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RESEARCH ARTICLE

IN VITRO SYNTHESIS OF LIGNIN-RESILIN BIOCOMPOSITES.

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Abstract

Lignin is one of the most abundant biopolymers in nature but is currently underused. Extensive research is to improve the market value of lignin and its utilization. This study presents an approach to alter the mechanical properties of lignin through its combination with recombinant resilin, a rubber-like protein. Different mass ratios of resilin were added to coniferyl alcohol, together with horseradish peroxidase and hydrogen peroxide, to generate a crosslinked lignin-like–resilin biocomposite, where the lignin-like component, the so-called dehydrogenation polymer, is the product of coniferyl alcohol crosslinking. Fourier transform infrared spectroscopy and western blot analysis indicated crosslinking of the resilin protein to dehydrogenation polymer. We show that resilin binding to dehydrogenation polymer leads to a reduction in the polymer particles size as well as an increase in mechanical stiffness. This study provides insight into the mechanisms of the in vivo and in vitro crosslinking of tyrosine rich structural proteins to lignin. The crosslinked lignin-protein complex may potentially impart improved properties to lignin and to the entire plant.

Introduction:-

Lignin is a branched aromatic polymer, the product of the oxidative combinatorial coupling of three main building blocks: coniferyl alcohol (C.A.), sinapyl alcohol and small amounts of *p*-coumaryl alcohol. Lignification occurs through the polymerization of these monomers, leading to the growth of the polymer, and branching occurs when two sequential reactions are possible at the phenolic end of the polymer. The phenylpropane units attach to one another by a series of characteristic linkages.(Ralph et al., 2004) Polymerization result in guaiacyl (G), syringyl (S) and p-hydroxyphenyl (H) units, along the lignin polymer.(Ralph et al., 2004; Vanholme et al., 2008; Van Acker et al., 2013) S lignin is unique to flowering plants, while H and G lignins are fundamental to all vascular plants. The S/G ratio varies greatly among plants and has an impact on lignin strength and internal crosslinks.(Weng and Chapple, 2010) Cell wall enzymes, either free or bound, including various types of peroxidase and oxidase enzymes, some of them with specific activity against coniferyl or sinapyl alcohol, have been shown to play a role in monolignol polymerization.(Donaldson, 2001)

Lignin is mainly formed in cells that are required to transport water and to withstand mechanical stress as lignification of the secondary wall layers of these cells increases their stiffness and strength. The number and distribution of

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lignified cells determines the growth phenotype and the mechanical properties of the plant.(Donaldson, 2001; Simmons et al., 2010) Previous attempts to alter the properties of the cell wall have mainly focused on reducing the amount of lignin or changing its structure in order to ease its extraction from the raw woody materials used in the pulp, paper, and biofuel industries.(Baucher et al., 2003; Vanholme et al., 2008)

Historically, lignin has been viewed as an industrial waste product and is mainly employed as a low-end by-product for energy applications. Recent studies have shown that lignin can be modified and utilized in an array of new products such as carbon fibers,(Kadla et al., 2002) bioplastics,(Calvo-Flores and Dobado, 2010) and thermoplastics.(Saito et al., 2012)

Lignin extraction from lignocellulosic materials usually requires severe conditions such as high temperature and pressure and the use of strong acids.(Tan et al., 2009) Therefore, studies on the polymerization mechanism are typically done by an in vitro system for artificial synthesis of dehydrogenation polymer (DHP), a lignin-like polymer that can be artificially synthesized in vitro by the dehydropolymerization of lignin precursors using peroxidase under oxidizing conditions.(Boerjan et al., 2003) Freudenberg et al. found that the dehydrogenation polymer (DHP) synthesized from the crosslinking of C.A. closely resembles spruce lignin in terms of functional group content, spectroscopic characteristics, and degradation products.(Freudenberg and Neish, 1968) However, DHP differs from natural lignin in terms of molecular weight, which is lower for DHP,(Tanahashi and Higuchi, 1981) and in chemical bond composition, in particular a lower β–O–4-ether content due to over-representation of dehydrodimerization reactions.(Ralph et al., 2004)

In this work, we propose a new way to manipulate the structure of lignin and its mechanical properties by introducing an elastic protein that can crosslink to the lignin monomers during lignin polymerization. In order to test this hypothesis, DHP was synthesized in the presence of the rubber-like protein resilin, which possesses a high tyrosine content that enables chemical crosslinking to the lignin monomers.

