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In-depth exploration of *Hevea brasiliensis* latex proteome and “hidden allergens” via combinatorial peptide ligand libraries

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ABSTRACT

The proteome of *Hevea brasiliensis* latex has been explored in depth via combinatorial peptide ligand libraries. A total of 300 unique gene products have been identified in this latex, whose proteome has been largely unknown up to the present. In search for unknown allergens, control latex and eluates from the ligand libraries have been fractionated by two-dimensional mapping, blotted and confronted with sera of 18 patients. In addition to the already known and named *Hevea* major allergens, we have unambiguously detected several others like, for instance: heat shock protein (81 kDa), proteasome subunit (30 kDa), protease inhibitor (8 kDa), heveamine A (43 kDa) and glyceraldehyde-3-phosphate dehydrogenase (37 kDa). Gene Ontology analysis of analyzed fractions has shown that major functions are substantially unchanged after sample treatment, while novel biological functions appeared that were undetectable in the crude sample.

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1. Introduction

Plant natural latex, involved in plant defense mechanisms, is the cytoplasmic content of laticifers or latex vessels (a network of tubes in the tree) and is the major location of cis-polyisoprene, a high-molecular mass natural polymer rubber.

Many of the latex-containing plants are important to the global economy, because for example (i) *Papaveraceae* are a source of opium, other pharmaceutically important alkaloids, with analgesic (morphine), antitussive (codeine) as well as

muscle-relaxing (papaverine) effects and (ii) *Hevea brasiliensis* latex is well known as a source of natural rubber.

Notwithstanding the importance of latex from *H. brasiliensis*, not much is known about its whole proteome. In 2002, Sookmark et al. [1] indeed performed a two-dimensional gel electrophoresis analysis of its cytosolic serum, but only for characterizing any polypeptide that might accumulate in a disease called “tapping panel dryness syndrome”. Due to the particular focus of their research, they only identified 3 proteins related to this disease. Even if relatively little

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knowledge has been developed on the *H. brasiliensis* latex total proteome, proteomics technologies have been used with the objective to detect and identify allergic antigens. In this domain, a deep knowledge has been accumulated over the years on the allergen content of *Hevea* latex, no doubt due to the wide-spread IgE-mediated sensitization in 5% to 18% of health care workers and in up to 50% of patients with spina bifida and other congenital anomalies. For the first time a protein called 27 KDa natural rubber latex protein was characterized as associated to latex induced allergy [2]. Then a milestone paper on this topic was published in 1997 by Posch et al. [3] who, contrary to all previous studies on allergens, exploiting one-dimensional techniques and immunoblotting, adopted 2D mapping, followed by blotting and immunorevelation with patients' sera. They were able to excise and identify by micro-sequencing 16 IgE-reactive latex polypeptides, corresponding to known and previously unreported allergens. Their study, as well as those of several other groups (www.allerdata.com), has led to an official list of 13 well characterized allergens and some other allergens still not included in the I.U.I.S. allergen nomenclature (www.allergen.org), like for instance, heveamine A [4].

Much deeper data came from genomic and transcriptomic analyses, rather than from proteomics. Thus, in the year 2000, Han et al. [5] constructed cDNA libraries from the latex of *H. brasiliensis* to investigate its gene expression: sequence analyses identified 245 expressed sequence tags (ESTs), of which 57% showed homology to previously described sequences in public databases. In a subsequent transcriptome analysis, the same authors [6] revealed some unique features of the repertoire of genes. Several of them, involved in the rubber biosynthesis, were expressed at low levels in the latex. In its latest report [7], this group further deepened its transcriptome analysis. This team thus generated a large number of unique transcripts; however, the functional classification that has been proposed according to the Gene Ontology convention, showed that 73.8% were related to genes of unknown function.

Contrary to transcriptomics detailed work that covered all the field, the detection and identification of low-abundance protein species from latex was mostly focused on allergy-inducing polypeptides. Thus the latex allergens Hev b 1, 2, 3, 4, 5, 6, 7 and 13 were detected in freeze-thawed and glycerinated latex serum at levels ranging from 75 (Hev b 6) to 0.06 nmol/mg total proteins (Hev b 4). The allergenicity of these different allergens is however not correlated with their concentration in sap. For instance, both Hev b 1 (rubber elongation factor) as well as Hev b 6 (prohevein) are considered as major *H. brasiliensis* allergens [8–10], while Hev b 6 concentration in the latex sap is up to a thousand times higher than some of the other latex allergens [11]. These data on allergen concentration were also confirmed by the abundance of mRNA transcripts. Interesting data on the presence of these allergens in latex mattresses [8,12] and gloves were also reported [13] in spite of harsh chemical treatments to transform initial latex into rubber. This last team concluded that IgE recognition of Hev b 4, Hev b 7b, Hev b 5 and Hev b 2 was most frequently encountered, with 75, 61, 31 and 28%, respectively, of the sensitized patient sera. Sensitivity to multiple latex proteins was common, and out of the 31 seropositive patients, 23 (74%)

had IgE against at least two latex allergens, while 12 (39%) had IgE specific for at least three allergens. In one instance, an in-depth structural analysis of the carbohydrate moiety of the Hev b 4 allergen was also performed [14]. More recently [15] proteomics technologies involving two-dimensional gel electrophoresis associated with immunoblotting have been used for the detection of *Hevea* latex allergens.

Aware of all these limitations, we have undertaken the present study with two aims: (i) exploring in depth the proteome of *H. brasiliensis* latex and (ii) trying to detect some of its low-abundance (hidden) allergens. For this purpose, we have exploited the combinatorial peptide ligand library technique, which has given remarkable results in the detection of the low-abundance proteome, as reported in several recent reviews [16–21], as well as in the detection of novel allergens in milk whey [22]. This approach could have important implications on allergy diagnosis.

2. Materials and methods

2.1. Chemicals and biologicals

The solid-phase combinatorial peptide library known under the trade name of ProteoMiner™ (Library-1), and its carboxylated version (Library-2, non-commercially available), as well as materials for electrophoresis such as gel supports and reagents were from Bio-Rad Laboratories (Hercules, CA, USA). N-ethylmaleimide, urea, thiourea, 3-[3-cholamidopropyl dimethylammonio]-1-propanesulfonate (CHAPS), tris(2-carboxyethyl) phosphine hydrochloride, bis-(2-hydroxyethyl)disulphide, isopropanol, acetonitrile, trifluoroacetic acid and sodium dodecyl sulphate were all from Sigma-Aldrich (St Louis, Mo, USA). The test used to determine serum-specific IgE (ImmunoCap®) was from Phadia AB (Uppsala, Sweden). Nitrocellulose and Optitran BA-S 83 nitrocellulose membrane were from Schleicher & Schuell (Dassel, Germany). Complete protease inhibitor cocktail tablets were from Roche Diagnostics (Basel, CH). Sequencing grade bovine trypsin was from Promega (Madison, WI, USA). Polyacrylamide gel (ExcelGel gradient 8–18%) and molecular mass standard protein mixture (MMr) were from GE Healthcare (Uppsala, Sweden). 3 µm ReproSil 100C18 chromatography support was from Dr. Maisch GmbH (Ammerbuch-Entringen, Germany). Alkaline phosphatase-conjugated goat anti-human IgE, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were purchased from Sigma-Aldrich, Saint Louis, USA. All other chemical were also from Sigma-Aldrich and were of analytical grade.

2.2. Equipment and software

Multiphor II chamber was from GE Healthcare (Uppsala, Sweden). Versa-Doc image system, Protean device, semi-dry blotting apparatus and PDQuest software were from Bio-Rad Laboratories (Hercules, CA, USA). StageTip devices, EasyLC chromatography system, nanoelectrospray ion source for mass spectrometer and fused silica capillary with 75 µm inner diameter and 360 µm outer diameter were from Proxeon Biosystems (Odense, Denmark). Speed Vac system was from SVPT s.r.l., Italy. LTQ-Orbitrap mass spectrometer was from

ThermoScientific (Bremen, Germany). A second hybrid quadrupole time of flight mass spectrometer API QStar PULSAR was from PE-Sciex (Toronto, Canada). The associated capillary chromatographic system Agilent 1100 Series equipped with a Nano Pump, Iso Pump, and Degaser was from Agilent (Santa Clara, CA, USA). Analyst QS 1.1 software was from Applied Biosystems (Foster city, CA, USA). Mascot search engine version 2.1.04 was from Matrix Science (London, UK) and Scaffold version 01_06_07 was from Proteome Software Inc. (Portland, OR, USA).

