CONVERSION OF B-TYPE TO A-TYPE PROCYANIDINS BY OXIDASES AND EVALUTION OF THEIR REACTION CONDITIONS

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ABSTRACT

A-type procyanidins (PCs) are polyphenols with anti-adhesive properties against gram-negative bacteria. As A-type usually occur in low content in plant matrices, for biotechnological applications, these PCs can be obtained by oxidizing B-type PCs through enzymatic processes. Thus, this study aimed to select commercial enzymes with potential for converting B-type to A-type PCs and to investigate their reaction conditions. Initially, the screening of four commercial enzymes showed that *T. versicolor* laccase and *A. bisporus* PPO demonstrated the higher conversion rates (53.8 and 67.7%) and were selected for further study. Subsequently, the enzymes' activity was assayed for optimal pH and temperature, as well as thermal and pH stability. While laccase showed higher activity in acidic conditions (pH 4) and across a wide temperature range, PPO exhibited maximum activity in neutral conditions and mild temperatures. The laccase also demonstrated better stability across a pH range of 4-7 and various temperatures. In contrast, the PPO maintained good activity stability in neutral pH but showed a significant activity decrease in acidic environments. These findings will be used to optimize the enzymatic conversion of B-type to A-type PCs, enabling a green process to obtain high-value bioproducts from underutilized natural sources of B-type PCs.

Keywords: Proanthocyanidins. Polyphenol oxidase. Laccase. Fungal enzymes.

1 INTRODUCTION

Procyanidins (PCs) are a class of polyphenols that occur naturally in plants and are present in varied concentrations in diverse food products. PCs consist of oligomers and polymers of (+)-catechin and (-)-epicatechin subunits, with variability in subunit composition, degree of polymerization and type of bond between subunits.[1] These compounds are classified into A-type and B-type: B-type PCs present subunits connected by a single C-C bond, while A-type PCs have at least one extra ether bond between two subunits, altering their three-dimensional structure and increasing rigidity.[2] A-type PCs exhibit distinct biological activities, such as the inhibition of adhesion of uropathogenic bacteria due to interactions with fimbriae structures,[3] which suggests their potential for biotechnological applications in preventing bacterial adhesion and biofilm formation. In nature, A-type PCs are significantly less abundant than B-type PCs. Accordingly, it is relevant to investigate other ways to obtain this bioproduct. One possibility involves the oxidation of B-type PCs, achievable via chemical or enzymatic pathways using oxidases. The enzymatic conversion involves the removal of a hydrogen atom from C2 of the upper subunit, forming and intermediary quinone. Subsequently, a nucleophilic attack by a hydroxyl group forms the characteristic extra ether bond of A-type PCs (**Figure 1**).[4]

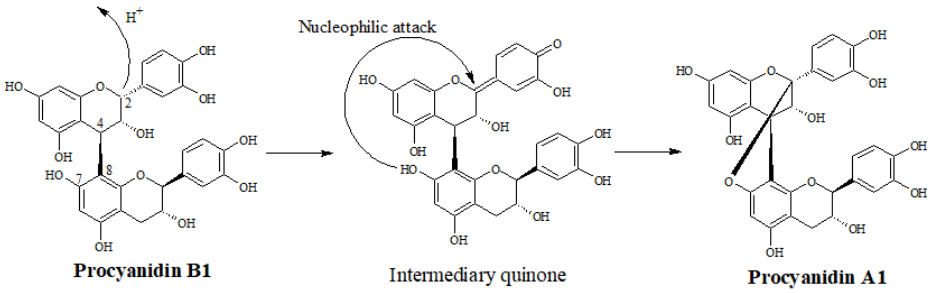

Figure 1 Proposed mechanism of the conversion of B-type to A-type PCs by oxidation.

Considering the increasing demand for green processes, the enzymatic conversion of B-type to A-type PCs offers a biocatalytic route to obtain a high-value product from natural sources rich in B-type PCs, such as agroindustrial residues like açaí (*Euterpe oleracea* Mart.) seeds.[5] Our group has previously demonstrated this conversion using a laccase from *Aspergillus* sp. However, there is a great variety of oxidases from bacteria, fungi and plants, with diverse oxidative potentials that are yet to be fully explored. Therefore, this study aims to evaluate the efficacy of four different commercial oxidases in converting B-type to A-type PCs, identify the most promising enzymes and study their optimal conditions of temperature and pH.

