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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), hevamine 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.

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, hevamine 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-propansulfonate (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