT

Combinatorial peptide ligand libraries (CPLs) have been adopted for investigating the proteomes of mango peel and pulp as well their peptidome content (the latter as captured with a C₁₈ resin). The aim of this study was not only to perform the deepest investigation so far of the mango proteome, but also to assess the potential presence of allergens and of peptides endowed with biological activities. The proteins of peel and pulp have been captured under both native and denaturing extraction techniques. A total of 334 unique protein species have been identified in the peel vs. 2855 in the pulp, via capture with CPLs at different pH values (2.2 and 7.2).

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1. Introduction

The mango is a fleshy stone fruit belonging to the genus *Mangifera*, consisting of numerous tropical fruiting trees in the flowering plant family Anacardiaceae. Mango is native to South Asia, wherefrom it has spread worldwide to become one of the most cultivated fruits in the tropics. While other *Mangifera* species (e.g. horse mango, *Mangifera foetida*) are also grown on a more localized basis, *Mangifera indica* – the ‘common mango’ or ‘Indian mango’ – is the only mango tree commonly cultivated in many tropical and subtropical regions. It is the national fruit of India, Pakistan and the Philippines, and the national tree of Bangladesh. The ripe fruit varies in size and color. Cultivars are typically yellow, orange, red or green, and carry a single flat, oblong pit that can be fibrous or hairy on the surface, and which does not separate easily from the pulp. Ripe, unpeeled mangoes give off a distinctive resinous, sweet smell. Inside the pit is a thin (1–2 mm thick) lining covering a single seed, 4–7 cm long. The seed contains the plant embryo [1].

Mango is used to make juices, smoothies, ice cream, fruit bars, *raspados*, *aguas frescas*, pies and sweet chili sauce, or mixed with *chamoy*, a sweet and spicy chili paste. It is popular on a stick dipped in hot chili powder and salt or as a main ingredient in fresh fruit combinations. In Central America, mango is either eaten green mixed with salt, vinegar, black pepper and hot sauce, or ripe in various forms.

When reading the literature on this tropical fruit, one has the impression that mango is almost a thaumaturgic foodstuff. In mango fruit pulp, the antioxidant vitamins A and C, vitamin B₆ (pyridoxine), folate, other B

vitamins and essential nutrients, such as potassium, copper and amino acids, are present. Mango peel and pulp contain other compounds, such as pigment carotenoids and polyphenols, and omega-3 and -6 polyunsaturated fatty acids [2,3]. Additionally, mango peel pigments seem to have important biological effects [4], including carotenoids, such as the provitamin A compound, beta-carotene, lutein and alpha-carotene [5], polyphenols [6,7] such as quercetin, kaempferol, gallic acid, caffeic acid, catechins, tannins, and the unique mango xanthonoid, mangiferin [8], any of which may counteract free radicals in various disease processes as described in some reports [9,10]. Phytochemical and nutrient content appears to vary across mango species [11]. Up to 25 different carotenoids have been isolated from mango pulp, the most abundant of which was beta-carotene, which accounts for the yellow–orange pigmentation of most mango species [12]. Peel and leaves also have significant polyphenol content, including xanthonoids, mangiferin and gallic acid [13]. The mango triterpene, lupeol [14], is an effective inhibitor in laboratory models of prostate and skin cancers [15,16]. An extract of mango branch bark called Vimang, isolated by Cuban scientists, contains numerous polyphenols with antioxidant properties in vitro [17] and on blood parameters of elderly humans [18].

Despite the large consumption of mangoes, especially in India, hypersensitivity reactions are distinctly rare. Allergy to mango can manifest in two forms: the immediate hypersensitivity reaction, presenting as anaphylaxis, angioedema, erythema, urticaria, wheezing dyspnoea, and the late reaction, presenting as contact dermatitis and periorbital edema [11]. Mango allergy, like that of poison ivy, is a version called also delayed hypersensitivity because the symptoms usually appear 48 or 72 h after exposure to the offending substance that triggers the response [19]. The fruit mango can cause immediate or delayed hypersensitivity reactions and allergic reactions without

Abbreviations: CPLs, combinatorial peptide ligand libraries; PM, ProteoMiner

* Corresponding author. Fax: +39 02 23993080.

E-mail address: piorgio.righetti@polimi.it (P.G. Righetti).

prior exposure, owing to cross reactivity and it may also manifest as “oral allergy syndrome”. It has been shown that pollens from trees (especially birch), grasses and weeds contain proteins of similar structure to those present in certain fruits, vegetables, nuts and spices. These proteins are recognized by the immune system and the ingestion of a foodstuff, which shares the same protein as the pollen, can result in triggering an allergic reaction in a susceptible individual. Mango allergens have been shown to cross react with *Artemisia* pollen, birch pollen, poison ivy, poison oak, mugwort, celery, carrot, pistachio nut, tomato, papaya and banana [20].

