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PROTEIN PROFILING OF Hevb1 AND Hevb3 IN *Hevea* SPECIES OBTAINED FROM THE 1995 GERMPLASM COLLECTION

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ABSTRACT

Most of commercial latex products originated from natural rubber produced by *Hevea brasiliensis*. *Hevea* latex contained of rubber particles that are linked with numerous allergens protein. The allergens protein associates with rubber particles can cause allergic reaction that range from contact and systemic urticarial to asthma, anaplyaxis and even death. Although allergen proteins are well known to be involved in rubber synthesis of *H. brasiliensis*, their presence in this species as well as other *Hevea* species is still need to be determined. For sustainability production of high demanding commercial latex, this study is conducted to investigate the protein profiling of two allergens on eight *Hevea* species obtained from the 1995 germplasm collection. Latex collected from eight *Hevea* species were prepared to detect allergens protein of Hevb1 and Hevb3 using SDS Polyacrylamide Gel (SDS-PAGE). Immunoblotting technique was used to evaluate compatibility of allergens protein to bind with monoclonal and polyclonal antisera. The results showed that both of Hevb1 and Hevb3 proteins were detected in eight *Hevea* species. However, Hevb1 and Hevb3 proteins showed different ability in binding the monoclonal and polyclonal antisera. Data on protein profiling of eight *Hevea* species constitute a potential source of good trait in future plant breeding or as a key to have a future rubber tree with less or allergen free latex.

Key words: *Hevea* species, latex, allergens protein, Hevb1, Hevb3

INTRODUCTION

Hevea species mainly *Hevea brasiliensis* has been cultivated worldwide for its natural rubber production known as latex. Latex is a white colloidal suspension that produced in laticifers, a specialized parenchyma cells in the bark of *Hevea* tree (van Beilen, 2007). After ultracentrifugation, the latex can be separated into three separate layers namely rubber particle, cytoplasmic C-serum and lutoid (Berthelot *et al*., 2014a; Hwee, 2014). These particles suspended freely in a liquid cytotosol or cytoplasmic matrix bound by a cytoplasm. Through ultrastructural analysis, the rubber particles from different species showed globular structures, which contains a homogeneous hydrophobic core of polyisoprene surrounded by monolayer contains of lipid and proteins (Wood & Cornish, 2000; Nawamawat *et al*., 2011; Li *et al*., 2014).

Based on *H. brasiliensis* bimodal size distribution, rubber particle mainly contain two components, which are small rubber particles (SRPs) and large rubber particles (LRPs) that showed differences in molecular weight distribution, molecular structure and in their surface proteins (Bahri & Hamzah, 1996; Ohya *et al*., 2000; Tarachiwin *et al*., 2005; Berthelot *et al*., 2014b). The size of rubber particles ranged from 0.08-2.00 µm and the thickness of monolayer ranged from 1.5-4.0 nm in *H. brasiliensis* (Cornish *et al*., 1999; Rochette *et al*., 2013). Presence of proteins in rubber particles is well known to constitute major latex allergens, which are necessary in rubber synthesis. Through proteome analysis, a total of 1300 proteins are presence in the latex and almost 600 are spotted in rubber particles (Wang *et al*., 2010). Later, Dai *et al*. (2013) only identified 186 different proteins in the rubber particles. Out of 186-rubber particle protein, at least 14 rubber particle proteins including Hevb1 until Hevb14 have been identified as source of allergens in *Hevea* latex (Dai *et al*.,

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2013; WHO-IUIS, 2013). From these, both Hevb1 (14kDa) and Hevb3 (24kDa) are abundant allergen protein in *Hevea* latex and known as rubber elongation factor (REF) and small rubber particle protein (SRPP), respectively (Dennis & Light, 1989; Oh *et al*., 1999; Berthelot *et al*., 2014b). These proteins are quite unique because they are insoluble in water compared to the rest. Due to this property they remained strongly attached to the surface of the rubber particles even after repeated attempt of washing (Bahri & Hamzah, 1996).

