AN ENZYME EXTRACT FROM DOUGLAS-FIR SAPWOOD AND ITS RELATIONSHIP TO BROWN STAINING[1]

Murray L. Laver
Associate Professor Forest Products Chemistry

and

Durria Abdel Azim Musbah
Ph.D. Graduate

Department of Forest Products
Oregon State University
Corvallis, OR 97331

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ABSTRACT

A chemical or enzymatic brown stain in sapwood of Douglas-fir has caused financial losses to lumber producers, particularly those producing valuable clear grades of export lumber. This work shows that the brown stain may be caused, at least in part, by an enzyme system in the sapwood. Buffered extracts of Douglas-fir sapwood showed enzyme activity when added to solutions containing *o*-diphenol and polyphenol compounds. No activity for monophenol substrates was detected. The enzymatic extract showed two pH optima for activity, one at pH 5.5 and one at pH 8.0, with the activity at pH 8.0 being somewhat greater. The activity was also temperature-dependent, with the highest activity at 35°C. The extract showed highest activities with the compounds (−)-epicatechin, dihydroquercetin, and 4-methylcatechol.

Keywords: Polyphenol oxidase, Douglas-fir sapwood, chemical stains, brown stains, protein extraction, enzyme activity.

INTRODUCTION

The stains that develop on commercial woods are problems of great economic importance, as they may degrade the appearance of the wood, lowering its value. Though stains are often caused by fungi, some nonfungal and usually brown stains, often referred to as "chemical brown stains" or "enzymatic stains," may appear on exposed surfaces of lumber (Miller et al. 1983). This type of staining of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco] has become a serious problem. We believe that it is caused, at least in part, by enzymes reacting on compounds naturally present in the wood. This is not unlike the reactions associated with the undesirable darkening of damaged tissue in fresh fruits and vegetables. Fruit and vegetable darkening is catalyzed by polyphenol oxidases (PPO), which are probably present in all plants (Mayer and Harel 1979; Wissemann and Lee 1981). Certainly, Douglas-fir wood contains polyphenol compounds, including (+)-catechin, (−)-epicatechin, and dihydroquercetin, upon which such enzymes may act. Similar-type reactions have been discussed for color changes in woods as exemplified by the report of Ota et al. (1991).

MATERIALS AND METHODS

Preparation of the enzyme extracts

A Douglas-fir tree 50-years-old (by ring count), 20.3 cm in diameter at breast height, and 9.1 m tall, was cut from McDonald Forest, Oregon State University, Corvallis, OR. The sample tree was selected as being representa-

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tive of Douglas-fir trees that are being harvested. Segments 15.2 cm long were immediately sectioned from the tree above breast height and stored at −25°C to prevent alteration of the enzymes and to reduce fungal growth.

The segments were later debarked by hand, and the frozen sapwood was shaved with a chilled hand-planer. The first 1 cm from the outside surface was discarded to prevent enzymes moving from the bark into the sapwood. We used portions of the remaining shavings to determine moisture content (56%) by drying them to constant weight in an oven at 105°C. Other sample shavings were immediately blended with buffer at low temperature to increase fracture of the rough cell walls and to expose the enzymes for solubilization.

The crude extract.—A potassium phosphate buffer-wood homogenate was maintained at ice-bath temperature to avoid denaturation of enzymes by heat. We selected pH 7.3, as low pH can inhibit enzymes, and pH 7.3 is near that used to extract enzymes from fruits and vegetables (Flurkey and Jen 1978; Interesse et al. 1980; Wissemann and Lee 1981). Potassium chloride was added to the buffer to assist separation of native proteins from nucleic acids.

Fresh buffer extractions of wood tissue were made by a modification of the method described by Flurkey and Jen (1978). Fresh extracts were prepared over the course of the work because many became brown upon standing, even at 4°C, indicating that they contained both an enzyme and a reactive substrate. A typical extraction was as follows: 25 g of fresh wood shavings (11.0 g dry wt) were blended in a Waring blender with 200 ml of 0.05 M potassium phosphate buffer (pH 7.3) containing 1 M potassium chloride (Gomori 1955) at 4°C for 4 min, or until the solids were fine. The homogenate was allowed to stand for 45 min at 4°C, then blended an additional 1 min in the same blender. The suspension was filtered through glass wool (4°C), and the filtrate was centrifuged in a Sorvall RC-5C (Rotor-600) refrigerated centrifuge (4°C) at 10,000 rpm for 45 min. The supernatant (150 ml) that formed the crude enzyme extract was recovered by decantation.