2.3. Human sera

Sera were obtained from 18 *H. brasiliensis* allergic patients from various French regions. Among the 18 patients, two of them presented some symptoms (pruritus and urticaria) linked to contact with latex manufactured material such as gloves. These patients presented positive skin prick test, but no specific IgE against *Hevea b. sap* (ImmunoCap=0), which has already been attested in the literature [23]. For the other patients' sera, the ImmunoCap values ranged from 3 (from 7.73 to 38.5 ng/mL of specific IgE) to 6 (>220.02 ng/mL of specific IgE). Patients were selected on the basis of symptoms, positive skin prick test results, and positive serum-specific IgE. All sera were from patients allergic to latex sap. One serum was from non-atopic donor. Sera were provided by biological analysis laboratories and represented residues of IgE titer evaluations. Among these 18 sera, 3 were chosen for their specific pattern and used for 2D immunoblot incubation.

2.4. Sample treatment

H. brasiliensis (para rubber tree) clone RRIM501 was used for latex collection. After tapping, latex was allowed to drop directly into liquid nitrogen in an ice kettle. The frozen latex grains were stored at -70°C until use. The frozen latex grains were centrifuged at $25,000\times g$ for 1 h at 4°C to separate the rubber fraction from the total serum. The clear serum ($V_{\text{tot}}=21\text{ mL}$), placed in a cooling bath, was added slowly with dry and pure ammonium sulphate (AR grade) under stirring. The final concentration of ammonium sulphate was 90% saturation and the mixture was maintained under stirring for 1 h. After one step of centrifugation (15,000 rpm for 1 h at 4°C), the supernatant was discarded and the protein pellets were resuspended in a total volume of 50 mL distilled water containing the protease inhibitor cocktail tablets. The pH and conductivity of the protein solution were measured (pH 6.85 and conductivity 61 mS/cm). An aliquot of 0.5 mL was kept and centrifuged (5 min at $15,000\times g$) and the total protein concentration of supernatant was measured with the Bradford assay at 10 mg/mL, corresponding to a total protein content estimated at 500 mg.

The protein solution was dialyzed (MWCO 3500 membrane) overnight at 4°C against PBS. The protease inhibitor cocktail tablets were added, and the starting material (pH 7.2, conductivity 14 mS/cm) was loaded at room temperature onto 2 successive 1 mL-columns of ProteoMiner (Library-1) and carboxylated ProteoMiner (Library-2). After an extensive wash with PBS, the columns were disconnected and separately eluted with 1 M NaCl, 2 M thiourea, 7 M urea, 3%

CHAPS (TUC), 9 M urea in 50 mM citric acid, pH 3.3 (UCA) and a hydro-organic mixture composed of acetonitrile, isopropanol, trifluoroacetic acid and water at final concentrations of respectively 16.6%, 33.3%, 0.5%, and 49.5%. After 2.5 M Tris-neutralization of UCA eluates, all fractions were dialyzed against a 100 mM ammonium bicarbonate solution. The dialyzed fractions were collected, and each fraction was separated in five equal parts. One fraction of each eluate was kept at -18°C for SELDI analysis and all others were frozen at -80°C for 2 h and lyophilized.

2.5. 1D-SDS-PAGE analysis

2.5.1. SDS-PAGE for sample comparisons

10 μL of each sample (corresponding to ca. 30 μg protein) was mixed with 10 μL of Laemmli buffer [24] (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl, pH approx. 6.8). The mixture was heated in boiling water for 5 min and immediately loaded onto the gel. The SDS-PAGE slab was composed of a stacking gel (125 mM Tris-HCl, pH 6.8, 0.1% SDS) with a large pore polyacrylamide (4%) cast over the resolving gel (8–18% acrylamide gradient in 375 mM Tris-HCl, pH 8.8, 0.1% SDS buffer). The cathodic and anodic compartments were filled with Tris-glycine buffer, pH 8.3, containing 0.1% SDS. Electrophoresis was at 100 V until the dye front reached the bottom of the gel. Staining and destaining were performed with colloidal Coomassie Blue [25] and 7% acetic acid in water, respectively. The SDS-PAGE gels were scanned with a Versa-Doc image system.

2.5.2. SDS-PAGE for 1D-immunoblot

The 3 different extracts of *H. brasiliensis* (untreated, Library-1 eluate and Library-2 eluate, 1 mg/mL) were mixed with 0.04 M Tris acetate buffer pH 6.8 containing 4% SDS and submitted to a 1D-SDS-PAGE separation performed in a polyacrylamide gel (ExcelGel gradient 8–18%). According to the manufacturer's instructions, the flat bed electrophoretic chamber was cooled at 18°C . Mr markers from 94 to 14.3 kDa were used as references.

2.6. 2D-PAGE analysis

The desired volume of non-treated sample and the mixture of all eluates were solubilized in the "2D sample buffer" (TUC, 40 mM Tris) to a final concentration of 2 mg/mL protein and the disulphide bridge reduction allowed to proceed at room temperature for 60 min by the addition of tris(2-carboxyethyl) phosphine hydrochloride at a final concentration of 5 mM. For alkylating the reduced $-\text{SH}$ groups, 150 mM De-Streak (bis-(2-hydroxyethyl)disulphide) (diluted directly from the stock 8.175 M) was added to the solution, followed by 0.5% carrier ampholytes (diluted directly from the stock, 40% solution) and a trace amount of bromophenol blue. All samples were analyzed in double: 7-cm long IPG strips (Bio-Rad Laboratories), pH 3–10 L, were rehydrated with 150 μL of protein solution, for 4 h. Isoelectric focusing (IEF) was carried out with a Protean IEF Cell in a linear voltage gradient from 100 to 1000 V for 5 h, 1000 V for 4 h, followed by an exponential gradient up to 5000 V, for a total of 25 kV/h. For the second dimension, the IPG strips were equilibrated for 25 min in a

solution containing 6 M urea, 2% SDS, 20% glycerol, 375 mM Tris-HCl (pH 8.8) under gentle shaking. The IPG strips were then laid on 8–18% acrylamide gradient SDS-PAGE gel slab with 0.5% agarose in the cathodic buffer (192 mM glycine, 0.1% SDS and Tris-HCl to pH 8.3) [26]. The electrophoretic run was at 5 mA/gel for 1 h, followed by 10 mA/gel for 1 h and 15 mA/gel until the dye front reached the gel bottom. One half of gels were incubated in a colloidal Coomassie Blue solution and destaining was performed in 7% acetic acid till clear background, followed by a rinse in pure water. The 2-DE gels were scanned with a Versa-Doc image system, by fixing the acquisition time at 10 s; the relative gel images were captured via the PDQuest software. After filtering the gel images for removing the background, spots were automatically detected, manually edited and then counted. The other half of gels was blotted on a CNBr-activated nitrocellulose (NCA) sheet performed with nitrocellulose according to Demeulemester et al. [27]. Nitrocellulose sheets were rehydrated firstly in PBS and, secondly, in anodic transfer buffer Tris (20 mM), glycine (150 mM), ethanol 20%, pH 10.5. The transfer of proteins was made by a semi-dry blotting apparatus for 1 h at 1 mA/cm² of gel.

2.7. Immunoblot

After 1D-SDS-PAGE separation, the proteins were transferred as described above. The nitrocellulose (NC) acetate sheets were then dried and the blotted sheets cut into strips (2.5 mm width). Each strip (1D) and sheet (2D) was blocked with PBS containing 0.3% Tween 20. One tenth of diluted patient's serum was individually incubated overnight at 20 °C with NC strips for 1D screening or with the whole NC sheet for 2D experiments. The 2D blots were incubated, in sealed bags, with 300 µL allergic patient serum diluted ×10 in PBS and 0.1% Tween. After washing, the NC strips and/or sheets were incubated with alkaline phosphatase (AP)-conjugated goat anti-human IgE for 2 h at 20 °C, followed by AP substrate 5-bromo-4-chloro-3-indolyl phosphate+nitroblue tetrazolium in 0.1 M Tris-HCl buffer, pH 9.5.