Hevb1 (REF) is a 137 amino acid protein (Dennis *et al*., 1989; Sussman *et al*., 2002) and is found tightly anchored on large rubber particles (LRPs, zone 1 with diameters over 0.4 μm) (Yeang *et al*., 2002a). Its specific role is still unknown but is believed to help in anchoring and positioning the phenyltranferase enzyme to the rubber molecule as well in positioning and protecting the growing pyrophosphate ends on the rubber molecule (Dennis *et al*., 1989). On the other hand, Hevb3 (SRPP) protein is tightly anchored with small rubber particles (SRPs, zone 2 with smaller than 0.4 μm in diameter) (Cornish, 2001; Yeang *et al*., 2002a; Singh *et al*., 2003). The protein is identified as prenyltransferase (Oh *et al*., 1999; Hamilton, 2002) that functions in catalysing the incorporation of isopentenyl diphosphate (IDP) into the rubber polymer (Dennis & Light, 1989; Oh *et al*., 2000; Singh *et al*., 2003).

Previous study reported that REF and SRPP were only detected on LRPs and SRPs, respectively. Later, studies conducted by several researchers reported that both REF and SRPP can be found in both types of particles when visualized under immunogold electron microscopy (Dennis & Light, 1989; Yeang *et al*., 1996; Bahri & Hamzah 1996; Singh *et al*., 2003). Although present of REF or SRPP are regarded as unnecessary for rubber synthesis in all latex producing plant, but they play significant role in rubber production in *Hevea* species (Singh *et al*., 2003; Wititsuwannakul *et al*., 2008). According to Yeang *et al*. (2002b), Hevb1 has been recognized being involved in rubber biosynthesis due to its amino acid similarities, thus presence of Hevb3 is also suggested to play the same role (Oh *et al*., 1999).

Several plant trees such as Russian dandelion (*Taraxacum koksaghyz* Rodin) and Guayule (*Parthenium argentatum*) are able to produce natural rubber (Ray, 1993; van Beilen & Poirier, 2007). In terms of physical properties, production of natural rubber from these species cannot be compensating with natural rubber qualities of *H. brasiliensis* for commercial purpose (van Beilen & Poirier, 2007). For this reason, *Hevea* latex has high marketable value and highly demand especially in industrial polymer. However, the presence of allergen proteins (Hevb1 and Hevb3) in latex products become a major concern especially for medical device (Brittner *et al*., 2016). Proteins eluting from commercial latex products such as glove, toys, balloons, baby pacifiers can cause immediate type I allergic reactions, and the symptoms ranged from contact and systemic urticarial to asthma, anaphylaxis and even death (Palosuo *et al*., 2011; Gawchik, 2011; Raulf, 2014).

In order to meet the global rubber demand, further improvement in term of breeding and selection of *Hevea* species is necessary. Fundamental knowledge on rubber biosynthetic process at the tissue, cellular and molecular level is crucial which directly affect rubber production and accumulation from this *Hevea* tree. Lack of study has been conducted on characterization and comparison of proteins associated with the rubber particle membrane in *Hevea* species. Besides that, do other *Hevea* species share similar rubber particle membrane proteins with *H. brasiliensis* are still unknown. Therefore, the objective of this study is to detect presence of potential allergens (Hevb1 and Hevb3) in eight *Hevea* species for further understanding rubber biosynthesis especially in latex allergens.

MATERIALS AND METHODS

Plant materials

Latex collected from eight *Hevea* species namely *H. brasiliensis, H. benthamiana, H. pauciflora, H. camargoana, H. nitida, H. guinensis, H. rigidifolia* and *H. spruceana* obtained from the 1995 germplasm collection, Bt. Arang, Selangor were used in this study. Small puncture to the bark on untapped *Hevea* trees was conducted to collect the latex. Milky sap oozed from trees was kept into 1ml Eppendorf tubes and ice-cold until it reaches to the laboratory.

Preparation of washed rubber particles

All latex samples were centrifuged at 19 000 r.p.m (43 000 g) in a Sorvall RC5B at 4ºC to separate rubber particles into three zones: rubber cream zone (zone 1 and zone 2 that centripetally separate) and 'bottom fraction' zone that mainly contain lutoids and the C-serum. The non-rubber fractions were discarded while zone 1 associated with LRPPs (top creamy rubber particles) and zone 2 associated with SRPs (transparent-bottom part of creamy rubber particles) was collected prior to protein analysis (Bahri *et al.,* 1993).