Resilin is an elastomeric protein found in the specialized regions of the cuticles of insects.(Weis-Fogh, 1960) Following its secretion into the extra cellular matrix, the protein binds to chitin, the main component of the arthropod cuticle and polymerizes through peroxidase-derived di-tyrosine bridge crosslinks. The integration between a tough polysaccharide scaffold (i.e., chitin) and an elastic protein (i.e., resilin) generates a biocomposite, which supports insects in a variety of functions that require efficient energy storage and long range elasticity.(Qin et al., 2009) As examples, resilin is responsible for the sound production of cicadas,(Young and Bennet-Clark, 1995) the flight system of locusts,(Weis-Fogh, 1960) the jumping mechanism in fleas and locusts,(Bennet-Clark and Lucey, 1967; Burrows and Sutton, 2012) and the durability of copepod opal teeth.(Michels et al., 2012) The resilin gene is comprised of three exons: exons I and III encode for elastic domains and exon II encodes for a chitin binding domain. The elasticity of the protein has been largely attributed to the exon I sequence.(Qin et al., 2012)

In vitro studies of resilin mechanical properties found that resilin is a soft elastomer, with a Young's modulus values of 50–300kPa, and ultimate tensile strength of 60–300kPa (depending of its source). In its native state, resilin outstanding ability to return to its original state following the removal of the applied stress (resilience) is of >92%, and the crosslinked protein can be elongated up to three times its original length before reaching mechanical failure.(Weis-Fogh, 1960) It was suggested by Bochicchio *et al*., that resilin elasticity is based on changes in entropy. When stress is applied resilin structure should be shifted from amorphous to a more organized structure leading to a significant decrease in entropy. When the stress is released, resilin is likely to return to its original relaxed state of high entropy.(Bochicchio et al., 2008)

In addition to its important role in the cuticles of insects, in vitro studies employing recombinant resilin (exon I) have found that resilin is able to modify and improve the mechanical properties of cellulose nanocrystal-resilin bionanocomposites,(Rivkin et al., 2015) epoxy-based structures(Verker et al., 2014) and collagen scapolds.(McGann et al., 2013; Sanami et al., 2015)

Other to polysaccharides and lignin, which make up the bulk of the plant cell wall, protein comprises approximately 0.5% of the dry weight of the cell wall.(Martius, 1992) There are indications that certain proteins may be crosslinked to cell wall components, such as lignin.(Showalter, 1993) Over the past few years, several studies have attempted an improved understanding of the mechanisms by which proteins are associated with lignin. These works have demonstrated the covalent crosslinking to DHP of amino acids and short peptides,(Cong et al., 2013; Diehl and Brown,

2014) and that the properties of the woody material and the extraction of reducing sugars can be altered by the expression of a tyrosine-rich peptide in the cell wall of a transgenic lines of poplars.(Liang et al., 2008) In this study, recombinant resilin (exon I) is crosslinked to DHP, which is a synthetic lignin-like polymer, to create a resilin-lignin biocomposite material. The results presented herein on the newly developed resilin-lignin biocomposite provide insight and direction toward a new way to modify lignin and tailor its properties both in vitro and in vivo.

Experimental procedure:-

2.1. Chemicals

The chemicals used in this work were purchased from Sigma-Aldrich. Recombinant resilin (Fig. 1) was produced according to the protocol described by Qin, G. et al.(Qin et al., 2011)

2.2. Synthesis of lignin DHP and DHP-resilin

10 mg of C.A. were dissolved in 0.5 mL acetone and added to three amounts of resilin: low (0.02 mg), medium (0.2-1.5 mg) and high (2-3 mg), HRP (64 units) and H_2O_2 (1.5 mM) in 12.5 mL of phosphate buffer (0.1 M, pH 7.4). The reaction mixture was incubated for 15 h at room temperature with shaking.

2.3. FTIR

Crude reaction products were centrifuged (18,000 ×*g*, 10 min, room temperature) and washed four times with deionized water. FTIR spectra of freeze-dried samples were obtained using a Nicolet 6700 (Thermo Fisher Scientific Inc., Waltham, MA). A mortar and pestle were used to combine 1mg of sample with 99 mg of KBr, which was then pressed into a pellet for FTIR spectroscopy.

