Development of a continuous δ-viniferin synthesis in a microreactor using immobilized horseradish peroxidase

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INTRODUCTION

δ-viniferin is a resveratrol dehydrodimer, an isomer of ε-viniferin, which widely exists in grapes, knotweed, peanuts, and red wine. It was found to have biological activities, such as antiviral, anti-inflammatory, antibacterial, anticancer, and antioxidation. It possesses strong antioxidant properties, which can help protect the body against free radicals and oxidative stress. Additionally, δ-viniferin has been found to have anti-inflammatory properties, aiding in reducing inflammation in the body. Some researchers have also suggested that δ-viniferin could have the potential to fight various diseases, including cancer, cardiovascular diseases, and neurodegenerative disorders.[1,2] However, at a cost often exceeding 300 euros for just 1 milligram, the expense associated with δ-viniferin may severely restrict research efforts and its global applicability.

AIM

- to develop a cost-effective and sustainable process for synthesizing δ-viniferin from bio-derived materials using horseradish peroxidase (HRP)
- optimization of crosslinked enzyme aggregates of HRP (CLEA-HRP) generation using microfluidic system[3]

EXPERIMENTAL

Optimization of CLEA-HRP generation (**Figure 1**)

Optimization focused on determining the optimal residence time for precipitation and crosslinking, screening various precipitation solvents, and adjusting the glutaraldehyde (GA) concentration for enzyme crosslinking at 25°C in PTFE tubes of various lenghts with 0.8 mm inner diameter.

Horseradish peroxidase (HRP) was dissolved in 0.1 M potassium phosphate buffer (pH 6.0).

Residence times in a microfluidic system obtained by changing the tube's lenghts: 3.77, 1.88, 0.94, 0.47 and 0.34 min

HRP inlet concentration: 0.02 mg/mL

HRP inlet solution flow rate: 50 µL/min

Organic solvents tested for precipitation: acetone, acetonitrile, isopropanol and ethanol.

Organic solvent flow rate: 50 µL/min

Crosslinking agent: 1, 1.5, 0.5, 0.1 mM glutaraldehyde (GA) solution

Crosslinking agent flow rate: 100 µL/min

The size of CLEA-HRP was assessed through dynamic light scattering analysis (DLS).

The activity of CLEA-HRP was measured spectrophotometrically using ABTS test[4] - the activity of CLEA-HRP was compared to the free enzyme (recovered activity).

Batch reaction[5] (**Figure 2**)

Various amounts (80 mg, 100 mg, 120 mg) of resveratrol were dissolved in 4.621 mL of citrate buffer (pH 5.0) with 50% (v/v) of acetone. 3,79% (v/v) of HRP in Milli-Q water (1 mg/mL) was added and the mixture was stirred for 30 min at tested temperature. Subsequantly, 1.43% (v/v) of H_2O_2 was added and the mixture was stirred for 1 h at tested temperature.

Temperature: 40°C

For quenching the reaction, the solution was placed in ice. The concentrations of resveratrol and δ-viniferin were analyzed using HPLC with Gemini-NX 3 µm C18 110 Å (150 × 4.60 mm) column and UV/VIS detector.

Continuous flow reaction (**Figure 3**): Work in progress

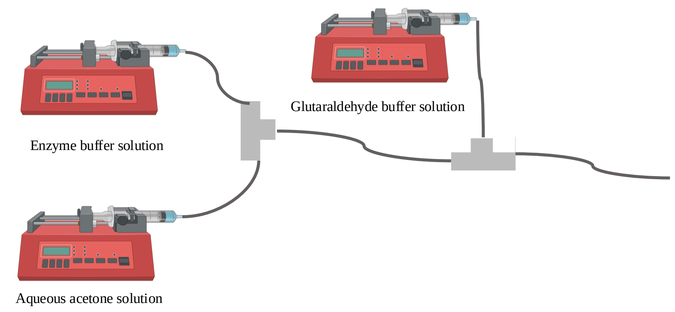

Figure 1: Scheme of a continuous CLEA-HRP production in a microfluidic system

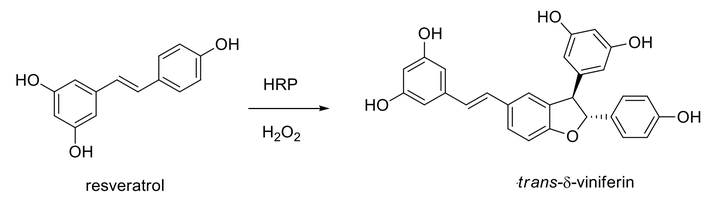

Figure 2: HRP-catalyzed synthesis of δ-viniferin from resveratrol

Figure 3: Scheme of a continuous δ-viniferin production in a microreactor with CLEA-HRP immobilized on the membrane surface