2 MATERIAL & METHODS

To evaluate enzymes from different sources, four commercial oxidases were acquired: laccase from *Aspergillus* sp. (ascomycete), laccase from *Trametes versicolor* (basidiomycete), polyphenol oxidase (PPO) from *Agaricus bisporus* (basidiomycete) and horseradish (*Armoracia rusticana*) peroxidase. The enzymes, as well as PC B1 ((−)-epicatechin-(4β-8)-(+)-

catechin) and A1 ((+)-epicatechin-(4β-8,2β-O-7)-catechin) analytical standards were obtained from Sigma-Aldrich (San Luis, Missouri, USA). To screen the four enzymes, reactions were performed using a 200 µg/mL sample of PC B1 analytical standard as substrate under orbital agitation for two hours, following temperature and pH conditions recommended by the literature and manufacturer. Conversion rates (%) of PC B1 to A1 were determined using a HPLC-DAD system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with a Symmetry C18 column (250x4.6 mm, 5 µm, Waters Technologies, Ireland) and a corresponding guard column at 30°C. The mobile phases were 0.5% formic acid in ultrapure water (A) and acetonitrile:phase A (80:20, v/v). Elution was conducted in gradient mode according to an adaptation of a previously published method[5]. Peaks for PCs B1 and A1 were identified and quantified using analytical curves (R^2 = 0.9996 for A1 and R^2 = 0.9823 for B1), enabling calculation of substrate consumption and conversion rate (%). All analyses were performed in triplicate. The two enzymes with the highest conversion rates were selected for further study.

For future optimization of reaction conditions, we assessed the activity of the enzymes under different pH and temperature conditions, as well as their thermal and pH stability. The enzymatic activity of the *T. versicolor* laccase was determined spectrophotometrically through the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), monitoring the reaction at 420 nm. One unit of enzyme (U) was defined as the amount capable of oxidizing 1 µmol of substrate per minute.[6] The enzymatic activity of the *A. bisporus* PPO was determined by the oxidation of L-Tyrosine, with one unit of enzyme (U) causing an increase in absorbance of 0.001 per minute at 280 nm.[7] To assess the behavior of the enzymes in different conditions, enzymatic activity was measured at pH levels 3, 4, 5, 6 and 7 (Laccase – 50 °C, PPO – 30 °C), and at temperatures of 30, 40, 50, 60 and 70 °C (Laccase – pH 4, PPO – pH 6). The stability of the enzymes was assessed by measuring enzymatic activity after exposure to the same conditions for up to 4 hours. All analyses were conducted in triplicate.

3 RESULTS & DISCUSSION

The screening results for the four enzymes are presented in **Table 1**. The *T. versicolor* laccase and the *A. bisporus* PPO exhibited the highest yields of A-type PC, of 53.8% and 67.7% respectively, with both converting over 60% of the available substrate. Consequently, these two enzymes were selected for further studies. However, neither enzyme fully converted PC B1 to PC A1, resulting in a varied percentage of unidentified compounds. These peaks likely represent reaction byproducts, intermediates or nonspecific reaction products, due to the general lack of specificity in oxidases and unknown purity levels of the commercial enzymes. Future experiments coupling with mass spectrometry and SDS-PAGE electrophoresis of the enzymes will help clarify the identities of these compounds and why they are being formed.

Table 1 Conversion of B-type to A-type PCs quantified by HPLC-DAD analysis by the four commercial enzymes evaluated.