Notwithstanding all data reported above on mango metabolites, not much is known on this fruit proteome. Only recently a two-dimensional (2D) mango pulp analysis has been reported, aiming at identifying modulation of protein expression associated with ripening [21]. A total of 373 spots could be visualized in the 2D map, leading to the identification of 51 unique gene products. In another report, via database searches of mango-derived ESTs and proteins along with proteins from six other closely related plant species, Renuse et al. [22] could identify 1001 peptides that matched to 538 proteins. However, this set of proteins applied to mango leaves, not to pulp nor peel. Given these scanty proteomics data, we have decided to perform an in depth exploration of both peel and pulp proteomes of mango via the well-ingrained combinatorial peptide ligand library (CPLL) methodology, a well-established tool for searching low- to very-low abundance proteins in any proteome, as amply illustrated in a number of reviews [23–27]. This project is aimed not only at offering an ample coverage of these proteomes, but also at detecting unreported allergens (if any) and to see if any of these species might have important nutraceutical effects. Additionally, in these two same biological compartments, we have performed a capture of peptides present in the intact fruit, via a C₁₈ resin, in future search of potential bioactive compounds.

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 and electrophoresis apparatus for one-dimensional electrophoresis were from Bio-Rad Laboratories, Hercules CA. β-Mercaptoethanol, dithiothreitol (DTT), ammonium persulfate, 3-[3-cholamidopropyl dimethylammonio]-1-propanosulfonate (CHAPS), acetonitrile (ACN), trifluoroacetic acid (TFA), sodium dodecyl sulfate (SDS), iodoacetamide (IAA), formic acid (FA) and all other chemicals used all along the experimental work were current pure analytical grade products and purchased from Sigma-Aldrich, St Louis, MO. C₁₈ resins were purchased from Pierce Chemical Company, Rockford, IL, USA. Complete protease inhibitor cocktail tablets and sequencing grade trypsin were from Roche Diagnostics (Basel, CH).

2.2. Plant materials and protein extraction protocols

Three mango fruits were bought in local supermarkets. After washing the surface in lukewarm 1% SDS solution, so as to eliminate bacterial and surface contamination from human hands, the peel was carefully excised and homogenized for 15 min in a fruit blender. For exploring the proteomes of both, peels and pulp, two extraction protocols were devised after full homogenizations of these two tissues. The native extraction buffer contained 50 mM Tris-HCl (pH 7.2), 50 mM NaCl and 2% (m/v) CHAPS, whereas the denaturing buffer contained additionally 1% (m/v) SDS and 25 mM DTT dissolved into the same buffer. Protease inhibitor cocktails were added to both extraction buffers in order to prevent protein degradation by protease action. In detail 5 g of minced peel was mixed with 10 mL of each extraction buffer and

they were gently shaken for 3 h at room temperature for native buffer and at boiling condition for denaturing one. As regards the pulp, 100 g of pulp was solubilized in 20 mL of each extraction buffer and treated in the same conditions reported for peel samples. Prior to capture with CPLLs, the homogenates were centrifuged at 18,000 rpm for 10 min to separate the insoluble debris from the clarified protein solution: the recovered volumes were 8 mL of each extraction for the peel and 18 mL for the pulp. While the native samples were immediately incubated with CPLLs, the denatured ones were diluted 1:10 (v/v) with a buffer containing 50 mM Tris-HCl (pH 7.2), 50 mM NaCl, 25 mM DTT and protease inhibitor cocktail in order to reduce the original 1% SDS amount to 0.1% (m/v) so as to allow an effective protein capture. Two technical replicas on the three different mango fruits were performed.

2.3. ProteoMiner and C₁₈ resins treatment

Each sample (both peel and pulp extracts) was titrated at pH 7.2 [28] and, subsequently, individually added with 100 µL of ProteoMiner beads overnight at room temperature under gentle shaking. The last CPLL incubation was performed by reducing the pH at a value of 2.2 with the addition of 0.1% TFA and formic acid and was always done overnight at room temperature under gentle shaking, in order to mimic reverse phase conditions for the capture of hydrophobic proteins. Additionally, for both peel and pulp, a third treatment with C₁₈ beads was performed, to harvest any peptide potentially present in these two tissues. After centrifuging at 4000 rpm for 5 min, the beads were washed with their respective buffers and the captured proteins were then desorbed (twice, with 100 µL each time) with a solution composed of 4% SDS and 20 mM DTT for 5 min, under boiling conditions [29]. Prior to SDS electrophoresis, the eluted proteins were alkylated with 50 mM iodoacetamide. The C₁₈ beads, conversely, were treated with pure acetonitrile and the eluted peptides, after solvent evaporation, sent directly to MS analysis. SDS-polyacrylamide gel electrophoresis and two-dimensional map analyses were performed as previously described [30].