2.4. Western blot analysis

Resilin was incubated overnight with C.A., HRP, and H_2O_2. Crude samples of resilin and coniferyl in mass ratios of 1:5 (high) and 1:50 (medium) were centrifuged (18,000 ×*g*, 10 min, room temperature) to isolate soluble and insoluble fractions. Samples were collected and analyzed by SDS-PAGE. In addition, a reaction mixture prepared without the addition of HRP and H_2O_2 was used as control. Proteins were transferred to nitrocellulose membranes using standard protocols. The proteins were detected using resilin polyclonal primary antibody and anti-rabbit alkaline-phosphates conjugated secondary antibodies. The membranes were developed with SIGMAFAST (Sigma-Aldrich) reagent.

2.5. DLS

Kinetic size measurements were performed on crude reaction products, after 30 min in a sonication bath (Elmasonic S10H, Elma Schmidbauer., Singen, Germany), by DLS using a Zetasizer Nano ZS (Malvern, UK). Crude DHP and resilin-DHP samples were measured three times at 25 °C in disposable polystyrene cuvettes. Zetasizer Software v 7.03 was used to analyze the data.

2.6. AFM

2.6.1 Imaging

The samples were imaged using a JPK Instruments (Berlin, Germany) NanoWizard3 AFM in tapping mode, with a Team Nanotec ISC75 cantilever.

2.6.2 Nanoindentation

The mechanical properties of DHP and resilin-DHP were evaluated on an Asylum Research MFP-3D-Bio AFM (Asylum Research, Santa Barbara, CA) in force mode using an AC240TS-R3 cantilever (k = 1.25 N/m, invOLS = 63.43 nm/V, set point = 1 V, probe radius = 9 ± 2 nm: cantilever was approximated as a punch with a power law of 1.0). All elasticity maps were taken over 5 × 5 μm^2 areas with a 20 × 20 $_{grid}$ of force curves per sample. Data was analyzed in Igor Pro 6.35A5 (Wavemetrics, Lake Oswego, OR, USA). Samples were prepared by drying a 50 µL drop of crude reaction products onto a glass slide.

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  1 RPEPPVNSYL PPSDSYGAPG QSGPGGRPSD SYGAPGGGNG GRPSDSYGAP GQGQGQGQGQ   60
 61 GQGQGQGQGQ GGYAGKPSDT YGAPGGGNGN GGRPSSSYGA PGGGNGGRPS DTYGAPGGGN  120
121 GGRPSDTYGA PGGGGNGNGG RPSSSYGAPG QGQGNGNGGR SSSSYGAPGG GNGGRPSDTY  180
181 GAPGGGNGGR PSDTYGAPGG GNNGGRPSSS YGAPGGGNGG RPSDTYGAPG GGNGNGSGGR  240
241 PSSSYGAPGQ GQGGFGGRPS DSYGAPGQNQ KPSDSYGAPG SGNGNGGRPS SSYGAPGSGP  300
301 GGRPSDSYGP PASGSGAGGA GGSGPGGADY DNDE
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Figure 1:- Amino acid sequence of synthetic resilin. Bold letters mark the tyrosine residues

Results:-

3.1 Resilin-DHP crosslinking

A series of resilin dilutions was combined with C.A. in order to prepare crosslinked resilin-DHP using a method described by Tanahashi in 1981(Tanahashi and Higuchi, 1981), with modifications. (The crosslinking of C.A. gives DHP, and thus the crosslinking of resilin and C.A. together is expected to yield resilin-DHP.) Fourier transform infrared (FTIR) was employed to verify the crosslinking reaction (Fig. 2). Figure 2 compares the spectra of DHP to the crosslinked mixture of resilin and C.A. at various mass ratios of protein to C.A. The introduction of protein is evident at the wavenumbers associated with amide functional groups, specifically, we observed an increase in the peak at 1660 cm^{-1} attributed to C=O stretching and the appearance of a shoulder peak at 1540 cm^{-1} due to N-H deformation with C-N stretching.(Diehl and Brown, 2014) Moreover, the trend becomes more pronounced as the protein concentration increases, providing further evidence for the presence of protein in the insoluble lignin-like fraction (similar to lignin, DHP is water insoluble).

