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Effects of lacquer polysaccharides, glycoproteins and isoenzymes on the activity of free and immobilised laccase from *Rhus vernicifera*

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ABSTRACT

The purified polysaccharides, glycoproteins, and isoenzymes of *Rhus* laccase, and crude enzymes, from Chinese lacquer (*Rhus vernicifera* sap) were used to determine their influence on the enzymic activity of *Rhus* laccase on several substrates (4-phenylenediamine, isoeugenol and coniferyl alcohol). No product identity changes were observed when these components were added singularly or in combination to the enzymic reactions (only relative product yields varied significantly), however, the polysaccharides (GP1 and GP2) and glycoprotein (stellacyanin, St) exhibited negative effects, and the two isoenzymes (L1 and L2) exhibited positive synergistic effects, on the activity of *Rhus* laccase. With respect to the activity of the crude enzymes, the negative effects of GP1, GP2 and St were greater than the positive effects of L1 and L2, compared with free *Rhus* laccase on its own (using 4-phenylenediamine as substrate), the estimated inhibitory effect (of GP1, GP2 and St) being by at least a factor of 50 (even with the positive effect of L1 and L2). This contributes to understanding of lacquer storage stability and drying rates. Immobilisation of crude enzymes using a variety of techniques (using natural and modified polysaccharides, and an inorganic support) where evaluated using isoeugenol as substrate. Agar embedding and zirconium chloride chelation methods resulted in the highest substrate conversion levels. The yields and products of isoeugenol catalysis using Vietnamese crude enzymes/purified *Rhus* laccase and commercial Denilite laccase were also compared and contrasted with their Chinese lacquer sap equivalents.

1. Introduction

Lacquer trees, *Rhus vernicifera*, grow widely in Asian countries and regions, especially in China and Japan [1], and are a kind of abundant natural resource [2–4]. When xylems of lacquer trees are injured or cut, they produce a milk white sap, named raw lacquer or Chinese lacquer, which contains urushiols (60–65%, w/w), *Rhus* laccases (RL, 0.3–0.5%, w/w), polysaccharides (5–7%, w/w), glycoproteins (1–3%, w/w), the pseudo-glycoprotein stellacyanin (St, ~1%, w/w) and water (20–30%, w/w), etc. (Fig. 1). All of these components have been separated and purified [1,5] and some of the components have been studied individually, such as the structural analysis of urushiols and laccols [6–11]; lacquer polysaccharides [4,12–14]; fast drying lacquer [15–19], and the morphology and components of lacquer film [20–23], however, their interactions have been scarcely investigated due to their complexity [24].

Some interesting phenomena have been observed, for example lacquer polysaccharides in the surface of the lacquer film act as a barrier layer against oxygen diffusion [25]. It has been hypothesised that this useful quality results from the complexation of polysaccharides, glycoproteins, and phenolic lipids [24]. Stereoselective bimolecular phenoxy radical coupling by an auxiliary protein without an active centre was found to assist laccases to synthesise enantiomeric pinoresinol [26], and bovine serum albumin (BSA) and St have a negative effect on the activity of these enzymes [27].

It is still difficult to make a study of the overall aspects of lacquer sap because of the complete reversed solubility of its components, such as laccase in water and urushiols in organic solvents, however, it is easier to investigate the water-soluble parts of the lacquer sap [1]. In this paper the effects of polysaccharides (GP1 and GP2), glycoprotein (St) and the isoenzymes L1 and L2 of RL, on the activity of free and immobilised *Rhus* laccase were studied, in order to systematically determine whether purified polysaccharide and/or glycoprotein and/or other components affect the catalytic properties of RL (free and immobilised) on particular substrates [1,5].

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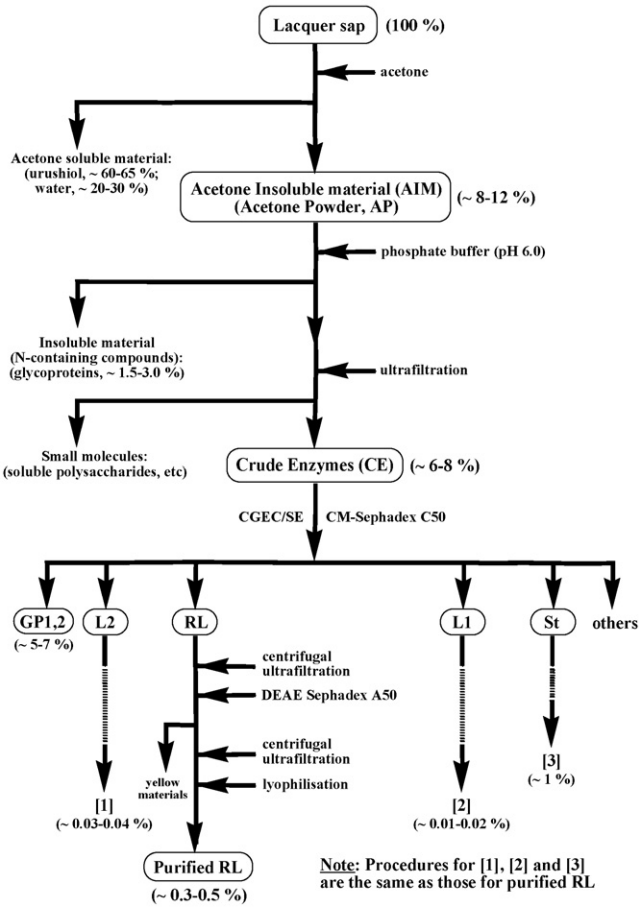

Fig. 1. Procedures utilised for the separation and purification of Chinese lacquer enzymes from *Rhus vernicifera* sap [1,5] (CGEC: continuous gradient elution chromatography; EC: stepwise elution; GP1, GP2: polysaccharides; St: stellacyanin; L1, L2: two isoenzymes of *Rhus* laccase; RL: purified *Rhus* laccase).