Ultraviolet-visible absorbance spectra of extracts

Ultraviolet-visible absorbance spectra were performed over the range 500 nm to 220 nm (Shimadzu UV-65FW double-beam recording spectrophotometer; Shimadzu Scientific Instruments, Inc., Columbia, MD) at a scan rate of 120 nm/min. The reference cuvette contained 2 ml of potassium phosphate buffer, the sample cuvette 2 ml of the crude enzyme extract.

Total protein assay

The crude enzyme extract was analyzed for total protein content by a standard Bio-Rad Microassay (Bio-Rad 1989, Richmond, CA). Bovine serum albumin (BSA) served as a standard (Spector 1979). The BSA, as purchased, was dissolved in 20 ml of 0.05 M potassium phosphate buffer, to yield an exact 1.41 mg/ml concentration. Several dilutions of this BSA standard, each containing between 5 and 20 μg/ml were prepared. Aliquots of 1.6 ml of each standard BSA concentration and each enzyme extract were placed into separate, clean, dry test tubes. A blank test tube was prepared with 1.6 ml of sample buffer only. Dye Reagent concentrate (0.04 ml Coomassie Brilliant Blue G-250, Bio-Rad) was added to each tube and mixed several times by gentle inversion. The reagent turned blue when added to the protein. After not less than 5 min and not more than 1 h, the absorbance (Shimadzu UV-65 FW) versus reagent blank was measured at 595 nm. The spectrophotometer computer plotted absorbance versus the known protein concentration of the BSA to give a straight line (method of least squares; $R = 98$), and from this line plot printed the protein concentrations of the enzyme extracts. Standard curves were prepared each time the assay was performed. All samples were measured in triplicate.

Determination of enzyme activity

Activities of the extracts with various substrates were determined spectrophotometrically by a method modified from that described by Interesse et al. (1980). The increase in absorbance was measured at maximal-absorbance wavelengths characteristic of reaction products of the various substrates. The wavelengths were determined by an initial spectrophotometric scan from 600 nm to 220 nm after a minimum of 5 min (Table 1). Unless otherwise noted, activities were then determined as follows:

The reference cuvette (quartz; 1-cm light path) contained 1 ml of 0.05 potassium phosphate buffer and 1 ml of 0.02 M substrate in the same buffer, the paired sample-cuvette 0.5 ml of 0.05 M potassium phosphate buffer and 1 ml of 0.02 M substrate in the same buffer. The cuvettes were saturated with oxygen for 5 min by means of capillary tubes, then placed in a temperature-controlled cell holder in the spectrophotometer and allowed to stand for 2–3 min until the solutions came to equilibrium at 35°C. The spectrophotometer was set to the previously determined wavelength for the enzyme-substrate reaction product [437 nm for (−)-epicatechin], the absorbance was set to zero, and the enzyme extract (0.5 ml) was added to the sample cuvette (zero time). As early analyses indicated a lag time of as much as 1–2 min before absorption changed, we read the spectrophotometer after 5 min (by stopwatch), then calculated the average change per minute. One unit of extract activity was given the definition similar to that of Flurkey and Jen (1978) and Wissemann and Lee (1981), as a change of 0.001 unit in absorbance per min per ml. Results are reported in units per milliliter. All compounds assayed were in aqueous buffer solutions freshly prepared before each activity determination, and each activity was determined in triplicate.

Substrate.—The extract activity, as related to the chemical structure of the substrate compound, was determined with monophenols (tyrosine and 4-hydroxycinnamic acid), diphenols (4-methylcatechol, catechol, and 3,4-dihydroxyphenylalanine) and polyphenols [(−)-epicatechin and dihydroquercetin]. The activities were assayed under the conditions already described, except that the (−)-epicatechin concentration was 0.01 M instead of 0.02 M because of limited solubility.