2.4. Mass spectrometry and data analysis

Ten microliter of tryptic 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 pre-concentration. After clean-up, the trap column was placed in series with a fused silica reverse-phase column (picoFrit column, C18 HALO, 90 Å, 75 µm ID, 2.7 µm, 10.5 cm length, New Objective). A nano chromatographic system (UltiMate 3000 RSLCnano System, Thermo Scientific) delivered a constant flow rate of 300 µL/min. The separating gradient ramped linearly from 1% acetonitrile to 40% acetonitrile in 30 min. The eluting peptides were on-line sprayed in a LTQ XL mass spectrometer (Thermo Scientific). Full scan mass spectra were acquired in the Orbitrap cell in the mass range of 350 to 1800 *m/z*. The nine most intense ions (minimum charge state 2+) were automatically selected and fragmented in the ion trap by collision-induced dissociation (CID). Target ions already selected for fragmentation were dynamically excluded for 60 s. The MS data were analyzed by Mascot search engine (Version 2.3.01), using Proteome Discoverer software (v. 1.2.0 Thermo) and consulting Uniprot_viridiplantae database. We set cysteine carbamidomethylation and oxidation of methionine residues as variable modifications; peptide mass tolerance was set to 1 Da, fragment mass tolerance to 0.8 Da and ion source cut-off of 20. The false discovery rate obtained consulting the decoy database was less than 0.05.

3. Results

Fig. 1 shows the SDS-PAGE profiles, in the case of mango peel, of the control vs. the eluates from various captures with CPLLs. PD (denatured peel) and PN (native peel) represent, respectively,

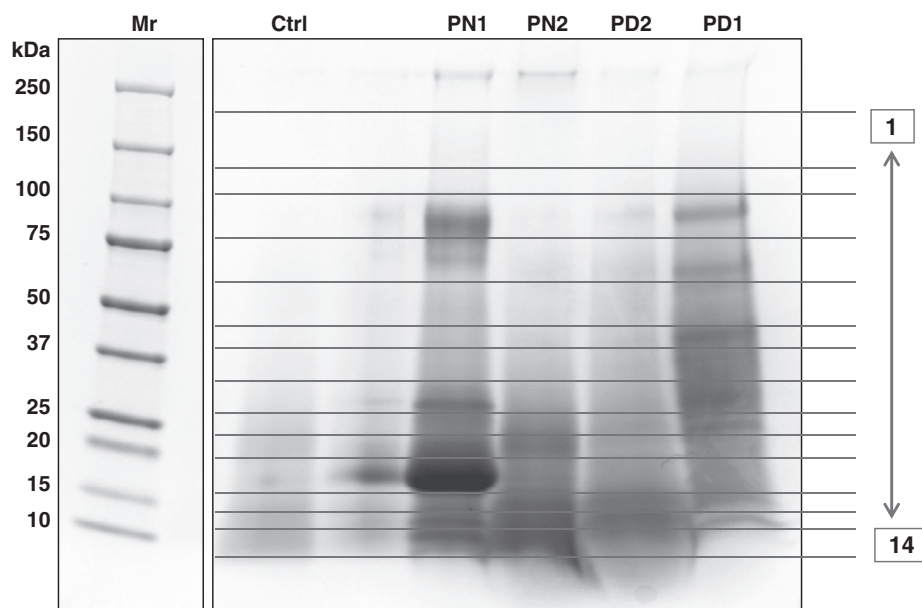


Fig. 1. SDS-PAGE of a mango peel extract. Lanes: Ctrl = 40 μ L of mix mango peel extracts (native buffer: 50 mM Tris–HCl pH = 7.2, 50 mM NaCl, 2% CHAPS and denaturing buffer: 50 mM Tris–HCl pH = 7.2, 50 mM NaCl, 2% CHAPS, 1% SDS, 25 mM DTT); PN1 = 25 μ L of first Laemmli buffer eluate of native mango peel extract treated with ProteoMiner library at pH = 7.2; PN2 = 25 μ L of first Laemmli buffer eluate of native mango peel extract treated at pH = 2.2 (0.1% TFA); PD2 = 25 μ L of first Laemmli buffer eluate of denatured mango peel extract treated with CPLs at pH = 2.2 (0.1% TFA); PD1 = 25 μ L of first Laemmli buffer eluate of denatured mango peel extract treated with ProteoMiner library at pH = 7.2. Colloidal Coomassie Blue staining.