2.8. Protein identification from library eluates by nanoLC-MS/MS

The various sample lanes of SDS-PAGE gels were cut after staining in 12 pieces of about 0.5 cm along the migration path, and proteins were reduced by 10 mM DTT and alkylated by 55 mM iodoacetamide. The gel pieces were shrunk in acetonitrile and dried under vacuum; proteins were digested overnight with bovine trypsin as previously described [28]. The tryptic mixtures were acidified with formic acid up to a final concentration of 10%. 10 µL of tryptic digest was loaded on StageTip and eluted by 30 µL of 80% acetonitrile and 5% formic acid in distilled water. The purified peptide mixture was then concentrated in a Speed Vac system and resuspended in 10 µL of 10% formic acid in distilled water. 5 µL of purified tryptic digest was injected in a capillary chromatographic system EasyLC. Peptide separations occurred on a RP homemade 10 cm reverse phase spraying fused silica capillary column (75 µm i.d. × 10 cm), packed with 3 µm ReproSil 100C18. A gradient of eluents A (H₂O with 2% v/v ACN, 0.1% v/v formic

acid) and B (ACN with 2% v/v H₂O with 0.1% v/v formic acid) was used to achieve separation, from 8% B (at 0 min 0.2 µL/min flow rate) to 50% B (at 80 min, 0.2 µL/min flow rate). The LC system was connected to an LTQ-Orbitrap mass spectrometer equipped with a nanoelectrospray ion source. Full scan mass spectra were acquired in the LTQ-Orbitrap mass spectrometer in the mass range *m/z* 350 to 1500 Da and with the resolution set to 60,000. For accurate mass measurements the lock-mass option was used [29]. The four most intense doubly and triply charged ions were automatically selected and fragmented in the ion trap. Target ions already selected for the MS/MS were dynamically excluded for 60 s. All analyses were performed in triplicate.

2.9. Protein identification from 2D spots by nanoLC-MS/MS

The spots cut from the 2D-PAGE were tryptic digested as described above. Mass spectrometry analysis was performed using a hybrid quadrupole time of flight mass spectrometer API QStar PULSAR, equipped with nanoelectrospray ion source. A total of 5 µL of trypsin digested sample was injected in a capillary chromatographic system Agilent 1100 Series. Peptide mixtures were separated on a 10 cm fused silica capillary with 75 µm inner diameter and 360 µm outer diameter, filled with Reprosil-Pur C18 3 µm resin. Peptides were eluted with a 60 min gradient from 92% buffer A (2% acetonitrile and 0.2% formic acid in water) to 80% buffer B (2% water and 0.2% formic acid in acetonitrile), at a constant flow rate of 200 nL/min. Analyses were performed in positive ion mode; the HV potential was set up at around 1.6–1.8 kV. Full scan mass spectra ranging from *m/z* 350 to 1350 Da were collected, and for each MS spectrum, the two most intense doubly and triply charged ions peaks in the mass range were selected for fragmentation. Tandem mass spectra were extracted by Mascot.dll (version 1.6.0.21) through Analyst QS 1.1.

2.10. Data analysis

The data were searched against the UniProtKB database (version 57.7), selecting *Viridiplantae* (Green Plants, 28203 sequences) as taxonomic reference base, with Mascot search engine. We used tryptic cleavage constraints with a maximum of 2 missed cleavages, cysteine alkylation and oxidation of methionine residues as variable modification, peptide mass tolerance was set to 5 ppm and fragment mass tolerance to 0.8 Da for Orbitrap data and to 100 ppm and 0.6 Da for peptide and fragment mass tolerance with QStar data. The criteria used for protein identification were: 20 ion score cut off, protein Mascot score more than 100 and at least 2 unique peptides sequenced. Scaffold-01_06_07, was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [30]. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The Gene Ontology analysis was

performed by using QuickGO interface (<http://www.ebi.ac.uk/QuickGO>) [31].

3. Results

Several authors describe the difficulties to treat and analyze the latex samples [32–34]; this was the reason why protein precipitation using ammonium sulphate was performed as a first operation. The soluble protein thus collected could be analyzed as appropriate for all analytical determinations. Amplification of low-abundance protein species was performed by two consecutive treatments with peptide ligand libraries as already proposed for other biological fluids [21,22] resulting in very complementary fractions easier to analyze. In practice, the starting material (dissolved in 150 mM NaCl in 20 mM phosphate buffer, pH 7.2, PBS) was loaded at room temperature onto 2 successive 1 mL-columns, the first filled with unmodified combinatorial peptide ligand library beads (Library-1) and the second filled with the carboxylated version (Library-2). After an extensive wash with PBS, the columns were disconnected and separately eluted with 1 M NaCl, TUC and UCA. A fourth elution was also performed using a hydro-organic solution as described in Section 2, but the collected fraction contained an extremely low amount of proteins and was thus discarded from further analysis.

All these fractions were analyzed by SDS-PAGE (Fig. 1). Although it is difficult to discern any special feature in the one-dimensional tracing, evidence was made on the enhancement of protein species that were not detectable prior to treatment with peptide libraries. As a second comment, the patterns evidenced the complementary composition of various fractions from the same library and also different patterns of the same type of elution from the two libraries. The only

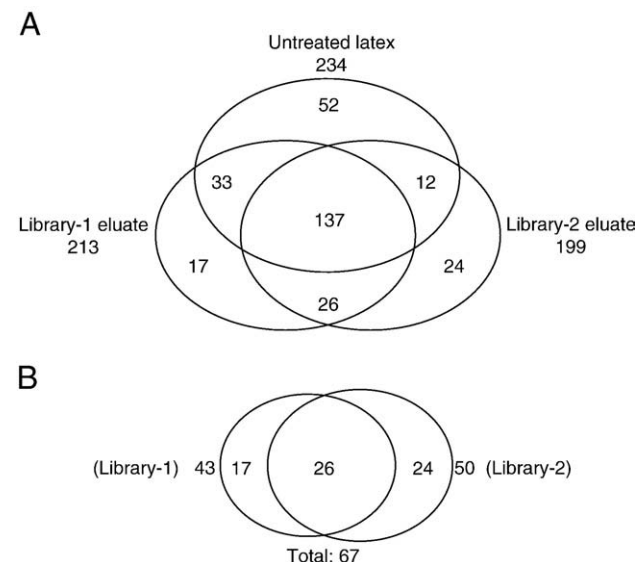


Fig. 2 – A: Overlapping Venn diagram of the total proteins detected (numbers outside the circles) in control (untreated) and in the two distinct eluates from Library-1 and Library-2. B: Overlapping Venn diagram of unique gene products detected only in the eluates of the two libraries.

relatively similar elution pattern from both libraries was the one obtained using TUC buffer (lanes 5 and 8 of Fig. 1). Sodium chloride elutions (lanes 4 and 7 of Fig. 1) revealed many undetectable species compared to the non-treated sample (lane 2 of Fig. 1). Conversely, as expected, the presence of high-abundance species from the crude extract (protein bands located between 5 and 10 kDa and between 10 and 15 kDa) was significantly reduced.

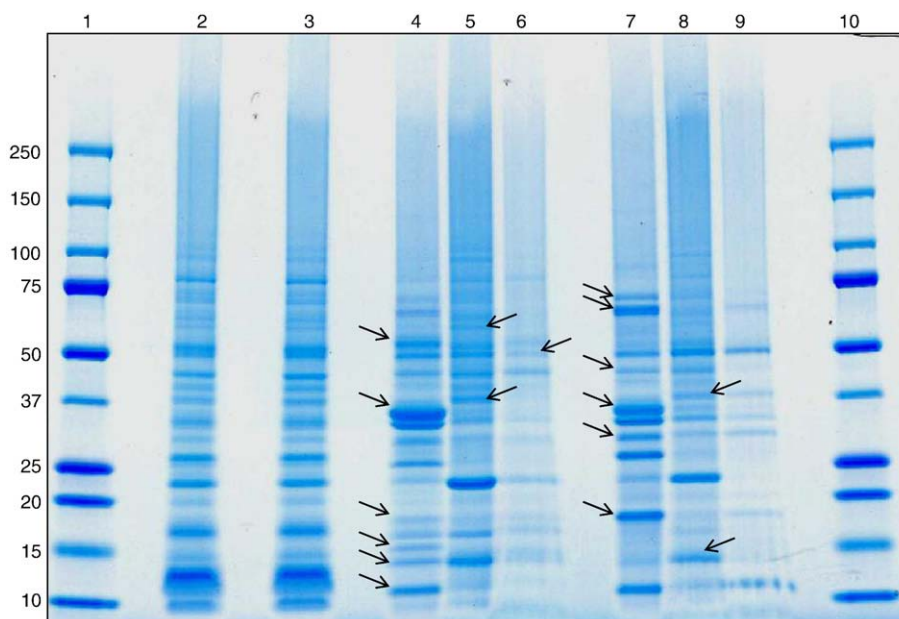


Fig. 1 – SDS-PAGE of control (lane 2) flow-through (FT, lane 3) and eluates from Library-1 (lanes 4, 5, 6) and Library-2 (lanes 7, 8, 9). Lanes 1 and 10 are Mr markers. NaCl elutions (lanes 4, 7), TUC elutions (lanes 5, 8) and UCA elutions (lanes 6, 9). Staining: Coomassie Brilliant blue. Arrows indicate major proteins enhanced upon the use of peptide libraries.

