A STUDY OF MACROMOLECULAR MODELS OF LIGNIN OBTAINED FROM FERULIC ACID

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Enzymatic dehydrogenation of ferulic acid has been carried out by the end-wise method with the aid of the hydrogen peroxide–horseradish peroxidase (E.C. 1.11.1.7) system. In vitro, high-molecular-weight analogs of lignin — dehydropolymers (DHPs) — have been obtained. It has been confirmed that the high-molecular-weight fraction of the DHPs is formed by the combination of subunits of approximately equal molecular weight (DP ~ 20). One of the factors determining the constancy of the molecular weight of the subunits is the achievement of a DP_{cr} at which the heterophase synthesis of DHPs begins. In addition to this, an important role is played by the surface charge of the subunits. For various reasons, of which the reactivity of the substrate is important, the end-wise method of synthesis can ensure the occurrence of the bulk mechanisms and conversely.

Phenylpropane units containing carboxy groups have been detected in pine, bamboo, and herbaceous lignins [1-3]. However, Nozu [4], while recognizing the possibility of the formation of free phenoxyl radicals in the enzymatic dehydrogenation of ferulic acid, rejects the possibility of their subsequent polymerization.

We have performed the in vitro synthesis of a macromolecular analog of lignin — adehydropolymer (DHP) — from ferulic acid (FA) as substrate. The synthesis was carried out by the "Zutropfverfahren" [5] (end-wise method [6]). The enzyme used was horseradish peroxidase (E.C. 1.11.1.7). In view of the fact that individual workers have put forward a hypothesis [7-9] of the necessity for the presence of carbohydrates in the polymerization of certain substrates, including ferulic acid, in our experiment pieces of Cellophane were placed in the reaction mixture. Interest is also presented by a check of the heterophase nature of the synthesis of the ferulic DHPs, which has been found in the synthesis of other DHPs [10], and the hypothesis that has been put forward that the growth of the macromolecule takes place through subunits with the same degree of polymerization of DP 20 [11]. The synthesis of the DHPs and the isolation of the products was carried out as in Scheme 1.

The properties of the DHPs obtained (EW-1, EW-2, and EW-3) were compared with the properties of milled wood lignin (MWL) isolated from the spruce *Picea abies*.

When the DHPS were washed, pronounced peptidization was observed, and to eliminate this subsequently we used dialysis. When EW-3 was dissolved in dimethyl sulfoxide (DMSO) some part of it (17% of the total) remained undissolved. The incomplete dissolution in dioxane of end-wise DHPs from coniferyl alcohol has been found by Lai and Sarkanen [12]. The macromolecular properties of the insoluble part of EW-3 are being subjected to further investigation.

Gel chromatograms of the DHPs obtained (Fig. 1) show their closeness in molecular-weight properties to MWL. The molecular weights (M_W) of the fractions calculated by the method of Alekseev et al. [13] and also found with the aid of a computer according to a Gaussian distribution of the area S of the peaks of the fraction is given below (Sephadex G-75):

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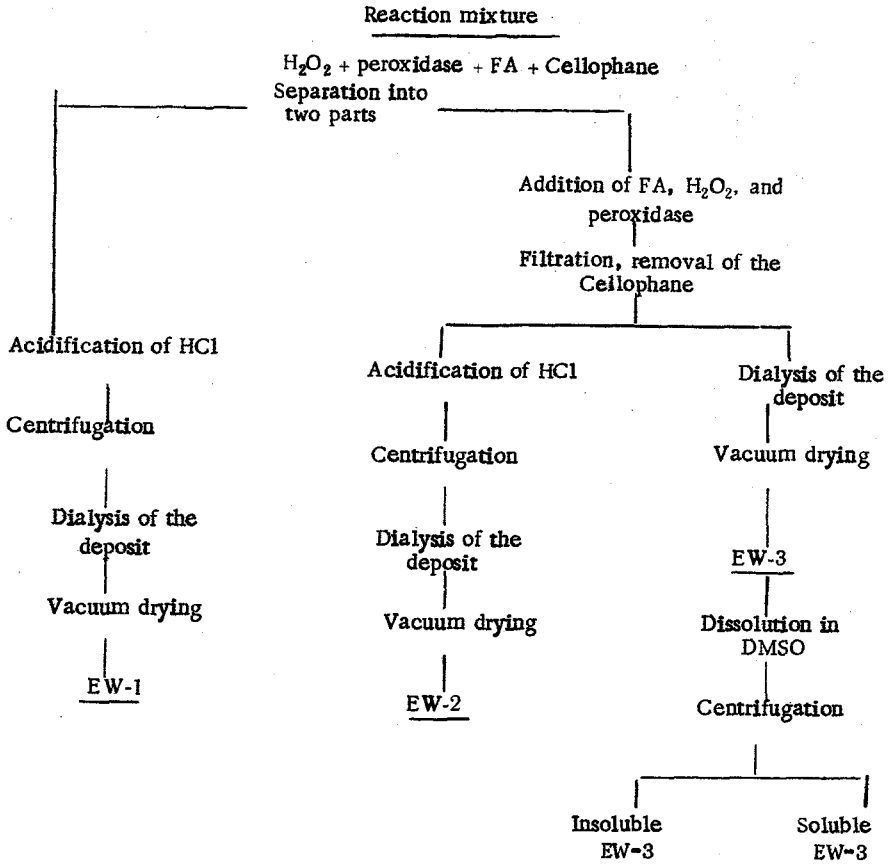
DHP	High-molecular-weight peak, $\overline{M}_W^B$	Low-molecular weight peak $\overline{M}_W^H$	$S_{M_W}^H / S_{M_W}^L$
MWL	15850	3710	0,12
EW-1	19600	3460	0,15
EW-2	14250	3110	0,12
EW-3 soluble in DMSO	23400	3110	0,19