the patterns of the control samples extracted either under denaturing or under native conditions. It can be appreciated that few bands can be seen in Ctrl track, the untreated sample named control. Conversely, the various eluates after CPL capture (from left to right, from PN1 to PD1; for identifications see the proper legend) exhibit a series of zones, variously populated, extending from ca. 10 kDa up to 100 kDa (with an extra band at ca. 250 kDa, which might represent aggregate material). Fig. 2 shows

the corresponding SDS-PAGE profiles of the mango flesh extracts. Here too the first two lanes on the left side (after the Mr ladder), labelled as FD (denatured flesh) and FN (native flesh) represent, respectively, the patterns of the control pulp samples extracted either under denaturing or under native conditions. Contrary to what occurs to the peel control lanes in Fig. 1, these three extracted samples show a number of well-distributed bands ranging from ca. 10 to 60 kDa. The remaining lanes to

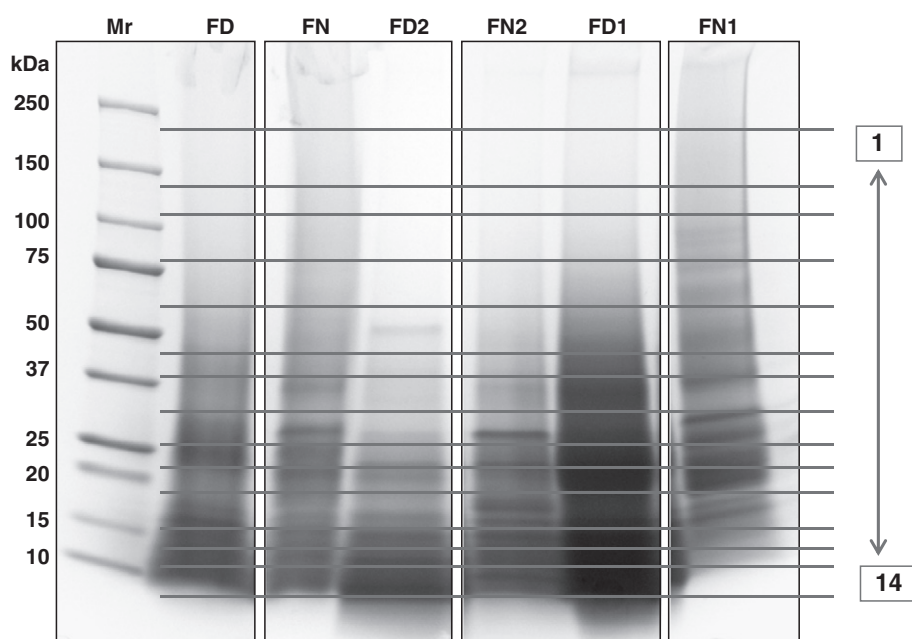


Fig. 2. SDS-PAGE of a mango pulp extract. Lanes: FD = 40 μ L of mango flesh extract (denaturing buffer: 50 mM Tris–HCl pH = 7.2, 50 mM NaCl, 2% CHAPS, 1% SDS, 25 mM DTT); FN = 40 μ L of mango flesh extract (native buffer: 50 mM Tris–HCl pH = 7.2, 50 mM NaCl, 2% CHAPS); FD2 = 25 μ L of first Laemmli buffer eluate of denatured mango flesh extract treated at pH = 2.2 (0.1% TFA); FN2 = 25 μ L of first Laemmli buffer eluate of native mango flesh extract treated at pH = 2.2 (0.1% TFA); FD1 = 25 μ L of first Laemmli buffer eluate of denatured mango flesh extract treated with ProteoMiner library at pH = 7.2; FN1 = 25 μ L of first Laemmli buffer eluate of native mango flesh extract treated with ProteoMiner library at pH = 7.2. Colloidal Coomassie Blue staining.

their right (from FD2 to FN1; for identifications see the proper legend) pertain to the various eluates after CPLL capture. It can be appreciated that the banding patterns are quite variegated and present band in common and bands typical of various extraction/CPLL capture conditions.