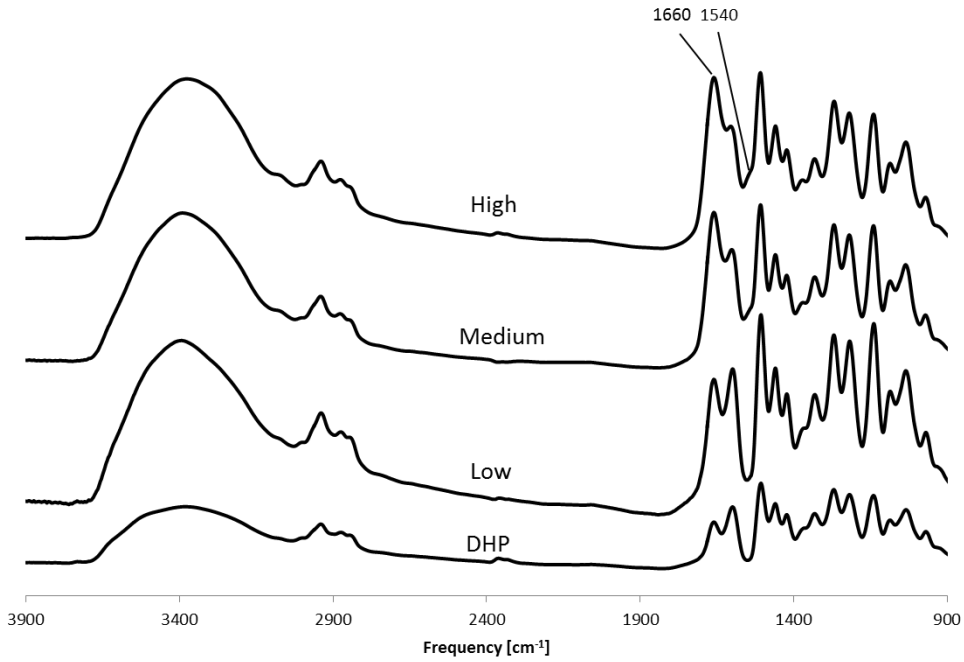

Figure 2:- FTIR spectra of DHP and cross-coupling products of resilin and C.A. at different resilin:C.A mass ratios: (bottom to top) DHP, Low, Medium, and High. The peak at 1660 cm^{-1} and the shoulder peak at 1540 cm^{-1} are emphasized by arrows.

3.2 Effect of crosslinking on the molecular weight of resilin

Western blot analysis was used to detect resilin and to determine its molecular weight at the end of the crosslinking reaction with C.A. (Fig. 3). After an overnight reaction, soluble and insoluble fractions were isolated and tested for the presence of resilin in order to determine whether resilin (soluble) was indeed included in the insoluble DHP fraction, indicative of a successful crosslinking reaction. Encouragingly, a majority of the resilin was detected together with the DHP in the insoluble fraction, at all protein concentrations. At the medium resilin:C.A. mass ratio, resilin was not at all detected in the soluble fraction, whereas the insoluble pellet showed two bands at 55 kDa and at 72 kDa (see Fig. 2; band at 55 kDa is indicated with an arrow). At the high resilin:C.A. mass ratio, monomeric resilin is detected both in the soluble and insoluble fractions (ca. 40 kDa), whereas the insoluble fraction displays additional bands at 55 kDa (denoted with an arrow in Fig. 2), 72 kDa, and 95 kDa, as well as a smear in the upper part of the lane due to a high molecular weight protein. A negative control reaction performed without hydrogen peroxide and horseradish peroxidase (HRP) showed most of the resilin located in the soluble fraction. As expected there was no evidence for the presence of resilin was observed in the soluble and insoluble fractions of pure DHP.