Scheme 1. Scheme of the synthesis and isolation of the products

We must mention the arbitrary nature of the value of M_W for the high-molecular-weight peak in view of its closeness to the volume V_0. This type of error is excluded in the determination of M_W of the low-molecular-weight peak. The constancy of the values of M_W of the low-molecular-weight fractions of the DHPs obtained is in harmony with Wayman and Obiaga's hypothesis [11] on the growth of DHP macromolecules from smaller subunits with M_W 3000-4000. Apparently, thanks to the high dilution of the reaction mixtures, the end-wise mechanism ensures the retention of a similarity of shape of the oligomeric subunits with their high intramolecular cyclization. This type of mechanism of the formation of macroscopic cross-linked systems is characteristic for synthetic polymers with a well-defined association of the initial monomers [14]. The high hydrophobicity of the lignin precursors is the reason for the preferential "monomer–monomer" interaction as compared with "monomer–solvent" interaction. It is obvious that, because of steric hindrance, the peroxidase does not take a direct part in the combination of the subunits. Thus, Nimz et al. [15] were unable to obtain a resin from lignosulfonates with the aid of the H_2O_2–peroxidase oxidizing system but fully succeeded in synthesizing it on the basis of inorganic one-electron oxidizing agents. According to Wayman and Obiaga [11] the subunits retained their individuality even after combination. An increase in the amount of units with M_W 4000 was observed when the destruction of MWL under the conditions of industrial processes was modelled [11].

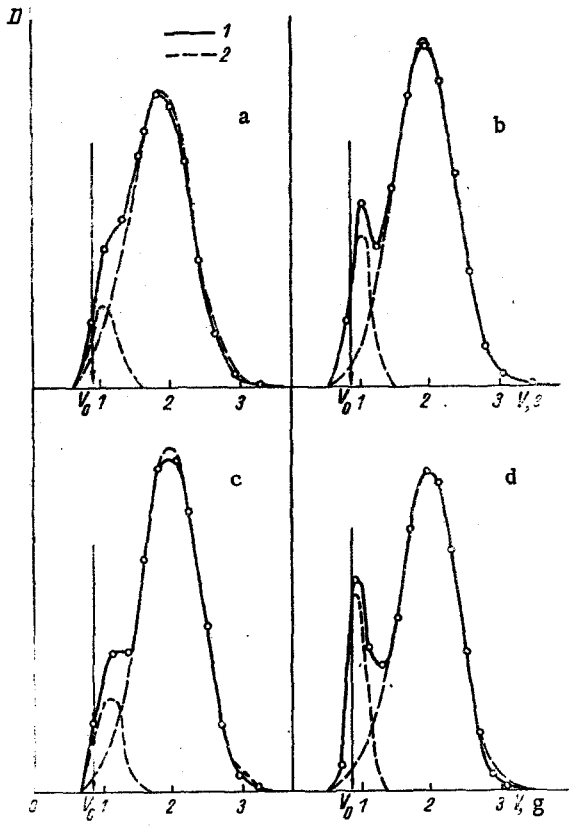


Fig. 1. Gel chromatography (G-75, eluent — DMSO) of milled wood lignin (a) and of the dehydropolymers obtained (b, EW-1; c, EW-2; d, EW-3): 1) experimental curves; 2) computer-calculated curve according to a Gaussian distribution.

The bimodality of the distribution confirms the opinion expressed previously [10] of the heterophase nature of the synthesis of DHPs. According to Gladyshev and Popov [16] the Flory-Huggins interaction parameter is determined by the expression

$$\mu_{cr}=1/2\ (1+1/DP_{cr}),$$

where DP_{cr} is the value at which the polymer precipitates.