We have also performed 2D map analyses of controls and pooled CPLL eluates of both peel and pulp, as shown in Fig. 3. The upper two panels refer to the peel, the bottom two to the pulp. On the left sides are the two control samples (MPC and MFC, respectively, for peel and flesh) and on the right hand the combined CPLL eluates. In the case of peel, one can appreciate the paucity of spots, although in the CPLL eluates a number of additional spots, in the low-Mr region, extending up to the alkaline pI values, are visible. Conversely, in the case of mango flesh, whereas the control is rather scarce in spots, the combined eluates are much richer, here too particularly in the low-Mr region and extending well into the most alkaline pI range. We attribute this strong difference between the peel and pulp (see also the data presented below) to the fact that, whereas the pulp can be easily homogenized in blenders, the peel is quite tough and cannot be properly dispersed even upon prolonged homogenization (up to 15 min of vigorous treatment).

Referring back to Figs. 1 and 2, the horizontal lines in the various sample lanes represent excised gel segments (a total of 14 in each case), whose protein content, after trypsin digestion, is sent to MS analysis in an LTQ-XL equipment. The streaking and hazy patterns in the various lanes are due to the fact that, in order to avoid losses of low-abundance proteinaceous material, we do not purify the various peel extracts for the removal of interfering plant polymers. Even though these patterns are not so aesthetically pleasing, the results in global proteome identifications have been quite robust (see below). The Venn diagrams of the number of proteins identified in peel and pulp extracts are shown in Fig. 4A and B, respectively. In Fig. 4A the upper diagram shows the contributions of the total CPLL (ProteMiner, PM) eluates, vs. the discoveries in the control, untreated sample. The lower diagram gives the distinct contributions of the native vs. the denaturing extraction conditions. Contrary to what could be expected (see also

Fig. 4B) the contribution to protein discovery upon a denaturing extraction is quite meager, since it amounts to only 9 additional species. It can be appreciated, nevertheless, that the contribution to protein discovery of all eluates from CPLLs represents an increment of 300% as compared to what had been found in the controls. By summing all contributions, the total description of the peel proteome amounts to 334 unique gene products. In Fig. 4B, the corresponding data for mango's flesh are given. Here it can be appreciated (lower diagram) that the extraction under denaturing conditions has given a robust contribution to protein discovery, since it permitted identifying 373 additional species. By summing all contributions, the total description of the flesh proteome amounts to 2855 unique gene products. The lists of total proteins found in the peel and pulp are provided as supplementary material as Tables S1 and S2, while Table 1 contains the list of peptides present in mango peel and pulp. A more complete study on biological functions of CPLL captured proteins was obtained by Gene Ontology (GO) analysis, performed by using the web available software QuickGO (www.ebi.ac.uk/QuickGO) and reported on Fig. 5.

4. Discussion

4.1. The title first

This is the third report on a series dedicated to proteomics of tropical fruits, the first two dedicated to the banana [31] and avocado [32]. These last two fruits can be classified as “recalcitrant tissues” in that scarce levels of proteins (typically below 1%) are dispersed in huge amounts of polysaccharides (banana) or solid oil (avocado). Nevertheless, by adopting the CPLL capture protocols and native/denaturing extraction conditions, we could identify in both these fruits >1000 unique gene products. Thus this third report on mango closes the circle on tropical fruits, although the term “samba” would rather refer to bananas, as the typical ornament of female dancers in the classical Copacabana Carnival in Rio. The elderly readers might even recall the superb ballets of Josephine Baker performing her Banana Dance at the Folies Bergère in