2. Materials and methods

2.1. Materials

Dried acetone insoluble material (AIM), also known in the field of lacquer chemistry as acetone powder (AP), was isolated from Chinese lacquer trees, *R. vernicifera*, in the Chengkou region of China. Crude enzymes (CE) were prepared from an aqueous solution of AIM (0.1 g/mL) after ultrafiltration, and *Rhus* laccases (RL—the simple purified *Rhus* laccase) was purified according to previously reported methods [1,5] (Fig. 1). Vietnamese laccase was separated from Vietnamese lacquer sap [28]. Denilite laccases are commercial enzymes purchased from Novozyme, Copenhagen, Denmark. Carrageenan, chitosan and agar were purchased from Nacalai Tesque Inc. (Kyoto, Japan), and sodium alginate was purchased from Kanto Chemical Co., Tokyo, Japan. Chitopearl SH-5010 and Chitopearl BCW-2510 were purchased from Fujibo Co., Ltd., Tokyo, Japan. The structures of these natural and modified polysaccharides are shown in Fig. 2. All other chemicals were of reagent grade and used as received unless otherwise stated.

2.2. Determination of protein concentration and enzyme activity

Standard spectrophotometric calibration curves were established by measuring optical density at 280 nm, using bovine serum albumin (BSA) and RL as standards. This permitted determination of enzyme concentration via reference to the generated standard curves [5]. RL activity (Table 1) was measured spectrophotometrically at 25 °C and 500 nm with 4-phenylenediamine as substrate (prepared with 0.1 mol/L sodium phosphate buffer, pH 7.5), isoeugenol and coniferyl alcohol at 258 and 322 nm, respectively (prepared with 1:1, v/v, water:acetone and 0.1 mol/L sodium phosphate buffer, pH 7.5). One unit of RL activity was defined as the change in optical density at 500 (258 or 322) nm effected per min per μmol of protein (using the molecular weight values reported in a previous paper [1]) added to 3 mL substrate solution in a 1 cm cell path length and incubated at 25 °C [5,29].

2.3. Immobilisation on natural and modified polysaccharides

Three immobilisation methods using natural polysaccharides, and one inorganic material – zirconium chloride–which was chosen for comparison, were applied as follows.

2.3.1. Immobilisation by embedding in carrageenan, sodium alginate, chitosan, and agar

Carrageenan (M_W 500 kDa, 0.5 g) was dissolved in deionised water (10 mL) with stirring at 80 °C, for 3 h. The solution was then cooled to 40 °C and crude enzyme (CE) solution (0.1 g/mL in 0.1 mol/L potassium phosphate buffer, pH 6.8) was gradually added with stirring. The resulting solution was left to stand for 10 min, and then stored at 4 °C until required.

Sodium alginate (0.5 g) was dissolved in deionised water (10 mL) with stirring at 50 °C for 4 h, and then cooled to room temperature (25–30 °C). CE solution (10 mL, as detailed above) was added dropwise with stirring. After standing for 1 h, 10% (w/v) aqueous calcium chloride solution was slowly added dropwise to the solution to induce localised precipitation and make small beads, which were then extracted by gentle filtration. The produced beads were steeped for 1 h in 10% (w/v) aqueous calcium chloride solution at 4 °C, and washed with deionised water (~200 mL) until washings were free from calcium (as tested for by adding alkali and observing absence of precipitated calcium hydroxide), and then stored at 4 °C until required [30].

Chitosan (small flake, M_W 600 kDa, 0.5 g) was dissolved in acetic acid (0.28%, w/v, 0.5 mL) with stirring at room temperature for 3 h. CE solution (10 mL, as detailed above) was added with stirring, the resultant solution left to stand for 1 h, and then degassed under vacuum for 1 h to remove dissolved air (particularly dissolved carbon dioxide which could react with the sodium dodecyl-benzene sulfonate used in the next step to induce bead formation). Aqueous sodium dodecyl-benzene sulfonate solution (0.3 mol/L, 15 mL) was slowly added dropwise to the solution to produce small beads, which were then extracted by gentle filtration, repeatedly washed with deionised water, and stored at 4 °C until required.

Agar (0.5 g) was dissolved in deionised water (10 mL) with stirring at 50 °C for 1 h. The resulting solution was then cooled to 40 °C and CE solution (10 mL) was added dropwise with stirring. The solution was then left to stand for 10 min, and was stored at 4 °C until required.

2.3.2. Immobilisation by adsorption onto Chitopearls

Chitopearls BCW-2510 and SH-5010 (4 g of each) were separately immersed in deionised water (~250 mL) with stirring for 24 h at room temperature. CE solution (10 mL) was separately added dropwise to each Chitopearl suspension and stirring continued for 2 h. The Chitopearl beads were then extracted by gentle filtration and stored at 4 °C until required.

2.3.3. Immobilisation by chemical derivatisation onto Chitopearls

Chitopearls BCW-2510 and SH-5010 (4 g of each) were separately immersed in deionised water (~250 mL) with stirring for 24 h at room temperature. Aqueous glutaraldehyde (2.5%, v/v, 100 mL) was added dropwise to the solutions with stirring. After 2 h, CE