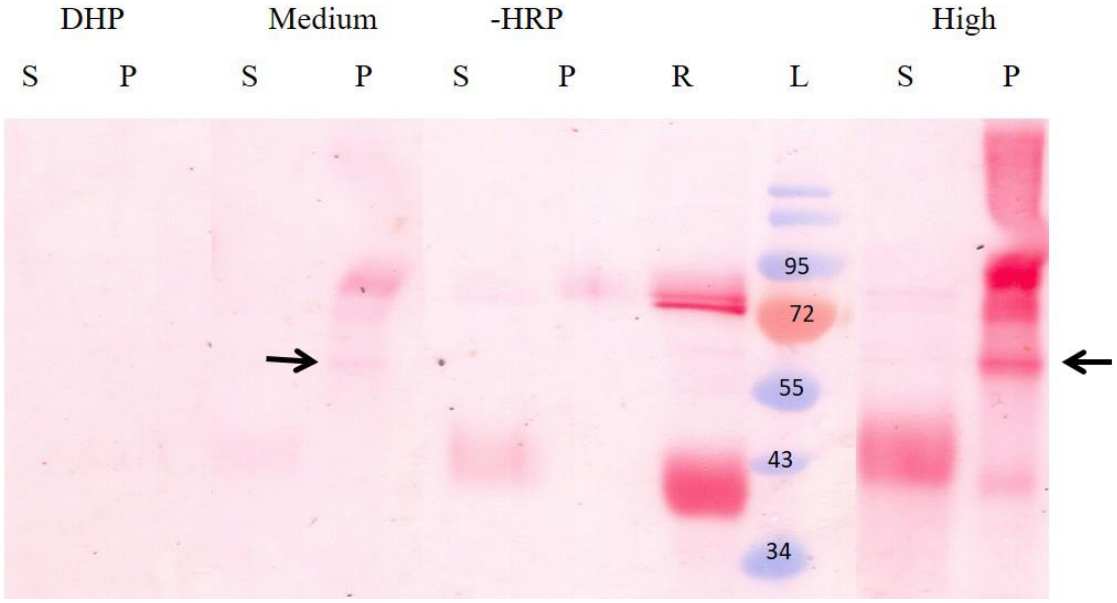

Figure 3:- Western blot analysis of soluble supernatant (S) and insoluble pellet (P) fractions from the crosslinking reaction of resilin and C.A. at resilin:C.A different mass ratios. From left to right: pure DHP, Medium, Medium without HRP and H_2O_2, protein ladder, bacterial resilin, and High resilin:C.A mass ratio. Resilin was detected using anti-resilin primary antibodies. Arrows mark a new band, located at approximately 55 kDa that was observed in the medium and high insoluble fractions.

3.3 Resilin-DHP cluster size

The effect of resilin content on the resilin-DHP suspension cluster size was evaluated by dynamic light scattering (DLS). Figure 4 shows the DLS sizes of DHP and of the crude reaction products of resilin and C.A. at different mass ratios. It appears that the cluster size is smaller in resilin containing samples compared with the pure DHP sample. Cluster size is smallest for the Low resilin:C.A. weight ratio, and increases with increasing resilin concentration in the sample.

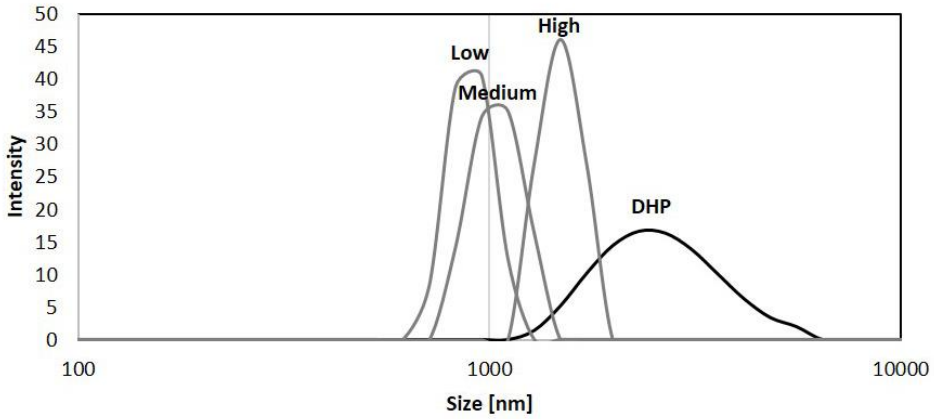

Figure 4:- Particle size distribution of DHP and crosslinked products of C.A and resilin in suspension at different mass ratios of resilin:C.A.

3.4 Effect of resilin on DHP morphology

The surface morphology of pure DHP and of resilin-DHP was studied by atomic force microscopy (AFM). Figure 5 presents surface scans (height mode) of films evaporated from pure DHP and crude resilin-DHP reaction products. Across the samples, there was no indication of a significant difference in either the size or shape of the particles. Spheres with diameters in the range of 300–600 nm were observed, regardless of protein content.