Detection of protein, SDS polyacrylamide gel (SDS-PAGE) and immunoblotting

Rubber particles proteins were solubilized by incubating in a detergent solution containing 0.1% (w/v) Triton X-100 and 1% (w/v) SDS. The detergent-treated suspension was centrifuged at 13 000 r.p.m for 10 min and the supernatant fraction was subsequently used for protein separation in SDS PAGE. Proteins of rubber particle membrane were separated through SDS-PAGE using 15% (w/v) gel. The electrophoresis gel was then transferred onto a nitrocellulose membrane (0.45 µm pore size) according to the method suggested by Mayer & Walker (1995). The membrane strip containing a duplicate lane was stained with Coomasie blue solution for successful transfer of proteins. Then, the membrane was blocked with 10% spray dried milk in phosphate buffered saline (PBS) for 30 min, followed by incubation with a polyclonal 14kDa and 24kDa antibodies (1:500) at room temperature with agitation for 1 h. After washing with PBS (4 x 10 min), the membrane was incubated with goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1: 10 000) for 30 min followed by another wash with PBS (4 x 10 min). The membrane was then transferred to a colour development buffer (PBS) for 30 min followed by a substrate solution (Sigma FAST DAB) to visualize the immunoreactive proteins (indicated by the colour development). For a monoclonal incubation, a rat monoclonal of 14 kDa (Clone 6H3)-DACI #J9221)/(1:1000)) supplied by The John Hopkins University and 24 kDa (mono R c2 hev b^3) supplied by Universiti Sains Malaysia (undiluted) were used.

RESULTS AND DISCUSSION

Profiling of two-membrane protein, Hevb1 and Hevb3 have been conducted in the latex from eight *Hevea* species. Protein band detection was done in rubber particles of both large rubber particles (LRP, zone 1) and small rubber particle (SRP, zone 2). From SDS-Polyacrylamide Gel analysis, the protein band was detected in all the species with molecular weight, 14 kDa (Fig. 1). All the species namely *H. brasiliensis, H. benthamiana, H. pauciflora, H. camargoana, H. nitida, H. guinensis, H. rigidifolia* and *H. spruceana* obtained from the 1995 germplasm collection strongly expressed the Hevb1 protein in both of the zones. Most latex producing plant especially *Hevea* genus are found to produce protein of Hevb1 and Hevb3 which are from rubber elongation factor (REF) and small rubber particles (SRPP) family (Berthelot *et al*., 2014b; Berthelot *et al*., 2014c).

These proteins are homologous, water insoluble acidic proteins, negatively charges and have a size of 14.6 and 24 kDa, respectively (Yeang *et al*., 1996). All *Hevea* species in this study produced Hevb3 protein band with molecular weight 24 kDa in zone 1 and only *H. spruceana* was absent in Hevb3 protein band in zone 2. Both of the proteins were strongly expressed in *H. brasiliensis* as compared to other *Hevea* species. In addition, *H. benthamina* also showed high band expression of Hevb3 protein only in zone 2. A study conducted by Berthelot *et al*. (2014a, 2014b) also showed both proteins of 14kDa and 24kDa were easily detected in *Hevea* latex using mass spectrometry. This might

