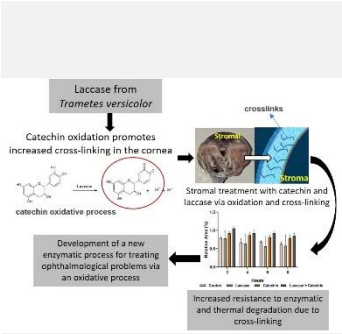
Characterization of sclera submitted to crosslinking using the oxidoreductase laccase

POSTER
Ph.D. Student: N
Journal: CEJ

***A.A. Morandim-Giannetti*[1],** *P.O. Gobira[2], J.C. Andrade Neto[2], P.A. Bersanetti[2]. (1) Centro Universitário FEI, Humberto de Alencar Castelo Branco 3972, São Bernardo do Campo, Brazil, andreia.moramdim@gmail.com. (2) Universidade Federal de São Paulo, Rua Pedro de Toledo, 669, São Paulo, Brazil.*



This study investigated the efficacy of *Trametes versicolor* laccase, an oxidoreductase used in several biotechnological processes combined with catechin, as a cross-linking agent for ophthalmological treatments employing porcine sclera. The ability of this combination to induce cross-links between collagen fibrils was analyzed, evaluating the resistance to enzymatic digestion by collagenase type I and thermal stability through Differential Scanning Calorimetry (DSC). The results demonstrated that the treatment with laccase and catechin enhances the formation of cross-links via the oxidative enzymatic process, conferring greater resistance to enzymatic degradation on the sclera and a significant improvement in thermal stability, evidenced by the increase in denaturation temperature.

Introduction

Vision can be compromised by various refractive errors that can reduce or lose visual acuity due to deformation in the cornea and sclera. [1] One of the main treatments used involves crosslinking, which promotes cross-links between the macromolecules in ocular tissues, such as collagen fibrils. This treatment promotes tissue stiffening and prevents deformation progression caused by refractive errors, such as myopia. [2] The oxidative enzymatic crosslinking induced by oxidoreductases, such as laccase, triggers a series of radical reactions that promote cross-links, a possibility for application in the ophthalmological field. [3] Therefore, the present work proposes the in vitro characterization of crosslinking via oxidative enzymatic process in porcine sclera using *Trametes versicolor* laccase, evaluating the efficacy by resistance to enzymatic digestion and Differential Scanning Calorimetry (DSC), aiming at possible ophthalmological treatments.

Material and Methods

Sclera samples were obtained through trephination of porcine eyes from slaughterhouses and kept in a 10% dextran solution in DMEM for 24 hours to ensure the uniform hydration of the sclera. Then, the samples were divided into 4 different groups: control (without treatment), treated with laccase (*Trametes versicolor*) 3 mg/mL, treated with catechin 4 mg/mL, and treated with laccase and catechin at the same concentrations as the previous groups. All solutions were prepared in DMEM containing 10% dextran and kept at 36 °C for 24 h, followed by the determination of the scleral area using a Zeiss Axio Scope A1 microscope with 10x magnification. Subsequently, each sample was subjected to treatment with collagenase type I from *Clostridium histolyticum* 2 mg/mL in DMEM with 10% dextran at 42°C for 8 h to assess resistance to enzymatic digestion, with the determination of the scleral area every 2 h. The evaluation of the thermal stability of the samples was determined through Differential Scanning Calorimetry (DSC) after 24 h of treatment, where about 2 mg of sclera were placed in a hermetically sealed aluminum crucible in an inert nitrogen atmosphere with a flow of 50 mL/min, with a constant heating rate of 10 °C/min from 25 to 100 °C using the Shimadzu DSC-60 equipment (Kyoto, Japan).

Results and Discussion

The results of the enzymatic digestion (Figure 1) show greater resistance to degradation for the group treated with laccase in the presence of catechin compared to the control sample. This greater resistance is due to the difficulty of collagenase finding cleavage points for its proteolytic action against the collagen fibers of the sclera. This difficulty is caused by the formation of cross-links via an oxidative process that made the sclera structure more rigid, hindering the permeability of collagenase. Therefore, when analyzing the relative area results for the group over

time, the parameter is seen at a much lower rate than the control. It was also observed that laccase alone did not show cross-linking action in the absence of catechin, as the digestion results of the control group were similar to the control group. However, in the presence of catechin, laccase promotes its oxidation and, after this process, an increase in cross-links between collagen fibrils intermediated by the oxidized catechin.

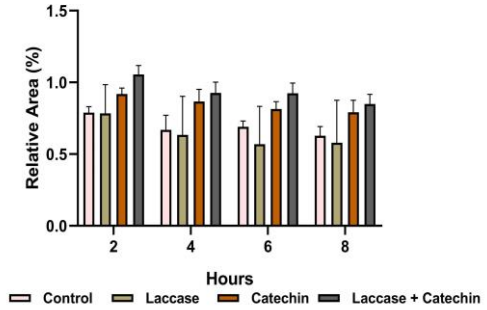


Figure 1. Digestion Results

The analysis via DSC (Table 1) showed that, when compared to the control group, the group treated only with laccase did not show a difference in denaturation temperature, indicating that the use of it without a carrier does not affect the formation of cross-links, since laccase, being a protein of considerable size, is not capable of permeating the tissue structure of the sclera and finding the possible preferential sites where it can catalyze the formation of radicals promoting crosslinking, corroborating with the results of resistance to enzymatic digestion. Therefore, when comparing the control group with the group treated with laccase in the presence of catechin, a percentage increase of 11.3% (7.3 °C) in the denaturation temperature is noted, indicating that the formation of cross-links in the scleral matrix conferring greater structural stability of the macronutrients that compose it, especially the collagen fibers, since these structures are bound by covalent bonds, requiring more energy to undo the three-dimensional structure, leading to an increase in the denaturation temperature, thus proving crosslinking.

These data corroborate the data evidenced during the digestion analysis, in which the greater effectiveness of cross-link formation mediated by catechin enzymatically oxidized by laccase was observed.

Conclusions

This work demonstrated the potential application of *Trametes Versicolor* laccase in the presence of catechin in scleral treatments via enzymatic catechin oxidation followed by cross-link formation. This potential was evidenced by the enhanced formation of cross-links in the sclera of porcine eyes, providing greater resistance to enzymatic degradation, as observed in the assay performed with collagenase, and greater thermal stability, demonstrated by the increase in denaturation temperature by 7.3°C. Based on these results, laccase and catechin can be considered promising cross-linking agents via the oxidation process for treating diseases such as keratoconus and myopia through crosslinking procedures.

Group	Denaturation temperature (°C)	Enthalpy (J/g)	Temperature increase (%)
Control	67.3 ± 1.2	10.8 ± 2.0	-
Laccase	67.28 ± 0.12	11.10 ± 0.65	-
Catechin	72.15 ± 0.40	10.29 ± 0.39	7.2 (+ 4.8 °C)
Laccase + Catechin	74.93 ± 0.73	15.35 ± 0.67	11.3 (+ 7.3 °C)

Table 1. Thermal Analysis Results by DSC

Acknowledgments

This work was approved by the Ethics Committee for the Use of Animals (CEUA) of the Federal University of São Paulo (number 3955010823).

References

[1] P. Kang, M. Boptom, G. Doig, F. Stapleton. *Clinical and Experimental Optometry, v. 103, (2020)*.
[2] L. Angelo, G. Boptom, M. Charles, Z. Mohammed*. Asia PacificJornal of Ophthalmology, v. 11 (2022).*
[3] V. Manhivi, E. Amonsou, T. Kudanga*. Elsevier, v. 264 (2018).*