ARTICLE IN PRESS

FEBS Letters xxx (2015) xxx–xxx

FEBS Letters

journal homepage: www.FEBSLetters.org

Borate–fructose complex: A novel mediator for laccase and its new function for fructose determination

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ARTICLE INFO

Article history:
Received 9 July 2015
Revised 14 August 2015
Accepted 17 August 2015
Available online xxxx

Edited by Judit Ovadi

Keywords:
Laccase
Borate–fructose complex
Mediator
Fructose determination

ABSTRACT

Laccase and borate–fructose complex were investigated by coincidence in a solid-state fermentation of *Edenia* sp. TS-76 under fructose oxidase screening. Laccase was purified to homogeneity with a 34-fold purification and 32% yield. Fructose had no significant effect on laccase activity, whereas borate reduced laccase activity by 60–90%; conversely, the borate–fructose complex increased laccase activity by nearly fourfold at pH 7.5. The complex caused a shift in the optimal pH for laccase from 5.0 to 7.5 and served as a highly efficient mediator. Borate complexed with fructose provides an alternative, time-saving, and specific method for serum fructose determination.

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1. Introduction

Laccase (EC 1.10.3.2), a phenoloxidase secreted by various plants, controls the degradation and formation of lignin in nature by oxidizing monolignols, naturally occurring phenol family and widely distributed in fungi [27,13,26], bacteria [9], insects [10], and algae [22]. It catalyzes one-electron oxidation of substrates, particularly phenolic derivatives [11,2], to their corresponding radicals.

Laccase substrate specificity has increased since the first use of 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) in laccase systems, in which either phenolic or non-phenolic lignin model compounds are oxidized by laccase [5]. The oxidation range can be expanded by these mediators acting as electron carriers between laccase and target compounds. Once the mediator is oxidized by laccase, it can move from the enzyme's active site and oxidize target compounds that are not substrates for laccase. Therefore, appropriate mediator selection plays a critical role in expanding laccase application. Several studies confirmed that laccase oxidizes non-phenolic aromatic, large molecular, or high redox potential substrates in the presence of suitable mediators. Most mediators are phenolic or nitrogen-based heterocyclic compounds [8]. The broad substrate specificity and oxygen utilization as an electron donor instead of hydrogen peroxide make laccase a promising candidate as a green catalyst for several applications. Interest in this enzyme for use in kraft processes, organic synthesis, renewable bioenergy industries, and other potential fields is growing.

Regarding global obesity epidemic etiology, a causal role of the increased fructose consumption was proposed [6]. Fructose intake effect in the form of sucrose or high-fructose corn syrup on carbohydrate metabolism and blood sugar levels have become increasingly important. Currently, fructose is indirectly determined using commercial assay kits (Sigma–Aldrich FA20; Abcam ab83380) requiring multi-enzymatic reactions and coenzymes. Although fructose oxidation can be specifically catalyzed by fructose 5-dehydrogenase accompanied by dye reduction, fructose 5-dehydrogenase is a membrane-bound enzyme that is difficult to purify to homogeneity.

OH groups of several glycosides can be oxidized by the laccase mediator system (LMS) of *Trametes pubescens* using TEMPO [4,20] or other components such as AZADO [30] as mediators. However, to date, no research has evaluated glycoside effect on laccase or its mediator system. We propose a new LMS for ABTS oxidation

Author contributions: Chih-Yu Cheng – author who participates in interpreting the data, drafting the article, revising it critically for important intellectual content. Chia-I. Liao – author who participated in all experiments and make contributions to acquisition of data and analysis of data. Shuen-Fuh Lin – author who makes substantial contributions to study conception and design and organized the study. Author who gives final approval of the version to be submitted and any revised version.

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http://dx.doi.org/10.1016/j.febslet.2015.08.032
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Please cite this article in press as: Cheng, C.-Y., et al. Borate–fructose complex: A novel mediator for laccase and its new function for fructose determination. FEBS Lett. (2015), http://dx.doi.org/10.1016/j.febslet.2015.08.032

with borate–fructose complexes. The complex is self-assembled by a well-known condensation reaction between boric acid and polyalcohol; it serves as a mediator between laccase and ABTS, the most commonly used colorimetric reagent for assaying laccase activity. This LMS illustrated the possibility of a novel primary mediator between laccase and ABTS, providing a new strategy for quantitative analysis.

Here, laccase was purified from *Edenia* sp. TS-76 and characterized. We also described the effect of borate–fructose complexes on this laccase and its possible utility for the quantitative fructose determination.

2. Materials and methods

2.1. Chemicals

Fractogel DEAE-650M and HW-55 gel filtration resins were purchased from Merck (Darmstadt, Germany). Molecular weight standards were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Toyopearl Phenyl-650M and Toyopearl MX-Trp-650M were purchased from Toyo Soda Manufacturing (Tokyo, Japan). Ultragel-HA was acquired from IBF Biotechnics (Pairs, France). Protein assay dye was obtained from Bio-Rad Laboratories (Richmond, CA). Other chemicals were of analytic reagent grade.