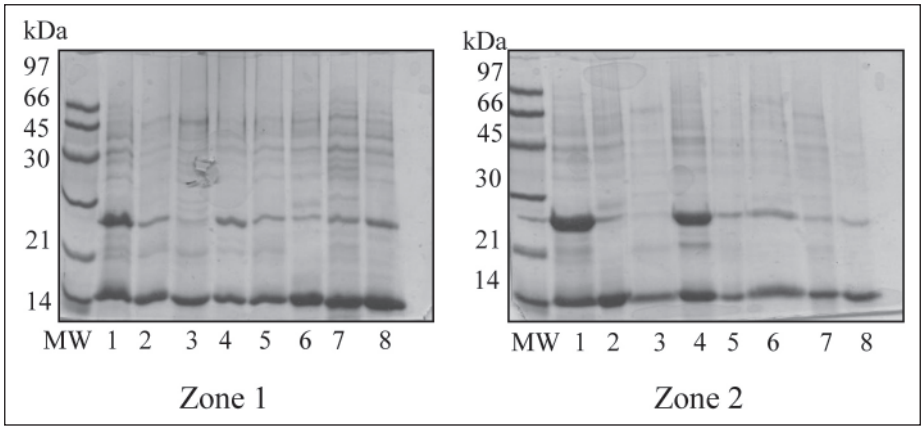

Fig. 1. SDS – Polyacryamide Gel Electrophoresis of rubber particle proteins from eight *Hevea* species from two centrifuged rubber fractions in zone 1 and zone 2. Lane MW: molecular weight marker, Lane 1: *H. brasiliensis*, Lane 2: *H. guainensis*, Lane 3: *H. spruceana,* Lane 4: *H. benthamiana*, Lane 5: *H. rigidifolia*, Lane 6: *H. camargoana* Lane 7: *H. nitida,* Lane 8: *H. pauciflora*.

be due to both *H. brasiliensis* and *H. benthamina* synthesis natural rubber at active rate compared to other species base on the intensity of protein present on the surface of their rubber particles.

High expression of Hevb1 band in *H. brasiliensis* is expected since it is the main protein in the latex. According to Berthelot *et al*. (2014b), Hevb1/REF is a predominant protein found in wash rubber particles, thus the protein is tightly bound to the rubber particle. Unlike Hevb3/SRPP, which is easily removed by simple washing. Presence of both proteins in most of *Hevea* species suggested that they are involved in rubber biosynthesis. Only Hevb1 protein has been identified to be involved in rubber biosynthesis due to its amino acid similarities (Yeang *et al*., 2002b). Although the role of Hevb3 is still unclear, it is suggested that presence of this protein is also associated with rubber production. Besides that, presence of both REF and SRPP on rubber particle was suggested due to its contribution in colloidal stability of the latex (Berthelot *et al*., 2014a).

In *Hevea* species, both proteins may help in increasing yield, molecular weight and quality of the natural rubber (Oh *et al*., 1999; Wagner *et al*., 1999). Similar to *H. spruceana*, absence of Hevb3 protein band is suggested that inactive of rubber biosynthesis activity in this species compare to other *Hevea* species. Considering negative detection of this allergen protein, *H. spruceana* has a good trait for breeding and its latex has good potential to reduce risk of latex-allergic products. In both zones of Fig. 1, we could also observed a few thinner bands between 14 to 24 kDa and above 24 kDa in all *Hevea* species, which could be attributed to protein contamination from the soluble C-serum and lutoids fraction (B-serum). This might be happened because omit of sucrose during rubber particle washing process due to insufficient amount of sample collected.

Immunoblotting analysis in this study used monoclonal and polyclonal antisera to compare their specificity against the rubber particle-associated membrane protein polypeptides. As shown in Fig. 2, monoclonal antiserum of 14 kDa obtained from the John Hopkins University and monoclonal antiserum of 24 kDa obtained from Universiti Sains Malaysia were binding specifically to 14kDa and 24