Table 1 – Gene products found only after treatment of the *Hevea latex* extract with peptide ligand libraries.

Identified protein	Access number
14-3-3-like protein C	P93343
14-3-3-like protein GF14-E	Q6EUP4
26S proteasome non-ATPase regulatory subunit 4	P55034
40S ribosomal protein S17-1	P49205
40S ribosomal protein S19-3	Q9FNP8
40S ribosomal protein S25	P46301
40S ribosomal protein S28-2	P34789
40S ribosomal protein S8	P49199
60S acidic ribosomal protein P0-1	O04204
60S ribosomal protein L22-2	Q9M9W1
60S ribosomal protein L23a-1	Q8LD46
60S ribosomal protein L26-1	P51414
60S ribosomal protein L27	Q05462
60S ribosomal protein L34	P40590
60S ribosomal protein L8-1	P46286
ABC transporter F family member 4	AB4F_ARATH
Actin-depolymerizing factor 2	Q39251
Alcohol dehydrogenase class-3	A2XAZ3
Alpha-soluble NSF attachment protein	P93798
Annexin D1	Q9SYT0
Caffeoyl-CoA O-methyltransferase	O65162
Calcium-dependent protein kinase 5	Q38871
Casein kinase I isoform delta-like	P42158
Casein kinase II subunit alpha-2	Q08466
Cell division control protein 48 homolog A	P54609
COP9 signalosome complex subunit 4	Q8LSU0
Delta-1-pyrroline-5-carboxylate synthetase	O04015
Elongation factor 1-alpha	O64937
Elongation factor 1-delta	O81918
Eukaryotic peptide chain release factor subunit 1-2	Q9LPV8
Eukaryotic translation initiation factor 3 subunit B	Q9C5Z1
Eukaryotic translation initiation factor 3 subunit B	P56821
Eukaryotic translation initiation factor 3 subunit D	P56820
Lipoxygenase	P38414
Mps one binder kinase activator-like 1	Q8GYX0
Nascent polypeptide-associated complex subunit alpha-like protein	NACA_PINTA
Nascent polypeptide-associated complex subunit alpha-like protein 2	NACA2_ARATH
Peptidyl-prolyl cis-trans isomerase 1	Q94G00
Probable methionyl-tRNA synthetase	Q9SVN5
Probable protein phosphatase 2C 59	Q8RXV3
Probable pyridoxal biosynthesis protein PDX1	Q39963
Profilin-2	Q9STB6
Proteasome subunit alpha type-1-A	P34066
Proteasome subunit alpha type-3	O23715
Proteasome subunit alpha type-4	O82530
Proteasome subunit alpha type-6	O48551
Proteasome subunit alpha type-7	O24030
Proteasome subunit beta type-1	P42742
Proteasome subunit beta type-5	O24361
Proteasome subunit beta type-7-A	O23710
Protein CYPRO4	P40781
Putative DNA repair protein RAD23-4	Q84L30
Putative quinone-oxidoreductase homolog, chloroplastic	Q9SV68
Ras-related protein RABA2a	O04486
Serine/threonine-protein kinase SAPK6	Q6Z144
Signal recognition particle 54 kDa protein 1	P49968
Signal recognition particle 54 kDa protein 2	P49972
SKP1-like protein 1A	Q39255
Soluble inorganic pyrophosphatase	Q43187

Table 1 (continued)

Identified protein	Access number
Vacuolar protein sorting-associated protein 2 homolog 1	Q9SKI2
Vacuolar protein sorting-associated protein 28 homolog 1	O65421
Vacuolar protein sorting-associated protein 32 homolog 2	Q9SZE4
V-type proton ATPase subunit E	Q9SWE7

The analysis of eluates by SELDI mass spectrometry (data not shown; see also Ref. [32]) between 8 and 15 kDa showed the complementary composition of fractions with low redundancy of species maybe except TUC eluates from both columns. As opposed to the paucity of signals of the control untreated latex the various eluates, except the hydro-organic one, from the two peptide library columns showed a considerable number of extra-peaks, especially in the m/z regions 9 to 10 kDa and 11 to 13 kDa, a zone difficult to explore by SDS-PAGE.

All eluates from each library were first pooled and re-fractionated in an SDS-PAGE gel, each lane was segmented into 12 pieces of about 0.5 cm along the migration path, and proteins were reduced by 10 mM DTT and alkylated by 55 mM iodoacetamide. The protein content of each slice was digested with trypsin and the resulting peptides were analyzed by nanoLC-MS/MS in an LTQ-Orbitrap mass spectrometer. After eliminating all redundancies from lane to lane, a total list of 300 unique gene products could be assembled (see Supplementary material). Several gene products have been attributed by homology to *H. brasiliensis* from other species. The Venn diagram of Fig. 2A gives the total discoveries of species in the untreated sample, as compared with the two eluates from the two libraries, together with the number of species common and unique to the various fractions. Out of 300 unique proteins, 137 (or 45.6%) were common to each sample; Library-1 contributed to the discovery of 17 undetectable species and Library-2 to the discovery of an additional 24 new species. These comments would not be complete without mentioning that 52 gene products detected in the untreated sample were not found in the library eluates. This could be explained by either the absence of peptides capable to capture them or the too weak elution stringency having left these species on the beads or present in the hydro-organic eluate. As a reminder the latter comprised a very low amount of proteins that could not be analyzed and possibly where missing proteins could be present. Fig. 2B gives the overlap among the unique captures of the two libraries, resulting in a net new discovery of 67 species that could not be detected in the starting material. This analysis could be of interest since it relates to the adsorption mechanism on the solid-phase library in relation with the desorbing agent. The behavior of each identified protein could give indications on the net charge of the identified protein and its hydrophobicity. For instance proteins desorbed with sodium chloride from the Library-2 are presumably associated with a dominant ionic exchange effect and are presumably cationic. Table 1 reports a list of identified proteins after treatment with libraries that are not detectable in the initial sample.

In addition to the global proteomic findings, we have next explored the presence of allergens on these *Hevea* extracts, not only to confirm the well-known and characterized ones, but especially to see if we could detect minor ones, since it is known that a number of health care workers show allergic reactions to still unidentified trace allergens in latex [35]. In a preliminary work, conventional SDS-PAGE gels were made, and the lanes containing the three types of samples (control and pooled eluates from Library-1 and Library-2, respectively) blotted and confronted with sera of the 18 allergic patients. From the data obtained (Fig. 3) one could see reactive zones in the Mr regions expected from the official allergen list (www.allergen.org), but also, especially in the Library-2 eluate, some additional reacting spots in a higher Mr region compared to the eluate from Library-1 (lanes 2 and 3 of Fig. 3). In several cases, a signal was detectable in the Library-2 sample but not associated with something for the Library-1 (lanes 4, 6 and 16 of Fig. 3). Nevertheless it appeared also that the positive reactions largely varied from serum to serum (hence from patient to patient) not only in intensity of the blot band, but also on the band positioning suggesting that a large number of antigens produce different allergic reactions. In order to properly identify such spots via MS analysis, the three samples (control and two library eluates) were further subjected to 2D mapping, one half of the samples (in triplicate) blotted for immuno-detection, while the other half of the gels (also in triplicate) stained with Coomassie blue for spot excision and MS analysis. Fig. 4 (A to C) shows three 2D maps, in which the spots positive to the immuno-detection on the blotted samples are marked and numbered. All these spots were excised, trypsinized and subjected to MS identification. Table 2 gives the list of allergens thus detected. It can be appreciated that, within the “canonical” 13 known allergens from the I.U.I.S. nomenclature, we could identify, in the

present sample, five antigens (Hev b 1, 2, 3, 6 and 9). However, and interestingly, in all of the three gels, we could also identify four additional allergens, for which data have already been reported in the literature [3,36,37] and of which one (glycer-aldehyde-3-phosphate dehydrogenase) had been identified as allergen in wheat flour [38] and in molds [39,40]. Although not identified from 2D excised spots but rather by LC-MS/MS from sliced SDS-PAGE, other mentioned proteins are comprised in the general MS protein list (Supplementary material). Two of them are already in the I.U.I.S. nomenclature (profilin and Mn-superoxide dismutase) (www.allergen.org). Two others are still not in the nomenclature but already recognized as allergens (hevamine A) [5,41] and citrate-binding protein [41]. None of the three sera, used for the 2D experiments, had specific IgE against these allergens.