Enzyme	PC B1 conversion (%)	PC A1 yield (%)	Unidentified byproducts (%)
Laccase (*Aspergillus* sp.)	19.0±0.7	17.2±0.6	1.7±0.1
Laccase (*T. versicolor*)	60.3±1.3	53.8±1.2	6.5±0.1
PPO (*A. bisporus*)	84.0±3.1	67.7±2.5	16.2±0.6
Horseradish peroxidase	47.8±0.7	27.3±0.4	20.6±0.3

We subsequently evaluated the pH and temperature conditions for the selected enzymes, with results presented in **Figure 2**. The enzymes exhibit distinct behaviors in response to pH and temperature variations. The laccase shows higher activity in acidic conditions, peaking at pH 4, with reduced activity in neutral environments. Conversely, the PPO demonstrates optimal activity in neutral (pH 6-7), with a marked decline in more acidic environments. Regarding temperature, laccase maintains activity above 40 U/mL across a broad range (30-60 °C), with the highest value at 50 °C. In contrast, PPO achieved peak activity at moderate temperatures (30-40 °C), with a significant activity loss at higher temperatures.

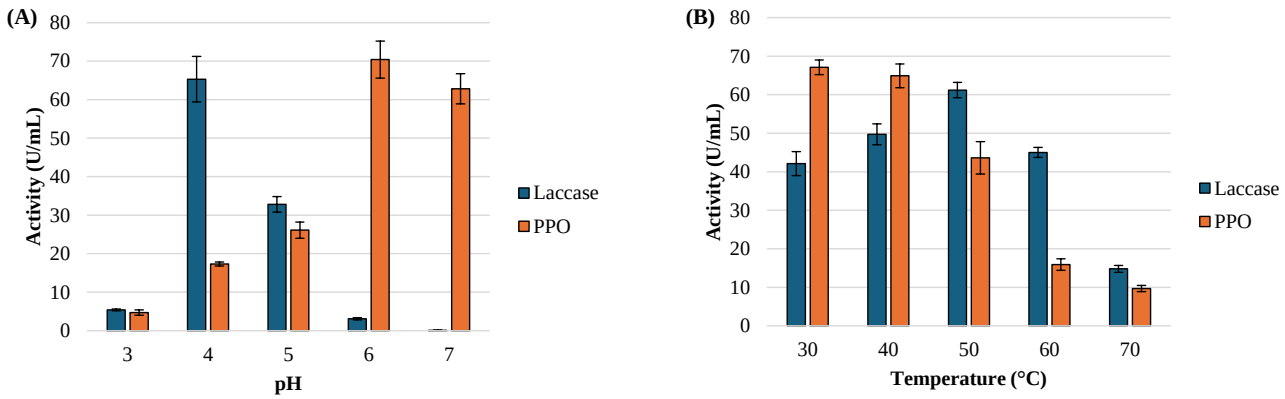

Figure 2 Optimal pH (A) and temperature (B) of the laccase from *T. versicolor* and the PPO from *A. bisporus*.

Then, we assessed the stability of both enzymes under different pH and temperature conditions (**Figure 3**). *T. versicolor* laccase maintained over 80% of its initial activity after 4 hours of exposure in pH 3-7 at 50 °C, though it showed very low activity in neutral media. This result suggests that *T. versicolor* laccase is suitable for reactions at pH 4-5 for prolonged reaction times.

A. bisporus PPO retained over 80% activity after 4 hours in pH 6-7 at 30 °C, the conditions where it also had the highest enzymatic activity. In more acidic conditions, PPO activity rapidly decreased, indicating that these conditions are unfavourable for conversion reactions with PPO. Therefore, neutral media are preferable for PPO over extended periods. Regarding the temperature stability, *T. versicolor* laccase showed decreased stability with increased temperature at pH 4. At its optimal temperature of 50°C, it retained 55% of its initial activity after 2 hours and 47% after 4 hours. Thus, the use of lower temperatures, like 30 or 40 °C should be more deeply investigated. In the case PPO, the enzyme presented good stability at 30 and 40 °C, maintaining over 80% residual activity after 4 hours, while activity rapidly decreased at 50-70 °C. These results suggest that mild temperatures (30-40 °C) are the best option for enzymatic conversion with PPO. Therefore, to fully unravel the operational conditions of these enzymes, optimizing reaction conditions based on pH-temperature interactions and product formation kinetics is still necessary.