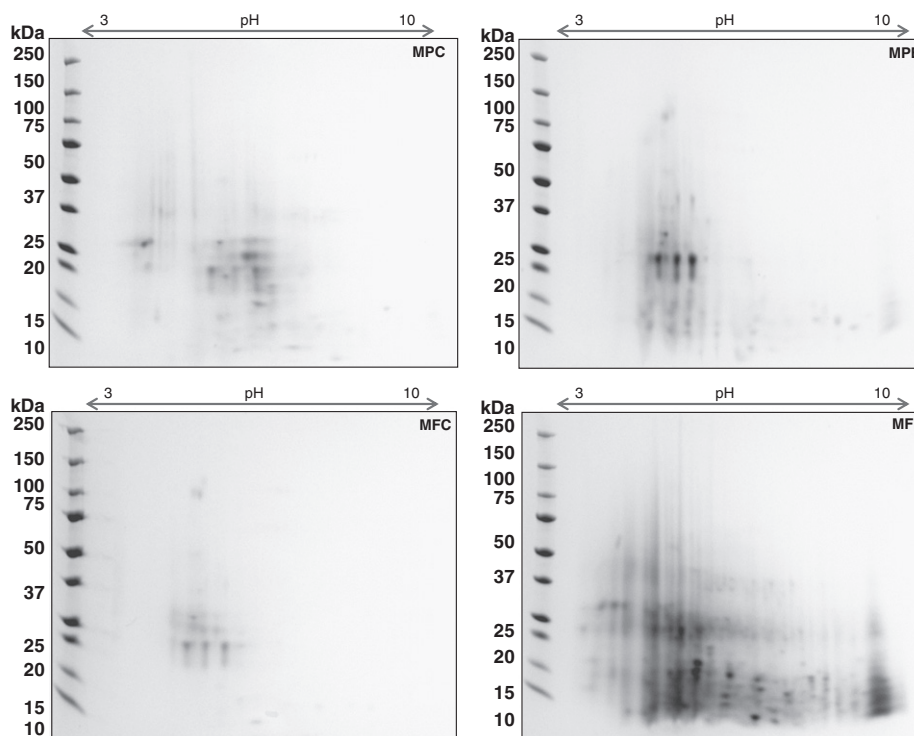


Fig. 3. Two-dimensional maps of mango peel and flesh extracts. Two upper panels: peel extracts. MPC: peel control, MPE: peel extract. Two lower panels: flesh extracts. MFC: flesh control; MFE: flesh extract. First dimension: pH 3–10 immobilized pH gradients; second dimension: 8–18% polyacrylamide gel gradient. Colloidal Coomassie Blue staining.

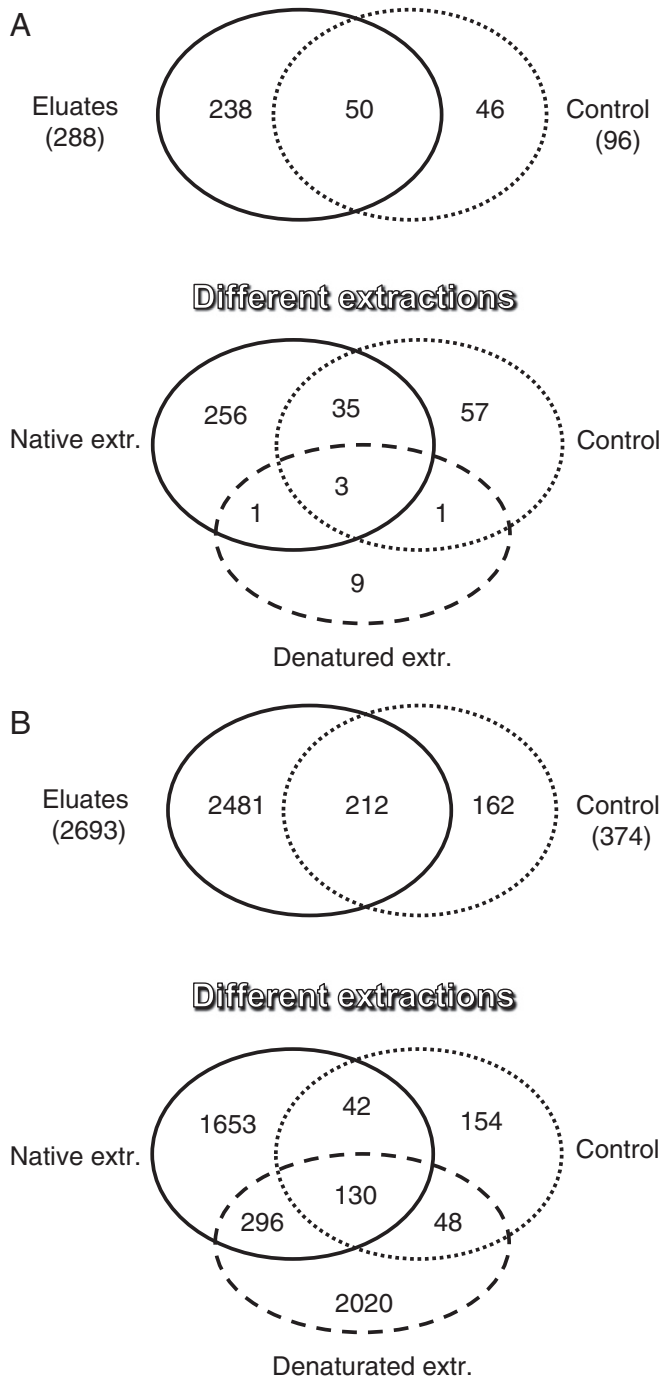


Fig. 4. Venn diagrams of the total protein identifications in A (peel compartment) and B (pulp). In both cases, the upper diagram gives the contributions to total discoveries of the untreated control vs. the CPLL capture. The lower graph gives the separate contributions of the two CPLL captures, under native and denatured conditions.

Paris, France, around 1927. There she wore a banana belt around her waist (it is hard to imagine her wearing a mango belt!).