From a general observation of 2D maps, one can find the confirmation that the eluate from Library-2 comprises allergens of higher masses than Library-1. Additionally, according to the isoelectric point localization, it can be observed that allergens from Library-1 are more acidic than those from Library-2. The high Mr reactive species was found to be a heat shock protein (81 kDa). It can also be appreciated that almost all of the allergen spots are found in a multitude of different spots. For instance glyceraldehyde-3-phosphate dehydrogenase is found in both Library-1 and Library-2 eluates and is present with seven spots total (see Table 2) located in various positions of molecular mass and isoelectric point. They may represent fragments of the same protein or truncated forms. Even if degraded, they interact with the peptide libraries by means of different epitopes and presumably different association constants [21,42]. The same behavior is also observed for other proteins. Table 1 shows additional data in which such spots are further subdivided into isoforms (i.e. species having the same Mr value) and into fragments

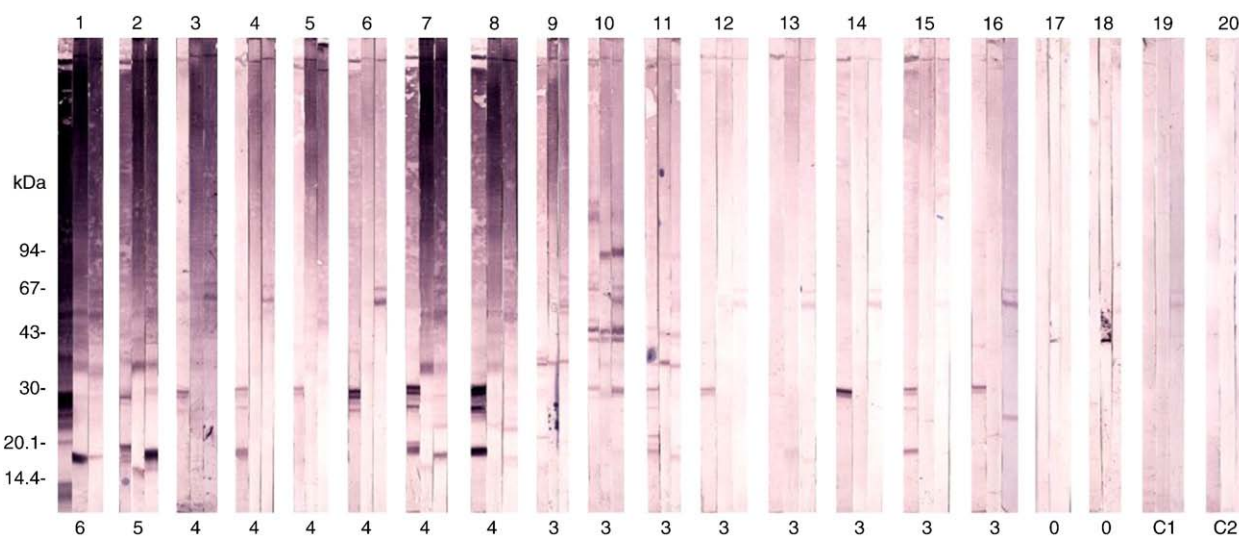


Fig. 3 – 1D SDS-PAGE immunoblots: Three nitrocellulose acetate strips blotted containing the three types of samples (control and pooled eluates from Library-1 and Library-2, respectively) were incubated with sera of 18 *Hevea b.* allergic patients (lanes 1 to 18) and one serum from a non-atopic donor, control 1 (C1, lane 19). A negative control was performed by PBS incubation (C2, lane 20). The bottom numbers represent the ImmunoCap® class specific to *Hevea b.* (from the sap extract). Class 0: IgE < 0.77 ng/ml; Class 1: 0.78 < IgE < 1.54 ng/ml; Class 2: 1.55 < IgE < 7.72 ng/ml; Class 3: 7.73 < IgE < 38.50 ng/ml; Class 4: 38.52 < IgE < 110.00 ng/ml; Class 5: 110.02 < IgE < 220.00 ng/ml; Class 6: IgE > 220.02 ng/ml.

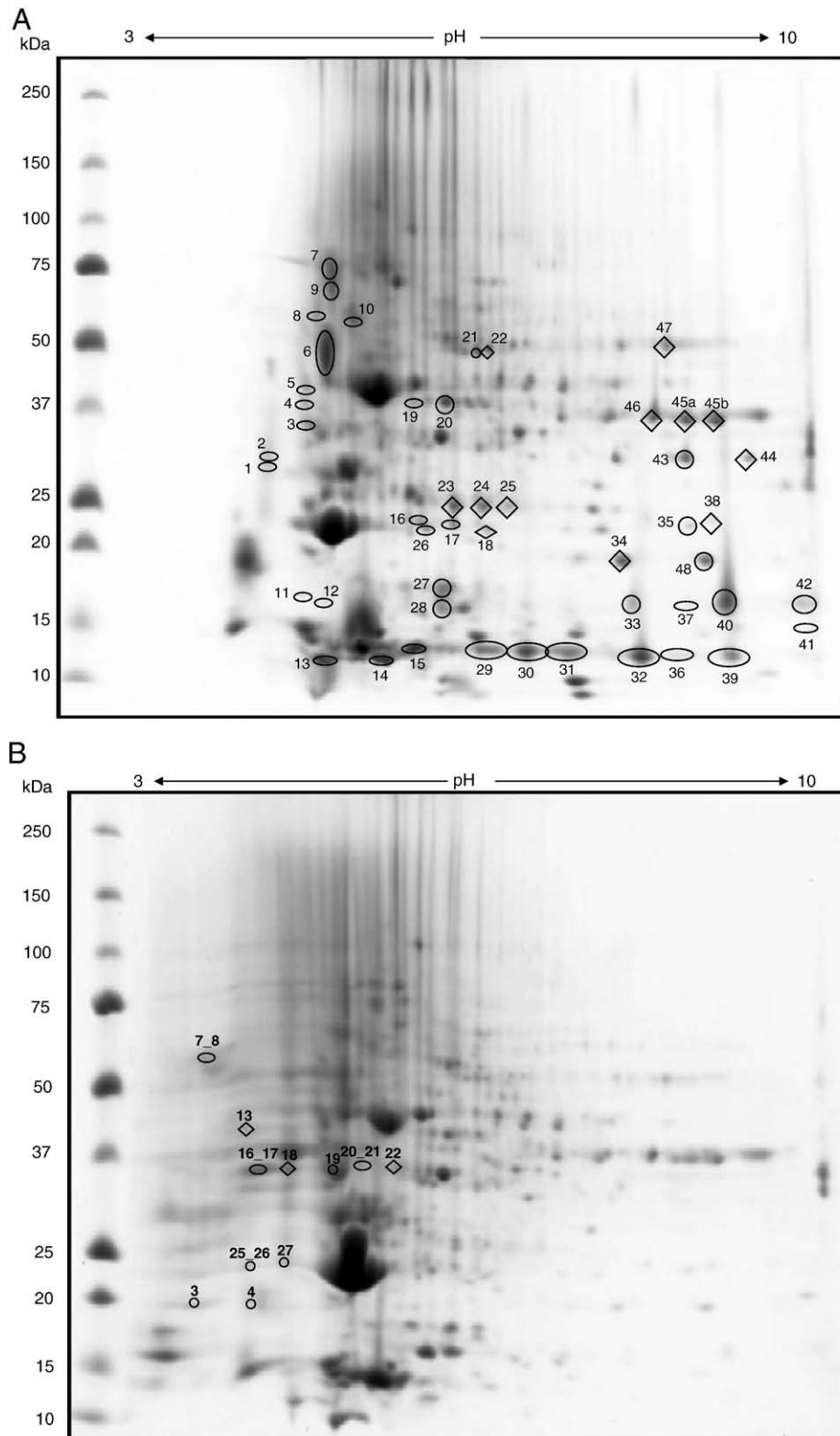


Fig. 4 – Two-dimensional (2D) maps of control sample (A) combined eluates from Library-1 (B) and combined eluates from Library-2 (C). The circles and relative numbers on selected spots represent polypeptide chains positive through immuno-detection performed on the blotted gel replicas and registered as allergens on www.allergen.org. Such spots have been subjected to MS analysis and their identities given in Table 2. Diamonds indicate spots positive through immune-detection but not classified in the I.U.I.S. nomenclature (see also Table 2 for protein names).