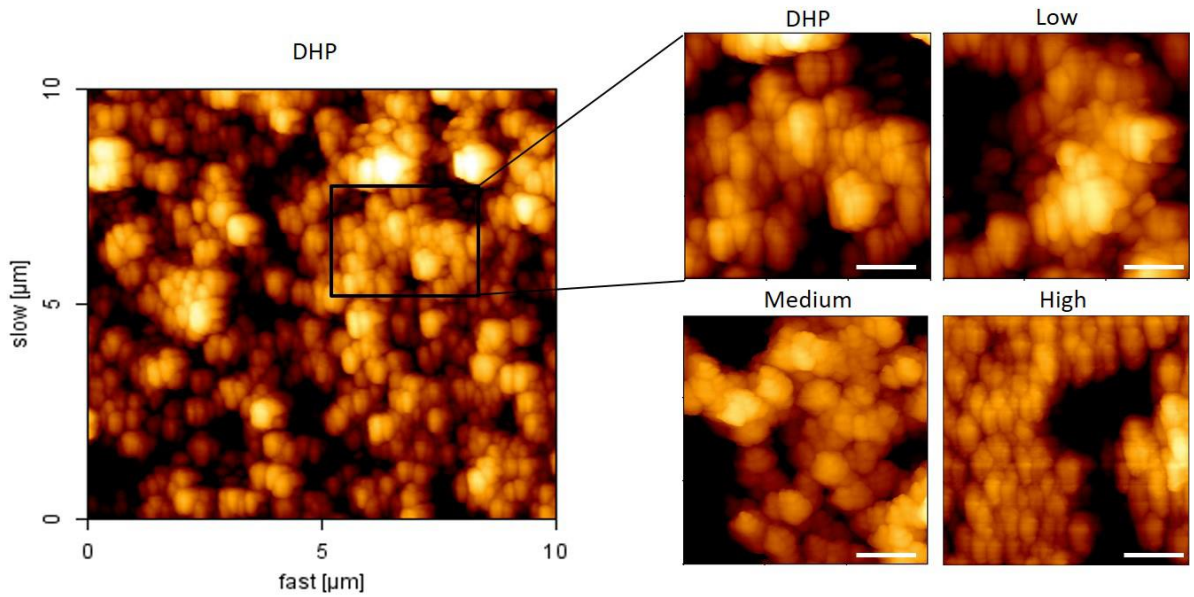

Figure 5:- AFM height images of films dried from pure DHP and from the crosslinked products of resilin and C.A. at different mass ratios of resilin:C.A. Bars represent 500 nm.

3.5 Effect of resilin content on the mechanical properties of DHP

Nanoindentation measurements were performed to quantify the mechanical properties of resilin-DHP. According to the results present in Figure 5, the elastic modulus increased by approximately 45% for the sample with the highest resilin content (1:5 resilin:C.A.) compared with pure DHP, however, no significant difference was observed between samples that contained low and medium contents and pure DHP.

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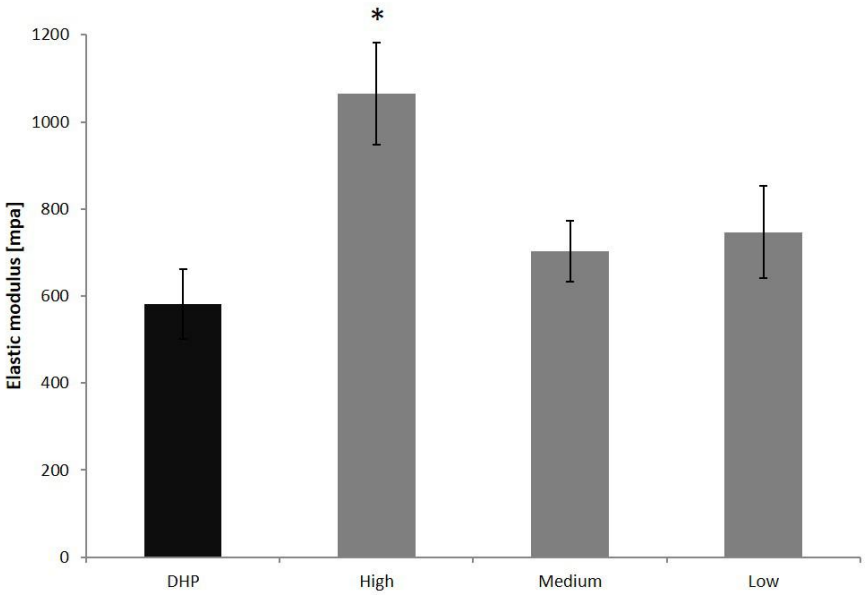