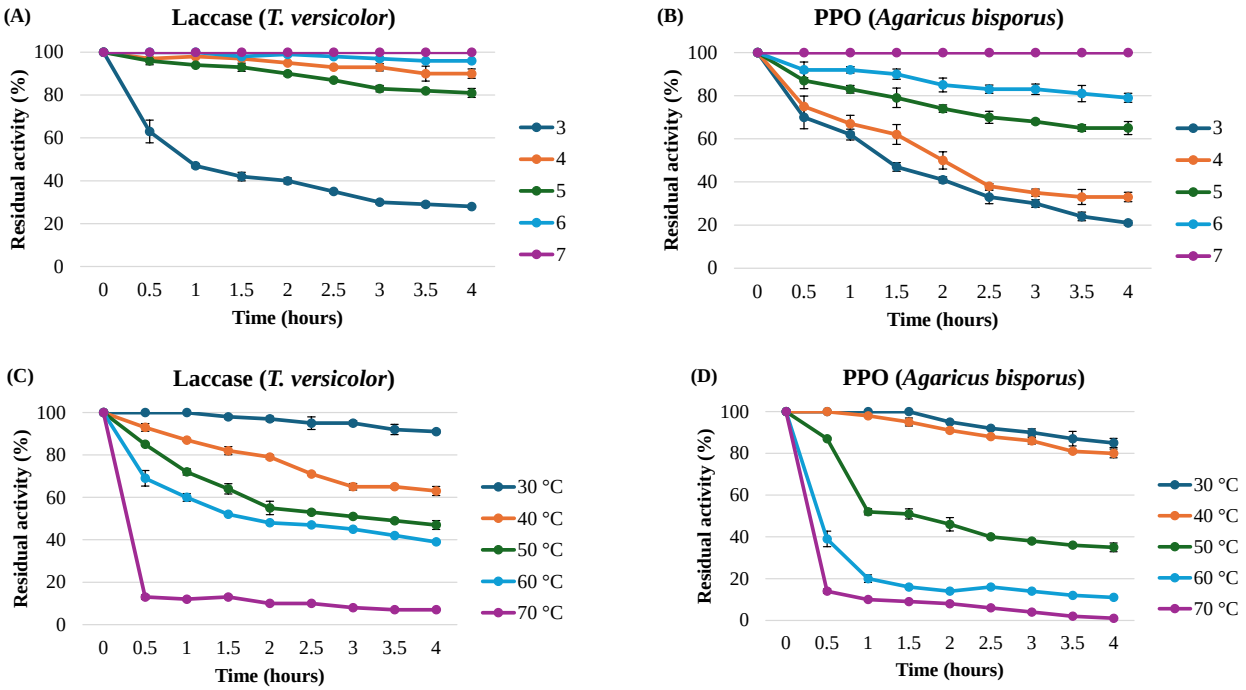

Figure 3 pH and thermal stability of *T. versicolor* laccase (A and C) and PPO (B and D).

The selected enzymes exhibited significantly different behaviors under various pH and temperature conditions. Oxidases produced by different organisms display diverse oxidative capacities and optimal conditions due to structural features such as complexity, flexibility, and the presence of copper atoms, as well as hydrophobic and hydrogen bonds. The evaluated PPO has a more flexible, less complex structure with fewer stabilizing interactions and copper atoms compared to the laccase, which which could be related to the enzyme's lower thermostability.

4 CONCLUSION

T. versicolor laccase and *A. bisporus* PPO were selected as candidates for the conversion of B-type to A-type PCs due to their high efficiency in this process. The evaluation of reaction conditions for both enzymes indicated that *T. versicolor* laccase should be studied at pH 4, preferably within a temperature range of 30-40 °C. In contrast, the optimization of B-type to A-type PC conversion by *A. bisporus* PPO should be conducted at pH 7, within the same temperature range of 30-40 °C. These findings will allow further optimization of the conversion reaction to maximize the yield of A-type PCs and to control by-product formation while studying the enzymes' kinetics. The enzymatic conversion of B-type to A-type PCs presents a promising green alternative for obtaining a high-value bioproduct from underutilized agroindustrial residues rich in B-type PCs, such as açaí seeds.

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