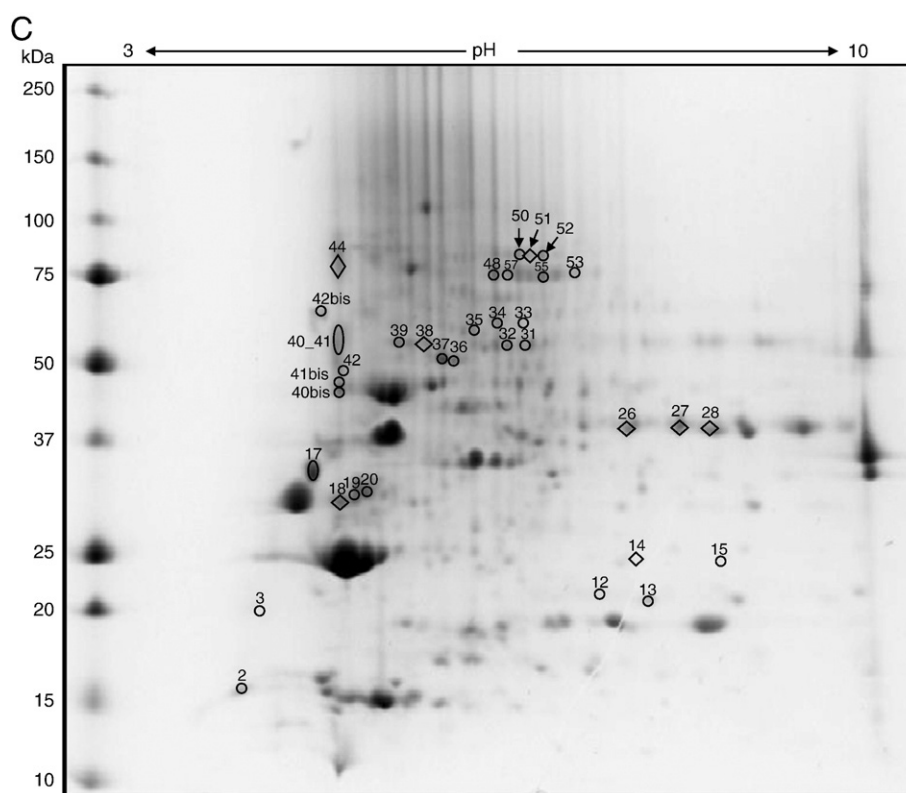


Fig. 4 (continued).

Table 2 – List of allergens detected in this study.

Registered <i>Hevea</i> allergens	Access number	Mass (kDa)	pI	Spot Nb (Fig. 5A)	Spot Nb (Fig. 5B)	Spot Nb (Fig. 5C)
Rubber elongation factor (Hev b 1)	P15252	22.34	4.8	13, 14, 15, 27, 28, 29, 34, 37		2
β -1,3-glucanase, basic vacuolar isoforms (Hev b 2)	P52407	41.39	8.95		16, 17, 19	
Small rubber-particle protein (Hev b 3)	O82803	22.34	4.8	1, 2, 3, 4, 11, 12, 19, 20, 26, 31, 43	4, 7, 8, 19, 20, 21, 25, 26, 27	3, 15, 17, 19, 20, 31, 36, 37, 39, 41, 41bis, 42, 42bis, 48, 50, 52, 53, 55, 57
Microhelix component (Hev b 4)	Q8WPJ2	40.95	6.67			
Acidic latex protein (Hev b 5)	Q39967	16.09	4.08			
Prohevein (Hev b 6.01), hevein (Hev b 6.02), prohevein C-terminal fragment (Hev b 6.03)	O49860	20.07	5.64	30, 32, 33, 39		
Patatin homologue from B-serum (Hev b 7.01), patatin homologue from C-serum (Hev b 7.02)	O04008	42.84	5.0			
Latex profilin (Hev b 8)	O65812	14.19	4.66			
Latex enolase (Hev b 9)	Q9LEI9	47.83	5.57			12, 33, 34, 35
Mn-superoxide dismutase (Hev b 10)	P35017	25.84	7.01			
Class I endochitinase (Hev b 11)	Q949H3	31.64	5.21			
Lipid transfer protein (Hev b 12)	Q8RYA8	11.83	9.47			
Latex esterase Early Nodule Specific Protein (ENSP) (Hev b 13)	Q7Y1X1	43.32	5.0			
Allergens non-classified in the I.U.I.S. nomenclature	Access number	Mass (kDa)	pI	Spot Nb (Fig. 5A)	Spot Nb (Fig. 5B)	Spot Nb (Fig. 5C)
Heat shock protein	P36181	80.63	4.94			44
Proteasome subunit	P34066	29.62	6.31	23, 24, 25	22	14
Protease inhibitor	Q6XNP7	7.57	5.1			18
Glyceraldehyde-3-phosphate dehydrogenase	P25861	36.8	8.3	22, 34, 45, 46, 47	13, 18	26, 27, 28, 38, 51

(degradation products from the original allergen, but still possessing the epitope responsive for immunoreactivity). It is not known at present if such degradation products had been present *in vivo*, in the freshly tapped latex, or had been produced upon storage (the protease inhibitors were added only upon arrival of the sample in our laboratory; the freshly tapped samples are usually added only with ammonia as stabilizer).

The Gene Ontology exploration of identified species is represented in Fig. 5. The largest group is represented by cytoplasm proteins as one expects. This category did not change substantially after treatment of the initial sample by both peptide ligand libraries. Also nucleotide-binding protein function remained practically constant in % for the analyzed samples. On the contrary, translation-involved proteins largely increased upon treatment with Library-2. The explanation is to be found in their capability to interact with nucleic acids as a result of a general alkaline isoelectric point. Therefore the presence of acidic carboxylated chemical functions on Library-2 very probably contributed to enhance this group of proteins. There were also protein categories that showed up *de novo* because of the treatment with peptide

Table 3 – Gene Ontology analysis of the identified proteins. The numbers in the boxes represent the % of total.

Gene Ontology category	Non-treated sample	Library-1	Library-2
Cytoplasm	21	17	21
ATP binding	15	10	14
Protein binding	10	6	11
Plasma membranes	8	7	6
Nucleotide binding	8	9	9
Metabolic processes	8	4	5
Translation	8	8	11
Catalytic activity	5	4	3
Oxidation reduction	5	0	4
Mitochondrium	4	0	0
Nucleus	4	5	6
Membrane	4	4	0
Ribosome	0	4	5
Protein phosphorylation	0	4	0
Aminoacyl-tRNA ligase	0	0	4

libraries. They are ribosome related proteins (for both libraries), proteins involved in phosphorylation processes upon treatment with Library-1 and aminoacyl-tRNA ligase activity after treatment with Library-2. Table 3 illustrates how the relative presence of protein categories changed after treatment by peptide libraries compared to the initial non-treated sample.

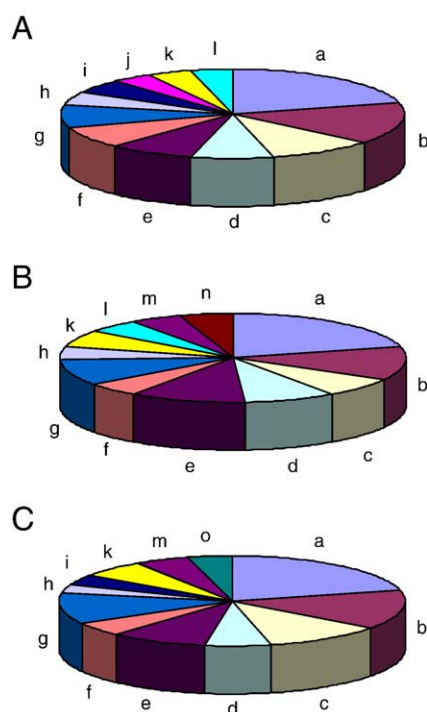


Fig. 5 – Gene Ontology classification of identified proteins from *H. brasiliensis*. A: GO pie of proteins found from the initial non-treated material; B: gene products found after treatment with Library-1; C: gene products found after treatment with Library-2. The gene products belong to the following categories: a: cytoplasm; b: ATP binding; c: protein binding; d: plasma membranes; e: nucleotide binding; f: metabolic processes; g: translation; h: catalytic activity; i: oxidation reduction; j: mitochondrium; k: nucleus; l: membrane; m: ribosome; n: protein phosphorylation; o: aminoacyl-tRNA ligase. For more detailed quantitative analysis see Table 3.