Figure 6. Elastic modulus measured by nanoindentation of dried films of pure DHP and resilin-DHP synthesized using different mass ratios of resilin:C.A. Each data point represents an average of 395-686 independent measurements. Error bars = $1.96 \times SD/n^{0.5}$. * = $P \leq 0.05$, ANOVA followed by Dunnett's method.

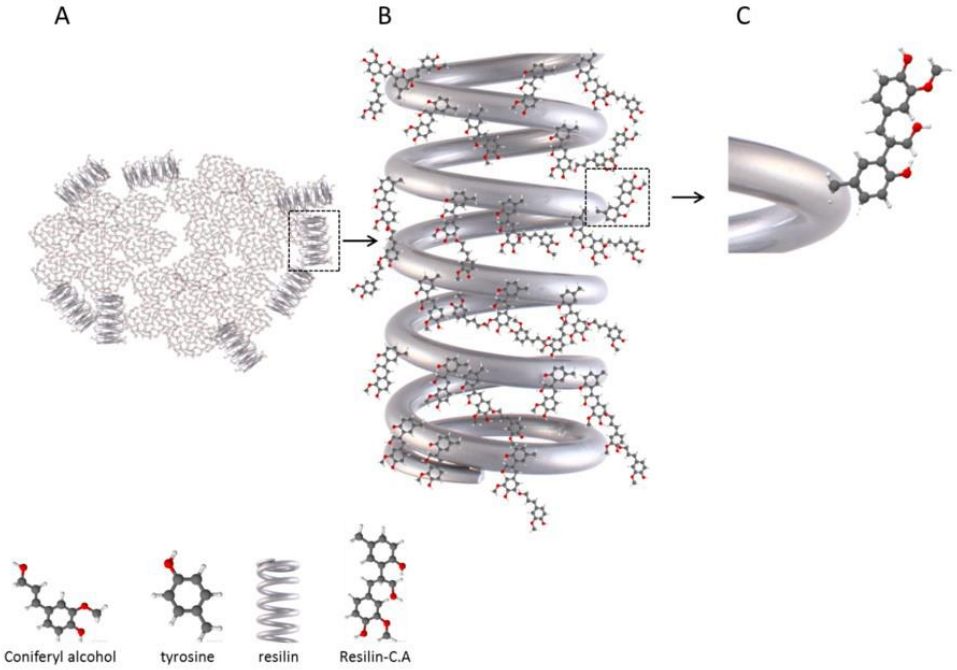

Figure 7. Hypothesized model of resilin-lignin (DHP) crosslinked polymer (A). Polymer is composed of crosslinked C.A. monomers and of resilin crosslinked to C.A. (B). C.A. covalently bonds with the tyrosine residues of resilin (C).

Discussion:-

Protein-lignin interactions are a topic of interest and have recently been demonstrated in vitro using DHP as a synthetic lignin analogue. Lignin monomers were crosslinked with amino acids bearing nucleophilic side groups via quinone methide polymerization,(Diehl et al., 2014) and cysteine and tyrosine rich peptides were shown to bind to lignin in the same way.(Cong et al., 2013; Diehl and Brown, 2014) The aim of the current work was to influence the mechanical properties of lignin through the incorporation of a full length resilin protein with rubber-like characteristics into the lignin structure. To achieve this, we crosslinked C.A. with resilin at different mass ratios, and analyzed the products of the reaction in order to determine resilin integration and its effects on the mechanical properties of DHP.