2.2. Microorganisms and culture conditions

The fungal strain TS-76 was isolated from the soil and identified as an *Edenia* species. The organism was maintained on YM agar slants (1% glucose, 0.5% peptone, 0.3% malt extract, and 0.3% yeast extract) at 30 °C. For enzyme production, the organism was grown in 1-L flasks, each containing 100 g of wheat bran supplemented with 100-ml water. After 5–7 days of cultivation at 30 °C, the entire culture was used for enzyme preparation.

2.3. Protein assay

Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. Protein assay reagents were purchased from Bio-Rad. Protein purity was determined by SDS–PAGE on 12.5% acrylamide slabs using a modified Laemmli buffer system [15]. Coomassie brilliant blue R-250 was used for staining.

2.4. Enzyme assay

Laccase activity was determined by monitoring ABTS oxidation at 420 nm. The assay was performed by mixing ABTS (10 mM, 100 μl), borate buffer (10 mM, pH 7.5, 700 μl), fructose (400 mM, 200 μl), and suitably diluted enzyme in 1-ml cuvettes. The initial absorbance changes of ABTS ($\varepsilon = 36\ mM^{-1}\ cm^{-1}$) were used to calculate the initial reaction rate υ. One-unit enzyme activity was defined as the amount of laccase that oxidized 1 μmol ABTS per min at 30 °C. Alternatively, oxidase activity was determined using the peroxidase-chromogen method. An enzyme aliquot was incubated at 30 °C in 1 ml of 50 mM Tris–HCl buffer (pH 7.8) containing 2% fructose, 2 units of peroxidase, 0.1 mM 4-aminoantipyrin (4-AA), and 1 mM phenol. Optical density was followed at 500 nm for 3 min. One-unit enzyme activity was defined as the amount of laccase that oxidized 1 μmol H_2O_2 per min at 30 °C.

2.5. Enzyme purification

A typical purification scheme was used, with all operations performed at 4 °C. To prepare the crude extract, TS-76 (100 g) wheat bran culture was soaked in 1 L of 20 mM borate buffer (pH 9.5, buffer A) with 0.1% Triton X-100 for 30 min and squeezed through a fine-mesh cloth. The aqueous extract was then centrifuged at 9000×*g* for 30 min to remove particles. The clarified aqueous extract (800 ml) was brought to 20% saturation with ammonium sulfate; the precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant to 90% saturation. The resulting precipitate was collected by centrifugation and dissolved in a minimal volume of buffer A. The enzyme solution was dialyzed overnight against the same buffer at 4 °C.

The dialyzed 20–90% ammonium sulfate fraction (200 ml) was applied to liquid chromatography in the order of a Fractogel DEAE-650M column (2.5 × 30 cm), Toyopearl Phenyl-650M column (2.5 × 15 cm), Toyopearl MX-Trp-650M column (1.5 × 15 cm), Fractogel HW-50 column (2.5 × 115 cm), and Ultragel-HA column (2.5 × 15 cm) (sMethod 1). The active fractions were pooled and stored at −20 °C.

2.6. General procedure for cyclic voltammetry studies

The working electrode was Pt; the reference electrode was 3 M $NaCl_{(aq)}$, and the auxiliary electrode was Pt wire. The cyclic voltammetry machine was a CH Instrument Model 600D series. Under argon, substrate (0.025 M) and $LiClO_4$ (0.1 M) were dissolved in 48-ml anhydrous methanol; the resulting solution was divided into four vials. Each vial was separately measured five times at a 0.025-VS^{-1} scan rate.

2.7. Quantitative analysis of fructose by the laccase-BF system and DNS

Fructose determination was performed by mixing ABTS (10 mM, 100 μl), borate buffer (10 mM pH 7.5, 700 μl), and suitably diluted enzyme in 1-ml cuvettes containing 0–100 mM fructose. The reaction mixture was measured spectrophotometrically at 420 nm. Fructose (0–100 mM) was analyzed alternatively after adding 1 ml dinitrosalicylic acid reagent [21], after which the mixture was boiled for 10 min and chilled. The resulting reducing sugar adduct was measured spectrophotometrically at 540 nm.

3. Results

3.1. Microorganism screening and enzyme purification

Laccase produced by *Edenia* sp. TS-76 was purified to homogeneity with a 34-fold purification and 32% yield (sTable 1). It was resolved as a single 73-kDa by SDS–PAGE (sFig. 1).

3.2. ABTS oxidation by laccase rather than fructose oxidase

First, the peroxidase-chromogen method was used to screen the fructose oxidase of TS-76 using fructose as substrate. Oxidase activity determined by spectrophotometry following 4-AA oxidation was feasible; however, no remarkable differences were observed with or without fructose (data not shown). This substrate-independent finding indicates that the oxidase activity was attributable to an oxidase that oxidizes phenol or 4-AA rather than fructose. The oxidation activity toward phenolic substrates was further attributed to a phenol oxidase using a typical ABTS method. Additionally, an unexpected enzyme activity increase was noticed in the presence of both fructose and borate, an alkaline buffer for enzyme extract. This is the first report on effects of borate, fructose, or both existing as a borate–fructose complex self-assembled via a well-known condensation reaction on laccase activity.

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