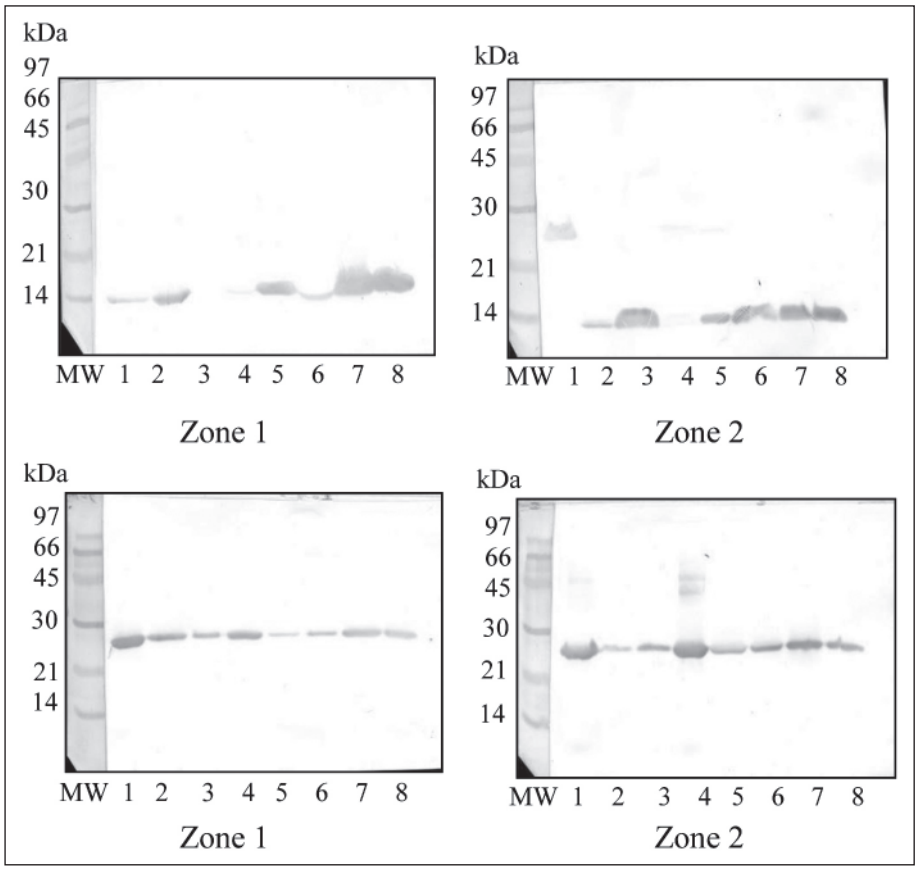

Fig. 2. Immunoblot analysis of antibody specificity probed with a monoclonal 14 kDa antiserum using Western blot in (a) zone 1 and (b) zone 2, and immunoblot analysis of antibody specificity probed with a monoclonal 24 kDa antiserum using Western blot in (c) zone 1 and (d) zone 2. Lane MW: molecular weight marker, Lane 1: *H. brasiliensis*, Lane 2: *H. guainensis*, Lane 3: *H. spruceana,* Lane 4: *H. benthamiana*, Lane 5: *H. rigidifolia*, Lane 6: *H. camargoana* Lane 7: *H. nitida,* Lane 8: *H. pauciflora*.

kDa *Hevea* proteins, respectively. In zone 1, monoclonal 14 kDa antiserum recognized the Hevb1 protein polypeptides in all *Hevea* species except in *H. spruceana*. There was no band detection in *H. spruceana* while a very faint band was observed in *H. benthamiana.* These results exhibited that the monoclonal 14 kDa antiserum did not bind with Hevb1 protein (14 kDa) in *H. spruceana* in large rubber particle while the same antiserum was slightly bound with Hevb1 protein in *H. benthamiana.* Negative result of protein binding in *H. spruceana* was obviously correlated with the result of protein fractionation from SDS-PAGE analysis as no potential allergen was presence in the rubber particle. In zone 2, the band was detected in all *Hevea* species except in *H. benthamiana*. In contrast, *H. spruceana* showed intense band as compare to zone 1 of the same species. This can be concluded that the allergen protein of Hevb1 was present only in zone 2 for *H. spruceana*. All the *Hevea* species have been confirmed to contain Hevb3 protein in large and small rubber particles (LRP, zone 1 and SRP, zone 2). Western blot from immunoblotting analysis showed clear band detection in all *Hevea* species for zone 1 and zone 2. This demonstrated that monoclonal 24 kDa antiserum was compatibly bound with Hevb3 protein in all *Hevea* species in both of the zones. Presence of Hevb1 and Hevb3 in most *Hevea* species can become a major concern with associated with latex allergy.

During polyclonal of 14 kDa and 24 kDa antibody were probed on to the blotted nitrocellulose membrane, a clear band of 14 kDa and 24 kDa molecular weight were detected in all *Hevea* species for both zone 1 and zone 2 (Fig. 3). This results demonstrated the specificity of antisera on the target protein despite they were polyclonal antisera. The similar results were reported by Bahri & Hamzah (1996) where polyclonal 24 kDa antiserum stained both the epitopes for 14 kDa and 24kDa protein polypeptides. Small rubber particles (SRP) and large rubber particle (LRP) are necessary components of rubber particles in *Hevea* latex that involved in rubber synthesis (Bahri & Hamzah, 1996; Sakdapipanish *et al*., 1999; Ohya *et al*., 2000;