Hence, at $DP_{cr} \sim 20$ the corresponding value of μ_{cr} is 0.61. Consequently, the value for a Θ-solvent is exceeded by 0.11. Apparently, in addition to the high dilution, the heterophase nature of the synthesis, when a solid phase separates out at a given DP_{cr}, also ensures a fairly constant DP of the initial particles. It is not excluded that the rapid precipitation of the particles is prevented by a surface charge, as is indicated by the observed peptidization effect.

The difference IR spectra (Fig. 2) of the DHPs obtained show a higher degree of condensation than MWL, which usually correlates with the degree of cross-linkage of the preparation. According to Lai and Sarkanen [12] bands at 1045 and 1280 cm^{-1} are characteristic for uncondensed phenylpropane units and bands at 1330 and 1600 cm^{-1} for syringyl and condensed guaiacyl units. As can be seen from Fig. 2, the DHPs obtained were characterized by a lower absorption at 1045 and 1280 cm^{-1} and a higher absorption at 1530 and 1600 cm^{-1} as compared with MWL. It is interesting to note that in situ lignin is created by a superposition on one another of end-wise and bulk mechanisms [6]. However, the polymers that we obtained by the "pure" end-wise method proved to be more bulk polymers than the MWL. A DHP obtained by the end-wise method using the O_2—$CuCl_2$—pyridine system [17] had approximately the same

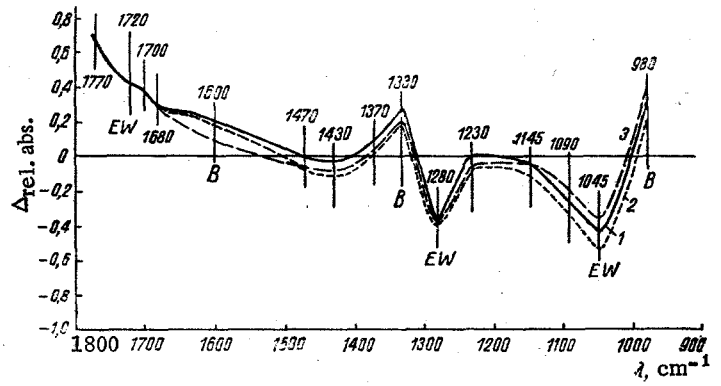


Fig. 2. Difference IR spectra of DHP preparations relative to spruce MWL: 1) EW-1; 2) EW-2; 3) EW-3.

nature of the change in the difference spectrum as the DHPs given in the present paper. Apparently it is convenient to compare preparations obtained from the same substrate but with the use of different methods of synthesis by Lai and Sarkanen's method [12]. However, a comparison of different preparations for the assignment of the contributions of the end-wise and bulk mechanisms, which in themselves are hypothetical, is impermissible. Consequently, it is better to be limited to the end-wise and bulk methods of synthesis. In essence, the in vivo synthesis of lignin takes place by the end-wise method but a locally high feed "by a pump" of substrates may lead to the realization of the bulk mechanism. It is not excluded, either, that the degree of condensation (proportion of bulk mechanism) both in vivo and in vitro largely depends on electronic effects of the substrates, i.e., on their reactivities.

A consideration of UV spectra (Fig. 3) taken in DMSO shows no qualitative differences between the preparations synthesized. The long-wave bands of FA are formed through the overlapping of the E_1, B, K ($\pi\rightarrow\pi^*$) and R($n\rightarrow\pi^*$) bands [18]. The bathochromic shift of the maximum and the increase in the intensity for FA in DMSO as compared with water are connected with the dipolar nature of the DMSO molecule. DMSO is a n-donor solvent possessing basic properties and entering into specific interaction with the solute corresponding to a Lewis acid-base interaction. The spectrum of MWL is formed by a complex overlapping of bands of different structural units of the macromolecule [18]. The spectra of the DHPs obtained differ from the spectra of the initial substrate and also from the spectrum of a MWL. To eliminate the possible influence on the UV spectrum of phenoxyl radicals trapped in the rigid structure of a DHP, vacuum-treated preparations were heated at 110°C for two hours. However, the spectra of the DHPs taken after the heating did not differ from the initial ones. The increase in the intensity of absorption on passing from EW-1 to EW-3 shows an increase in the degree of condensation, which increases the probability of the formation of cross-linked systems. Absorption in the visible region is due to a long chain of conjugation and $n\rightarrow\pi^*$ transitions, and also to some contribution of an $S_0 \rightarrow T_1$ transition [18].

It is known that the glass temperature T_g characterizes the segmental mobility of a macromolecule on passing from the vitreous state to the highly elastic state. The T_g values of various dry preparations of lignins isolated from wood are between 160 and 180°C [19]. Investigations with the aid of a differential scanning calorimeter of EW-1 showed no temperature transitions in this region. No decomposition processes were observed, either. According to DTA results, the DHP obtained from FA by the use of the $CuCl_2$—pyridine catalytic system begins to decompose at 204°C [17]. It is not excluded that temperature transitions are not observed because of the small change in heat flow in the region of T_g which are at the limits of sensitivity of the instrument used.