4. Discussion

The present data represent the first in-depth exploration of *H. brasiliensis* latex proteome as a whole since, as stated in Section 1, no such study has been reported before on this specific subject. The only proteomic study of any latex appears to be the one by Cho et al. [43] on lettuce latex. Latex is not an easy material to deal with because it contains polymerized large molecules on which proteins may be associated. Treatments that are necessary to (i) get a clear protein solution and (ii) remove material that could interfere with peptide library treatment [32], may also be a significant source of protein losses. In spite of these difficulties the identification of no less than 300 unique gene products reported in the present work expands considerably the knowledge of this proteome, in which the identification of only a part of the potential allergens had been reported so far. However, compared to transcriptomics investigations what is the depth of this exploration remains to be seen. According to Chow et al. [8] thousands of unique transcripts could be obtained in *Hevea* latex, which in principle should be assumed to contain just as many protein species. Where this is the case, it would mean that we have been able to detect a fraction of the entire proteomic asset of this latex. However, according to the same authors [8], the Gene Ontology convention showed that a large majority of such transcripts were related to genes of unknown function. Moreover, Ko et al. [7] state that, unexpectedly, several genes involved in the rubber biosynthesis are expressed at very low levels in the latex, suggesting that a

large number of proteins might be present well below the detection limit. It might be argued that the peptide library as a means to increase the concentration of low-abundance proteins should solve this problem, as indeed it occurred in several applications, in which the number of proteins detected after library treatment was two to five folds higher than in the control [17–21]. Here, however, the extra number of proteins detected after library treatment has been of only 64 species, resulting in an increment of 25% as compared to the untreated sample. This might be due to two reasons. To start with, latex contains many hydrophobic proteins that may have been present in the hydro-organic ignored fraction from peptide library elution. This fraction was ignored because it did not contain enough proteins for an extensive proteomics analysis, but trace compounds present could contain hydrophobic proteins that escaped the identification. As a second possible explanation, in general we have always operated the library beads under strong overloading conditions, almost in conflict with “good laboratory practice” in protein chromatography. This has permitted to capture enough low-abundance material so as to facilitate its detection. As an example, in the case of the human red blood cell proteome, in which we could detect as many as 1578 unique gene products [44], as compared to barely 252 in the control, the two libraries used were massively overloaded with 6g total lysate! In the present case, we were limited in the amount of sample we could obtain (barely 500 mg). In addition, plant material suffers from severe interference with separation materials [45–47], requiring purification from polymeric material (e.g., polyphenols, polysaccharides, and all polymer-like acidic materials) that could strongly stick to the library beads hampering thus the binding of proteinaceous species [32]. The protocol adopted in the present work (precipitation in ammonium sulphate saturated solution) might have not been sufficient to remove polymeric material on the one hand and might not have been stringent enough for precipitating low-abundance proteins, on the other hand, which might have thus been lost in the supernatant. These concomitant factors could have substantially reduced the capture on the peptide library beads.

Nevertheless the treatment with peptide libraries revealed a number of interesting species not yet described in *H. brasiliensis* latex proteome. From the ontology graphic observation (Fig. 5) it can be said that the categories that are mostly modified in their relative concentration were proteins involved in translational processes, ribosome proteins, amino acid phosphorylation proteins and aminoacyl-tRNA ligase activity. The last three categories, that were detectable only after treatment with the peptide libraries, represented about 13% of the total findings. Translated into a number this accounts for 32 new gene products, which is about one half of new discovered species. When comparing the Gene Ontology classes absent from the initial non-treated biological sample to the list of gene products discovered upon library treatment (see Table 2), a good coherence could be underlined.

Outside and in addition to the in-depth mapping of latex proteome, we have explored the presence of potential allergens in these samples, with the aim of tracking down trace species that have most often escaped detection so far. It is in fact known, among workers in the field of rubber production and in the medical field, that beyond the 13 latex allergens officially

accepted for diagnostic, the latex allergome could comprise more protein species, including isoforms as here described. It has been reported that for a complete diagnosis of allergy against *Hevea* latex the concomitant characterization of the allergens from the official list is not enough for a proper understanding [48]. It is in this context that more recently it has been proposed to produce an allergen-enriched *Hevea* protein fraction obtained by ion exchange chromatography on a strong cationic resin [49]. Indeed the authors demonstrated the significant increase of signals from a 2D electrophoresis exposed to several sera from allergic individuals. Unfortunately no identification of proteins was made from those obtained fractions.

The combinatorial peptide ligand library technique allowed detecting both dominant and low-abundance (hidden) proteins. Even if some of the allergens were highlighted in all samples (starting material, Library-1 and Library-2): Hev b 3, proteasome subunit and glyceraldehyde-3-phosphate dehydrogenase, some others were detectable (“un-hidden”). Indeed, from our experiments with the three patient sera, unambiguously and at once, we can detect through the use of peptide ligand libraries several *H. brasiliensis* allergens: two listed in the I.U.I.S. nomenclature (Hev b 2 in the Library-1 and Hev b 9 in the Library-2) and two out of the nomenclature (heat shock protein and protease inhibitor in the Library-2). Moreover, via the MS analysis, some other allergens have been characterized: the pan-allergen profilin (Hev b 8 in the Library-1 and the Library-2), Mn-superoxide dismutase (Hev b 10) and the citrate-binding protein. Another interesting aspect of the present work is the extreme heterogeneity of most of the allergens listed in Table 2, both in terms of charge (isoforms) and in terms of size (most likely proteolytic fragments generated either *in vivo* or *in vitro*). It has to be mentioned that some *H. brasiliensis* allergens already described in the literature, as for instance, the isoflavone reductase, thioredoxine and triose-phosphate isomerase [3,41,50] are not revealed by our series of experiments with the combinatorial peptide ligand library technique.

5. Conclusions

The treatment of *H. brasiliensis* latex proteins with peptide libraries allowed evidencing a significant number of so far undescribed gene products complementing thus the list of proteins expressed in this biological fluid. A major interest of exploring this latex proteome was the identification of often “hidden” allergens with the aim of confirming and elongating the list of such important antigens for diagnostic applications. Actually it is known that the number of allergens is large (perhaps several dozens), but diagnostic tests are most of the time based on the first 13 allergens from the official list. Among the found allergens, it was possible to identify, in the same experimental process, a substantial number of already known species plus additional other gene products, most of them present in a large number of isoforms. For instance glyceraldehyde-3-phosphate dehydrogenase was never described except in wheat flour and molds that are, phylogenetically, very distant from *H. brasiliensis*. It is here suggested that the use of peptide library could be a possible tool to enhance the presence of some latex allergens that are otherwise very difficult to detect. Such

an approach would increase the probability of finding allergenic reaction outside the well-known thirteen *Hevea* antigens. The preparation of latex sample by treatment with peptide libraries appears in fact far simpler than a fractionation by anion exchange chromatography followed by a salt gradient elution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:[10.1016/j.jprot.2010.03.002](https://doi.org/10.1016/j.jprot.2010.03.002).