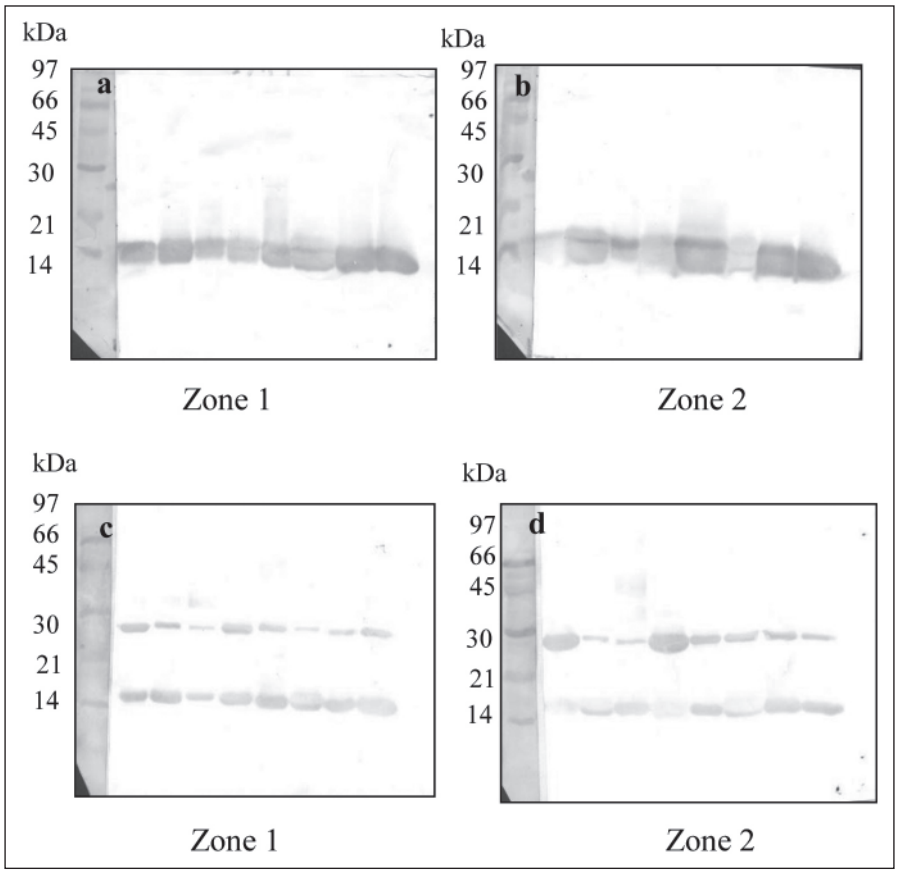

Fig. 3. Immunoblot analysis of antibody specificity probed with a polyclonal 14 kDa antiserum using Western blot in (a) zone 1 and (b) zone 2, and immunoblot analysis of antibody specificity probed with a polyclonal 24 kDa antiserum using Western blot in (c) zone 1 and (d) zone 2. Lane MW: molecular weight marker, Lane 1: *H. brasiliensis*, Lane 2: *H. guainensis*, Lane 3: *H. spruceana,* Lane 4: *H. benthamiana*, Lane 5: *H. rigidifolia*, Lane 6: *H. camargoana* Lane 7: *H. nitida,* Lane 8: *H. pauciflora*.

Tarachiwin *et al*., 2005). Although Hevb1 and Hevb3 are tightly associated with LRPs (zone 1) and SRP, (zone 2) (Cornish, 2001; Yeang *et al*., 2002b; Singh *et al*., 2003), presence of both Hevb1 and Hevb3 proteins in both zones for most of *Hevea* species was further confirmed that the proteins can be found in both types of the particles.

CONCLUSION

Protein profiling in eight *Hevea* species using SDS-PAGE analysis showed different profile of rubber particle membrane proteins present in all the species. *H. brasiliensis* and *H. benthamiana* showed higher intensity of both Hevb1 (14 kDa) and Hevb3 (24 kDa) protein whereas *H. spruceana* has very little or void of Hevb3 (24 kDa) protein in its latex. Immunoblotting analysis revealed that protein Hevb1 (14 kDa) is absence in *H. spruceana* (zone 1), which can be suggested that this species has high potential to produce latex with less or allergen free latex. For sustainability and health impact due to allergic reaction, selection of *Hevea* species with low intensity of allergen protein can be used as an alternative to meet global demand on latex products. This study also promotes the manipulation of different protein profiling in eight *Hevea* species to improve breeding selection in latex productivity.

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