4.2. Now the peel

At first sight, it would appear strange to perform a proteomic/peptidomic exploration of mango's peel. Yet, precisely in view of its extraordinary content of metabolites of importance to human health, we thought it would be nice to dig deep also on its protein content, considering that, just like for lemon peel in the Mediterranean area, mango's peel is extensively exploited in the Far East cuisine for flavoring

foods. In fact, we have recently reported an in depth analysis of lemon peel (the flavedo, not the underlying spongy albedo), since this biological tissue is used < not only for cooking, but also for preparing a special Italian liqueur called Limoncello [33]. Yet, in lemon peel we could identify more than 1000 proteins, whereas here only a bit more than one third identifications have been obtained. We believe this is due to the fact that, whereas lemon peel is soft and spongy, thus easily amenable to proper homogenization, mango's peel is rather tough and has probably not been properly homogenized by the blender which we routinely exploit for vegetable tissues. The results of identified proteins were surprising for two reasons. First of all for mango peel we could recognize more than 300 proteins even if a low content is normally reported in literature. Secondly for mango pulp we have made a list of 2855 proteins confirming the data reported in literature. Despite the high number of identified unique gene products, the number of specific proteins for *M. Indica* is quite low, due to the incomplete knowledge of mango genome and consequently of its proteome. But our results would represent the initial step to improve the information about this fruit nutritional properties. In order to complete our research project we have performed a GO analysis to compare the biological functions of proteins found both in mango peel and pulp. In mango peel they are mostly connected with defensive processes and membrane activities. In fact they are components of integral structures and plasma membrane and they play an important role in the transferase activity and in the transport. Instead the proteins of mango pulp are present in chloroplasts and nucleus and they are connected with metabolic functions like heme binding, glycolysis, carbohydrate metabolic processes and response to oxidative stress. Finally we also reported a list of peptides found both in mango peel and pulp, which were not recognized as part of known proteins due to the lack of complete mango proteome knowledge. It is interesting to underline that we found more unrecognized peptides in the peel than in the pulp because the number of identified peel proteins is lower than in the pulp so it is likely that there would be a greater number of peptides which are not associated to a known protein. Among these peptides, some could possibly be connected with nutritional and allergenic functions: it could be interesting to perform experiments to test biological activities.

As regards to potential mango allergies, the responsible allergens are not yet identified, due also to the fact that mango's proteome is not completely known because its genome has not yet been sequenced. For this reason in allergens databases we could not find specific proteins referred to mango but in our identified species we could verify the presence of well-known allergens, referred to the same plant order (*Sapindales*), belonging to the same taxonomic group (*Plantae Magnoliopsida*). By consulting the IUIS allergen nomenclature databases (<http://www.allergen.org/>), we obtained a list of allergens, of which some are present in our list. In particular in CPLL eluates we recognized: non-specific lipid transfer protein, superoxide dismutase, germin-like protein and profilin. The non-specific lipid-transfer protein transfers phospholipids as well as galactolipids across membranes, so it plays a role in wax or cutin deposition in the cell walls of expanding epidermal cells and certain secretory tissues. Superoxide dismutase destroys radicals which are normally produced within the cells and which are toxic to biological systems. For this reason it is involved in cellular response to UV-B, to copper ions, to light intensity and to salt stress. Finally germin-like protein plays a role in broad-spectrum disease resistance and profilin in actin polymerization and depolymerization affecting the structure of cytoskeleton. In detail, some of them were found in mango's peel and flesh eluates like profilin and superoxide dismutase, while in peel's eluates only non-specific lipid transfer protein and germin-like protein were identified according to their defensive and transfer functions.

It was demonstrated that profilins are allergens responsible for cross reactivity between botanically unrelated pollens and fruits. Allergy to mango has also occasionally been reported in people