EXPERIMENTAL

Ferulic acid was obtained by condensing vanillin with malonic acid by Knoevenagel's method [20]. mp 172°C.

MW lignin was isolated as described previously [21].

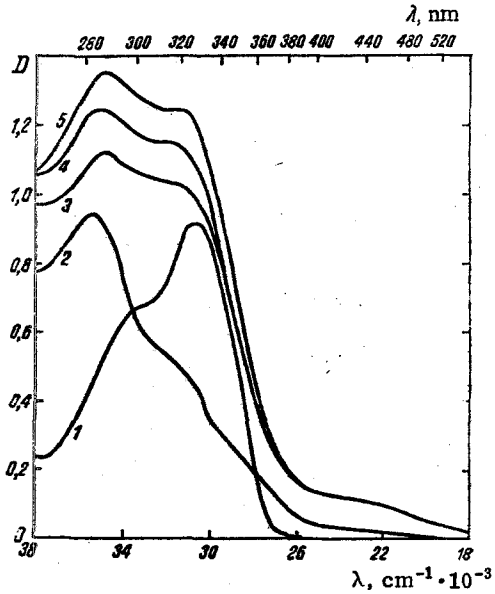


Fig. 3. UV spectra taken in DMSO: 1) ferulic acid ($5 \cdot 10^{-5}$ M); 2) spruce MWL ($2.3 \cdot 10^{-4}$ M); 3) EW-1 ($2.2 \cdot 10^{-4}$ M); 4) EW-2 ($1.9 \cdot 10^{-4}$ M); 5) EW-3 ($2.3 \cdot 10^{-4}$ M).

Synthesis of the DHPs. In a two-liter round-bottomed flask, 0.05 g of horseradish peroxidase (Reanal, RZ 0.6; activity by the o-dianisidine method 350-500 units/mg) was dissolved in 480 ml of phosphate buffer (pH 5.9) containing strips of Cellophane. Over 90 min at 25°C, 800 ml of a 0.1% solution of the substrate and 680 ml of a 0.1% solution of hydrogen peroxide were added simultaneously in drops. During the addition of the components, the color of the solution rapidly changed from colorless to brownish pink. After the addition of the components, the solution was stirred for two hours, which corresponds to the method of Nakatsubo and Higuchi [22].

The reaction mixture was then divided into two parts. One part was acidified with hydrochloric acid (pH 2). The gel-like red-brown precipitate was centrifuged off (6000 rpm). After dialysis, the residue was dried in a vacuum chest at 40°C (EW-1). To the second part, over 90 min with stirring, were added 0.025 g of peroxidase, 0.266 g of ferulic acid, and 340 ml of 0.1% H_2O_2. After the end of the synthesis, the solution was filtered. EW-2 was isolated from the filtrate by a method similar to that for the isolation of EW-1. The precipitate dried on the filter, EW-3, was dissolved in DMSO. From a centrifugate was obtained soluble EW-3 and from the precipitate insoluble EW-3. The yields of the products were as follows: EW-1 0.15 g (14%), EW-2 0.17 g (16%), EW-3 0.12 g (11%). The total yield of DHPs was 41% on the amount of the initial FA.

Gel chromatography was effected on Sephadex G-75 in a 5 × 130 mm column. The sample consisted of 0.1 ml of a 0.8% solution in DMSO. The value of V_0 was determined with dextran blue (molecular weight - $2 \cdot 10^6$). The M_W values of the corresponding peaks were calculated from the formula $K_{av} = A - B \log M_W$, where A = 2.9, B = 0.065 [13]. To calculate K_{ev} from the weight of the collected fractions we passed to milliliters.

IR spectra were taken on a UR-20 spectrometer. Tablets were molded with KBr. The difference IR spectra were calculated by the method of Smilga et al. [23].

UV spectra were taken on a Specord UV-VIS spectrometer.

Thermograms were recorded on a DSK-2 differential scanning calorimeter at a rate of scanning of 12.5°C/min. The scanning range was 50-230°C. The sensitivity of the instrument at a signal/noise ratio of 1 was not less than 0.3 mW.

SUMMARY

1. The possibility of the enzymatic synthesis of a dehydropolymer based on ferulic acid has been confirmed.

2. The increase in the high-molecular-weight fraction of the DHP takes place by the combination of subunits of approximately the same molecular weight.

3. The heterophase nature of the biosynthesis is one of the factors determining the constancy of the molecular weight of the subunits.

4. The DHPs obtained possess a high degree of condensation.

5. For certain reasons, the end-wise method of synthesis may lead to the occurrence of the bulk mechanism, and conversely.

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