TABLE 1. *Wavelengths used for determining activities of the crude extract of Douglas-fir with various substrates.*

Substrate	Other studies (nm)[1]	This study (nm)
Monophenols		
Tyrosine	415, 430, 472, 490	400
4-hydroxycinnamic acid	410	410
Diphenols		
4-methylcatechol	395, 400, 410, 415, 420, 496	395
Catechol	420[2]	420
DL-3,4-dihydroxyphenylalanine	475, 410, 415, 420, 430, 460, 475, 480	475
Polyphenols		
(−)-epicatechin	395, 440	437
Dihydroquercetin	—	395

[1] Reported by Interesse et al. (1980) unless otherwise indicated.
[2] Wissemann and Lee (1981).

pH.—The activity as a function of pH was determined for the extract with 0.02 M 4-methylcatechol as the substrate (395 nm wavelength) in 0.05 M sodium-acetate buffer in the pH range 4.0-6.0, in 0.05 M potassium phosphate buffer in the pH range 6.5–7.5, and in 0.05 M tris-hydrochloric acid for higher pH ranges.

Temperature.—Activity was also determined for the extract at various temperatures from 5°C to 40°C ± 1°C, with 0.02 M 4-methylcatechol as substrate (395 nm wavelength) in 0.05 M potassium phosphate buffer.

RESULTS AND DISCUSSION

An ultraviolet-visible scan of the crude extract showed a maximum at 282.4 nm, a slight shoulder at 290.0 nm, and a peak at 321.2 nm (Fig. 1). These results agree with those of a study of *Neurospora* tyrosinase (Guttridge and Robb 1975) except for a slight difference at peak 321.2. The spectrum in our study is typ-

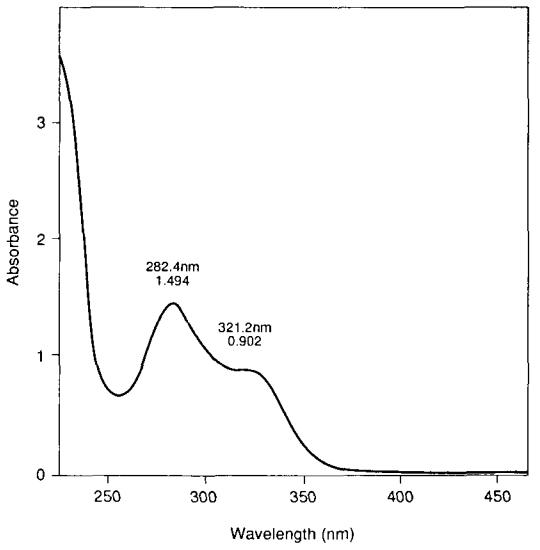

FIG. 1. Ultraviolet-visible absorption spectrum of the crude extract of Douglas-fir sapwood in 0.05 M potassium phosphate buffer.

ical of a protein extract except for the distinct peak at 321.2 nm, which appeared to be an oxygenated species similar to the oxytyrosinase inferred by Jolley et al. (1974) from studies of mushroom tyrosinase.

The properties of a typical crude extract prepared from fresh wood shavings (1.0 g dry weight of wood) were as follows: volume of the extract 13.6 ml; activity (units/ml) 114; total units of activity 1,550.4; total protein 390.1 mg; specific activity (units/mg protein) 4.0. The activities were determined at pH 7.3 with (−)-epicatechin as substrate. The absorbances were measured at 437 nm. Activities reported are the averages of triplicate determinations.

The results of extract activity related to the chemical structure of substrate compounds are shown in Table 2. The extract was not active towards the monophenols, tyrosine, or 4-hydroxycinnamic acid, but showed activity towards all *o*-diphenols and polyphenols tested. The highest activity levels were shown towards (−)-epicatechin followed by dihydroquercetin and 4-methylcatechol.

The effect of pH on the extract activity showed two optima, at pH 5.5 and pH 8.0 (Fig. 2). The activity at pH 8.0 was some 3 times that at pH 5.5.