At the end of the reaction and after centrifugation, two phases were achieved: an insoluble fraction that contained hydrophobic DHP and a water soluble fraction. Purified recombinant resilin is fully soluble under aqueous conditions,(Rivkin et al., 2015) and therefore unreacted resilin was expected to be located in the soluble fraction, whereas resilin crosslinked to DHP was expected to be found in the insoluble pellet.
Comparison of the FTIR spectra of pure DHP and the insoluble fraction obtained in the crosslinking reaction between resilin and C.A. indicated that DHP was indeed formed by the crosslinking reaction (Fig. 2). Furthermore, the incorporation of resilin gave rise to strengthened vibrational bands assigned to the amide functional groups associated with the protein, similar to the results obtained by others.(Cong et al., 2013; Mcdougall et al., 1996) Unreacted resilin also contains amides, however, the samples were thoroughly washed in order to remove unbound protein, and the amide bands at 1540 cm^{-1} and 1660 cm^{-1} became more prominent with increasing protein concentration. FTIR does not give direct evidence of covalent crosslinking. Permanent association between resilin and DHP is strongly suggested according to Western blot analysis (Fig. 3), in addition to being incorporated into the insoluble fraction after the crosslinking reaction, in some cases the molecular weight of the protein increased. For instance, a new band was observed at 55 kDa in the insoluble fraction of the medium resilin:C.A. mass ratio reaction. The increase in resilin molecular weight is likely due to additional weight from DHP molecules linked with resilin.

The presence of monomeric resilin was detected in the soluble fraction of the higher mass ratio reaction (1:5 resilin:C.A.) and is attributed to an excess loading of protein that was uncrosslinked and therefore remained soluble. Here, the insoluble fraction gave rise to several bands, including a monomeric band, which may represent protein bound to few molecules of C.A. These molecules reduced resilin solubility but did not grossly alter its molecular weight. In addition to the 55 kDa band that was also seen in the medium reaction ratio, evidence of high molecular weight resilin-derivatives was observed in the upper part of the lane. The high molecular weight derivatives are attributed to resilin crosslinked to C.A. and to other resilin monomers since similar to C.A., resilin also self-polymerizes in the presence of HRP and H_2O_2.(Qin et al., 2011)

Additionally, most lanes contained a 72 kDa band, including the pure resilin lane, indicating that it is apparently not a feature of the crosslinking reaction. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (data not shown) was conducted under denaturing conditions, that do not allow reversible adhesion of particles, therefor resilin and DHP association is most likely a covalent one. Finally, resilin was found entirely within the soluble fraction of a negative control reaction performed in the absence of HRP and H_2O_2 since the crosslinking reaction did not occur.

The presence of resilin in the crosslinking reaction resulted in a reduction of the size of the clusters as demonstrated by DLS measurements of the reaction mixtures suspension (pre-purification). Since DHP is highly hydrophobic, the presence of a hydrophilic protein may effectively terminate its growth by polymerization due to repulsion between hydrophobic DHP particles and hydrophilic protein. The size difference between the clusters obtained by crosslinking with different resilin contents may possibly be related to an increase in the hydrodynamic radius of the resilin-DHP particles when a high amount of hydrophilic protein is incorporated (see Fig. 7). However, in contrast to the DLS results, AFM of dry films prepared from the crosslinking products of the reaction between coniferyl alcohol and resilin at different mass ratios did not indicate any obvious visual difference in surface topography compared with pure DHP samples. AFM height images indicated similar cluster sizes for all samples with no apparent impact due to the resilin content in the sample. Possibly, cluster size differences are obscured in this technique due to cluster adhesion driven by the removal of water molecules as the samples were dried.

In addition to crosslinking and characterizing the products of the reaction between resilin and C.A., the main goal of this study was to validate the hypothesis that it is possible to change the mechanicals properties of lignin through the incorporation of a rubber-like protein into the polymer. Therefore, once evidence was obtained for the formation of a resilin-DHP biocomposite, nanoindentation was employed to determine the mechanical properties of these

biocomposites as a function of resilin content. According to nanoindentation, a high resilin content led to an increase in the elastic modulus of the biocomposite, indicating that the composite became stiffer. The increase in rigidity is likely derived from the multiple potential crosslinking interactions that can occur between resilin and DHP. due to the 21 tyrosine residues present in resilin.(Carrillo et al., 2005)

Conclusions:-

The current study demonstrates the ability to influence the mechanical properties of lignin by insertion of a rubber-like protein into the polymer structure. The research findings presented herein of the in vitro DHP model support the hypothesis that the mechanical properties of lignin may be altered in vivo by the expression of a recombinant protein that can crosslink with lignin monomers within the cell wall of transgenic plants. Such plants may provide improved woody raw materials for different industrial applications. Future work is currently underway to explore the in vivo insertion of recombinant resilin into plants and to test whether the mechanical properties of these transgenic plants is improved.

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