REFERENCES

- [1] Sookmark U, Pujade-Renaud V, Chrestin H, Lacote R, Naiyanetr C, Seguin M, et al. Characterization of polypeptides accumulated in the latex cytosol of rubber trees affected by the tapping panel dryness syndrome. *Plant Cell Physiol* 2002;43:1323–33.
- [2] Alenius H, Kalkkinen N, Lukka M, Turjanmaa K, Reunala T, Makinen-Kiljunen S, et al. Purification and partial amino acid sequencing of a 27-kD natural rubber allergen recognized by latex-allergic children with spina bifida. *Int Arch Allergy Immunol* 1995;106:258–62.
- [3] Posch A, Chen Z, Wheeler C, Dunn MJ, Raulf-Heimsoth M, Baur X. Characterization and identification of latex allergens by two-dimensional electrophoresis and protein microsequencing. *J Allergy Clin Immunol* 1997;99:385–95.
- [4] Chardin H, Raulf-Heimsoth M, Chen Z, Rihs HP, Mayer C, Desvaux F-X, et al. Interest of 2-D electrophoretic analysis for the characterization of the individual sensitization to latex allergens. *Inter Arch Allergy Immunol* 2002;128:195–203.
- [5] Han KH, Shin DH, Yang J, Kim IJ, Oh SK, Chow KS. Genes expressed in the latex of *Hevea brasiliensis*. *Tree Physiol* 2000;20:503–10.
- [6] Ko JH, Chow KS, Han KH. Transcriptome analysis reveals novel features of the molecular events occurring in the laticifers of *Hevea brasiliensis* (para rubber tree). *Plant Mol Biol* 2003;53:479–92.
- [7] Chow KS, Wan KL, Isa MN, Bahari A, Tan SH, Harikrishna K, et al. Insights into rubber biosynthesis from transcriptome analysis of *Hevea brasiliensis* latex. *J Exp Bot* 2007;58:2429–40.
- [8] Chardin H, Chen Z, Raulf-Heimsoth M, Mayer C, Sénéchal H, Desvaux F-X, et al. Identification of Hev b 1 in natural latex mattresses. *Int Arch Allergy Appl Immunol* 2000;121:211–4.
- [9] Chen Z, Posch A, Cremer R, Posch A, Raulf-Heimsoth M, Rihs HP, et al. On the allergenicity of Hev b 1 among health care workers and patients with spina bifida allergic to the natural rubber latex. *J Allergy Clin Immunol* 1997;100:684–93.
- [10] Chen Z, Posch A, Lohaus C, Raulf-Heimsoth M, Meyer HE, Baur X. Isolation and identification of hevein as a major IgE-binding polypeptide in *Hevea* latex. *J Allergy Clin Immunol* 1997;99:402–9.
- [11] Yeang HY, Hamilton RG, Bernstein DI, Arif SA, Chow KS, Loke YH, et al. Allergen concentration in natural rubber latex. *Clin Exp Allergy* 2006;36:1078–86.
- [12] Chardin H, Desvaux FX, Mayer C, Sénéchal H, Peltre G. Protein and allergen analysis of latex mattresses. *Int Arch Allergy Immunol* 1999;119:239–46.
- [13] Yeang HY, Chow KS, Yusof F, Arif SA, Chew NP, Loke YH. Appraisal of latex glove proteins in the induction of sensitivity to multiple latex allergens. *J Investig Allergol Clin Immunol* 2000;10:215–22.
- [14] Kolarich D, Altmann F, Sunderasan E. Structural analysis of the glycoprotein allergen Hev b 4 from natural rubber latex by mass spectrometry. *Biochim Biophys Acta* 2006;1760:715–20.
- [15] Gonzales-Buitrago JM, Ferreira I, Isidoro-Garcia M, Sanz C, Lorente F, Davila I. Proteomic approaches for identifying new allergens and diagnosing allergic diseases. *Clin Chim Acta* 2007;385:21–7.
- [16] Righetti PG, Boschetti E, Lomas L, Citterio A. Protein Equalizer technology: the quest for a “democratic proteome”. *Proteomics* 2006;6:3980–92.
- [17] Boschetti E, Lomas L, Citterio A, Righetti PG. Romancing the “hidden proteome”, Anno Domini two zero zero seven. *J Chromatogr A* 2007;1153:277–90.
- [18] Guerrier L, Righetti PG, Boschetti E. Reduction of dynamic protein concentration range of biological extracts for the discovery of low-abundance proteins by means of hexapeptide ligand library. *Nature Protocols* 2008;3:883–90.
- [19] Boschetti E, Righetti PG. The ProteoMiner in the proteomic arena: a non-depleting tool for discovering low-abundance species. *J Proteomics* 2008;71:255–64.
- [20] Righetti PG, Boschetti E. The ProteoMiner and the FortyNiners: searching for gold nuggets in the proteomic arena. *Mass Spectrom Rev* 2008;27:596–608.
- [21] Boschetti E, Righetti PG. The art of observing rare protein species in proteomes with peptide ligand libraries. *Proteomics* 2009;9:1492–510.
- [22] D'Amato A, Bachi A, Fasoli A, Boschetti E, Peltre G, Senechal H, et al. In-depth exploration of cow's whey proteome via combinatorial peptide ligand libraries. *J Proteome Res* 2009;8:3925–36.
- [23] Blanco C, Carrillo T, Ortega N, Alvarez M, Dominguez C, Castillo R. Comparison of skin-prick test and specific serum IgE determination for the diagnosis of latex allergy. *Clin Exp Allergy* 1998;28:971–6.
- [24] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.
- [25] Righetti PG. Immobilized pH gradients. Theory and methodology. Amsterdam: Elsevier; 1990.
- [26] Hamdan M, Righetti PG. *Proteomics Today*. Hoboken: Wiley; 2005. 346–8.
- [27] Demeulemester C, Peltre G, Laurent M, Marchand F, David B. Cyanogen bromide-activated nitrocellulose membrane: a new tool for immunoprinting techniques. *Electrophoresis* 1987;8:71–3.
- [28] Simó C, Bachi A, Cattaneo A, Guerrier L, Fortis F, Boschetti E, et al. Performance of combinatorial peptide libraries in capturing the low-abundance proteome of red blood cells. 1. Behavior of mono- to hexapeptides. *Anal Chem* 2008;80:3547–56.
- [29] Olsen JV, de Godoy LM, Li G, Macek B, Mortensen P, Pesch R, Makarov A, Lange O, Horning S, Mann M. Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. *Mol Cell Proteomics* 2005;12:2010–21.
- [30] Craig R, Beavis RC. TANDEM: matching proteins with tandem mass spectra. *Bioinformatics* 2004;20:1466–7.

- [31] Binns D, Dimmer E, Huntley R, Barrell D, O'Donovan C, Apweiler R. QuickGO: a web-based tool for Gene Ontology searching. *Bioinformatics* 2009;25:3045–6.
- [32] Boschetti E, Bindschedler LV, Tang C, Fasoli E, Righetti PG. Combinatorial peptide ligand libraries and plant proteomics: a winning strategy at a price. *J Chromatogr A* 2009;1216:1215–22.
- [33] Aamir R, Safadi GS, Melton AL, Wagner WO, Pien LC, Cornish K, et al. Shared IgE-binding sites among separated components of natural rubber latex. *Int Arch Allergy Immunol* 1996;111:48–54.
- [34] Barbas C, Castro M, Herrera E. A simplified measurement of protein in natural latex. *Allergy* 1998;53:720–2.
- [35] Baur X, Chen Z, Raulf-Heimsoth M, Degens P. Protein and allergen content of various natural latex articles. *Allergy* 1997;52:661–4.
- [36] Kanokwiroon K, Teanpaisan R, Wititsuwannakul D, Hooper AB, Wititsuwannakul R. Antimicrobial activity of a protein purified from the latex of *Hevea brasiliensis* on oral microorganisms. *Mycoses* 2008;51:301–7.
- [37] Guarneri F, Guarneri C, Guarneri B, Benvenga S. In silico identification of potential new latex allergens. *Clin Exp Allergy* 2006;7:916–9.
- [38] Sander I, Flagge A, Merget R, Halder TM, Meyer HE, Baur X. Identification of wheat flour allergens by means of 2-dimensional immunoblotting. *J Allergy Clin Immunol* 2001;107:907–13.
- [39] Benndorf D, Müller A, Bock K, Manuwald O, Herbarth O, von Bergen M. Identification of spore allergens from the indoor mould *Aspergillus versicolor*. *Allergy* 2008;4:454–60.
- [40] Hong SG, Cramer RA, Lawrence CB, Pryor BM. Alt a 1 allergen homologs from *Alternaria* and related taxa: analysis of phylogenetic content and secondary structure. *Fungal Genet Biol* 2005;42:119–29.
- [41] Yagami T, Haishima Y, Tsuchiya T, Tomitaka-Yagami A, Kano H, Matsunaga K. Proteomic analysis of putative latex allergens. *Int Arch Allergy Immunol* 2004;135:3–11.
- [42] Li L, Sun CJ, Freeby S, Yee D, Kieffer-Jaquinod S, Guerrier L, et al. Protein sample treatment with peptide ligand library: coverage and consistency. *J Proteomics and Bioinformatics* 2009;2:485–94.
- [43] Cho WK, Chen XY, Uddin NM, Rim Y, Moon J, Jung JH, et al. Comprehensive proteome analysis of lettuce latex using multidimensional protein-identification technology. *Phytochemistry* 2009;70:570–8.
- [44] Roux Dalvai F, Gonzalez de Peredo A, Simó C, Guerrier L, Bouyssie D, Zanella A, et al. Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry. *Mol Cell Proteomics* 2008;7:2254–69.
- [45] Rabilloud T. Solubilization of proteins for electrophoretic analyses. *Electrophoresis* 1996;17:813–29.
- [46] Gegenheimer P. Preparation of extracts from plants. *Methods Enzymol* 1990;182:174–93.
- [47] Isaacson T, Damasceno CMB, Saravanan RS, He Y, Catala C, Saladie M, et al. Sample extraction techniques for enhanced proteomic analysis of plant tissues. *Nat Protocols* 2006;1:769–74.
- [48] Arif SA, Hamilton RG, Yusof F, Chew NP, Loke YH, Nimkar S, et al. Isolation and characterization of the early nodule-specific protein homologue (Heb b13) an allergenic lipolytic esterase from *Hevea brasiliensis* latex. *J Biol Chem* 2004;279:23933–41.
- [49] Wagner S, Bulbin M, Hafner C, Kopp T, Allwardt D, Seifert U, et al. Generation of allergen enriched protein fraction of *Hevea brasiliensis* latex for in vitro and in vivo diagnosis. *Allergy and Immunology* 2007;143:246–54.
- [50] Conti A, Giuffrida MG, Hoffmann-Sommergruber K, Wagner S, Amato S, Mistrello G, et al. Identification of latex UDP glucose pyrophosphorylase (Hev b UDPGP) as a novel cause of latex fruit allergy syndrome. *Eur Ann Allergy Clin Immunol* 2007;39:116–8.