TABLE 2. *Substrate studies of the crude extract of Douglas-fir sapwood.*

Substrate[1]	Activity (units/ml)	Relative activity (%)
Monophenols		
Tyrosine	0	0
4-hydroxycinnamic acid	0	0
Diphenols		
4-methylcatechol	20.6	21.2
Catechol	3.9	4.0
DL-3,4-dihydroxyphenylalanine	3.9	4.0
	10.0	10.3
Polyphenols		
(−)-epicatechin	97.2	100.0
Dihydroquercetin	48.9	50.3

[1] Substrate concentration was 0.02 M with the exception of that for (−)-epicatechin, which was 0.01 M.

The optimum temperature for maximum activity was 35°C (Fig. 3). At temperatures of 10°C and lower, there was less than 5% of maximum activity, little or no reaction occurring at low temperatures. Activity increased with increasing temperature until 35°C, then decreased sharply. At 40°C, activity was only 41.7% of the maximum at 35°C. At 30°C and 40°C, the activity was almost the same (39.1% and 41.7% of maximum, respectively); thus, there is a definite optimum at or near 35°C (Fig. 3).

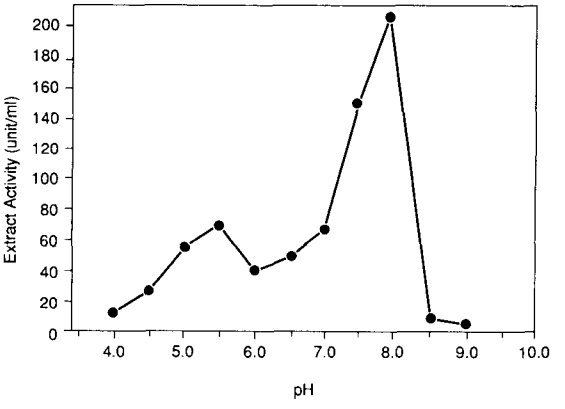

FIG. 2. Optimum pH of Douglas-fir sapwood extract activity with 4-methylcatechol as substrate. Absorbance was measured at 395 nm.

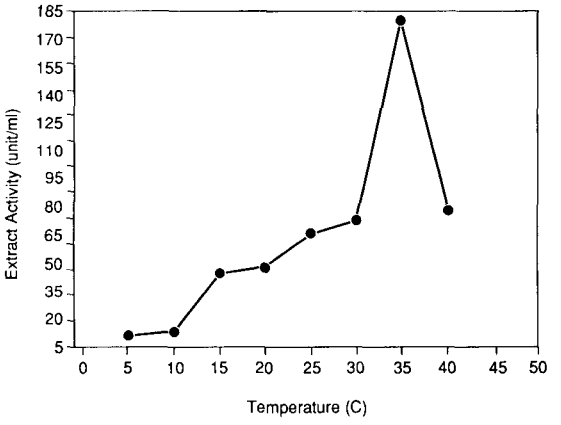

FIG. 3. Optimum temperature of Douglas-fir sapwood extract activity with 4-methylcatechol as substrate. Absorbance was measured at 395 nm.

CONCLUSIONS

Douglas-fir sapwood contains proteins that display enzyme activities that undoubtedly contribute to the formation of chemical or enzymatic brown stains. Extracts of the proteins showed high activity with (−)-epicatechin and dihydroquercetin, two polyphenolic extractives that occur naturally in the wood. Song (1988) and Arvey (1993) have established that dihydroquercetin, in particular, was a precursor to stain formation; thus, both the precursors to stain formation and an enzyme that reacts with the precursors are naturally present in Douglas-fir. When conditions are suitable for enzymatic activity, discoloration occurs.

The enzyme system showed two pH optima for activity, one at pH 5.5 and one at 8.0, the activity at pH 8.0 being somewhat the greater. The activity was also temperature-dependent, the highest activity occurring at 35°C. Stain formation might therefore be controlled by drying the freshly sawn lumber as quickly as possible at temperatures well above 35°C. Miller et al. (1988) showed that steaming Douglas-fir sapwood to 100°C was the most effective stain-preventive treatment. The steam and the high heat may have contributed to denaturation of the enzymes.

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