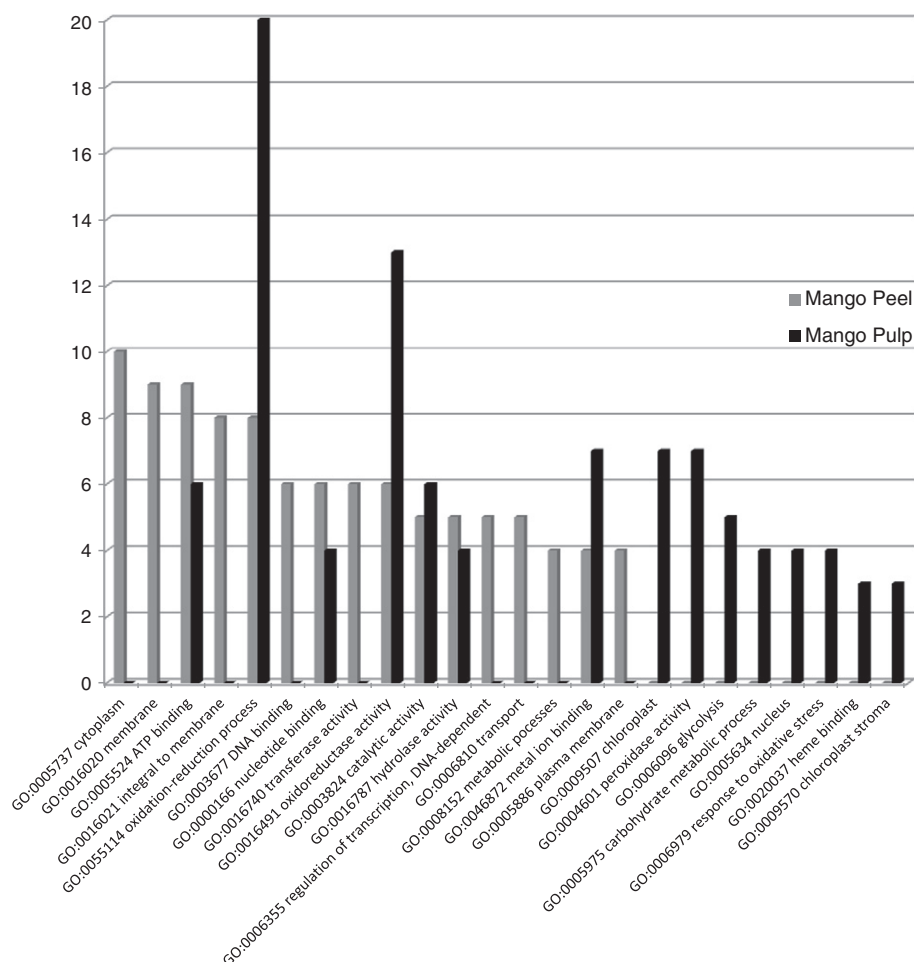
Table 1

List of peptides found in both mango peel and pulp.

Peptide_sequence	Peptide_query	Experimental_Mr	Calculated_Mr	Mascot_score
<i>Mango pulp peptides</i>				
HRGVSSQPESQPVN	10810	1521,19	1520,73	43
PPPNTAKK	6838	852,12	851,48	42
DPTLSG	3199	588,39	588,27	42
GMYCIEDIEGISAMSKLTF	14333	2082,2	2082,89	41
<i>Mango peel peptides</i>				
VEVFSAPKDYIMDQGTK	15520	1943,68	1942,93	50
KLTDALQMFQNL	13374	1712,54	1711,79	45
LESDSGEIWGKVEWF	15473	1937,5	1936,93	44
CNIKCMKKGQHCQAY	15268	1927,31	1926,83	43
GNHGFAGRGTSVGGH	11953	1465,91	1466,67	43
TVMYQTFEKLKGYCGS	15244	1927,22	1926,88	43
SHGLGEEALKVFCEMPLSGSTK	18020	2391,51	2392,14	43
AGPICASMSMSSNPPYDLTSF	17588	2334,03	2334,98	42
PIMGKVMEIFRPG	12105	1489,33	1489,77	42

with latex (*Hevea brasiliensis*) hypersensitivity. Rubber elongation factors, hevein and prohevein present in natural latex are the allergens thought to be responsible for this latex allergy which usually manifest as contact dermatitis, oropharyngeal allergy syndrome and rarely as anaphylaxis, as reported in our previous manuscript [34]. The cross reactivity between latex and various fruits like chestnut, peach, avocado,

passion fruit, fig, grape and orange has been termed as “latex-fruit syndrome” [35]. In CPLLs eluates of mango peel and flesh elongation factors were found as possible responsible of “latex-fruit syndrome”. The final step would be the detection of allergens, by incubation of blotted 2D maps of CPLL eluates with sera of allergic patients, and their identification through MS analysis.

**Fig. 5.** Gene ontology analysis of CPLL captured proteins of mango peel vs. CPLL captured proteins of mango pulp.

5. Conclusions

This is the third report on the investigation of trace proteins (and peptides in the case of mango) in tropical fruits in order to detect trace allergens (if any) and/or proteins/peptides endowed with nutraceutical effects important for human health. Overall our data suggest a predominant role of the CPLL technology in the investigation of vegetable proteomes as an instrument that is able to overcome most limits of proteomic analysis in the detection of low-abundance proteins, thus permitting exploration of foodstuff composition in order to verify the existence of proteins/peptides endowed with potential health effects.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbapap.2013.09.004>.

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