RESULTS

Optimization of CLEA-HRP generation

Table 1: Results of testing different residence times; organic solvent: pure acetone, 1 mM GA

Tube length, cm	τ, min	Recovered activity, %	St. dev., %
100	3.77	74.96	3.10
50	1.88	85.48	4.13
25	0.94	93.15	2.37
12.5	0.47	93.32	1.68
9	0.34	98.36	1.67

Tube with a length of 9 cm and a residence time of 0.34 min was selected for further testing due to its highest achieved recovered activity (**Table 1**).

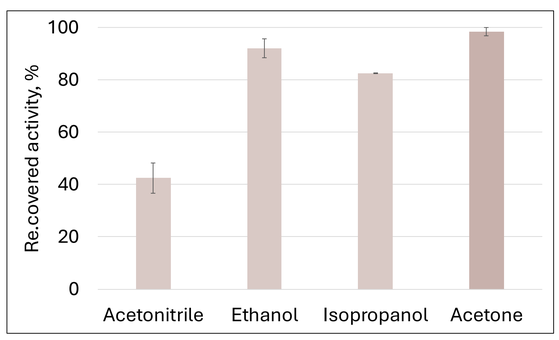

Figure 4: The effect of tested organic solvents as precipitation solvents on recovered activity; 1 mM GA, residence time 0.34 min

Acetone was selected among the tested organic solvents as the best-tested solvent for precipitation because of the highest achieved recovered activity (**Figure 4**).

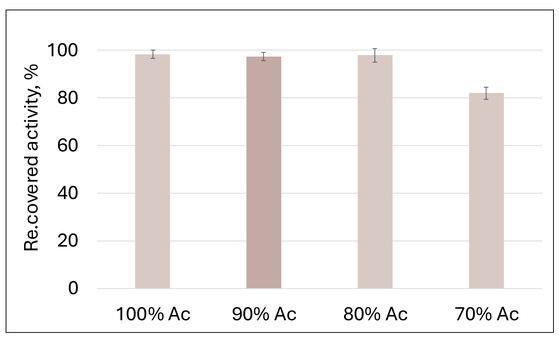

Figure 5: Results of testing different acetone:buffer ratios; 1 mM GA, residence time: 0.34 min

The final choice of organic solvent was 90% (v/v) acetone due to its highest recovered activity (**Figure 5**).

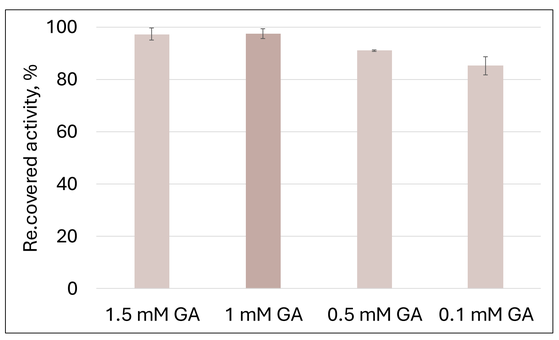

Figure 6: Results of testing different concentrations of glutaraldehyde; organic solvent: 90 vol.% acetone, residence time 0.34 min

1 mM was selected as the best GA concentration for cross-linking enzyme because the highest recovered activity was retrained (**Figure 6**).

DLS

The resulting CLEA-HRP exhibited an average particle radius of 150 nm (**Figure 7**).

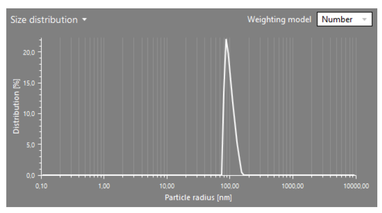

Figure 7: Size distribution of CLEA-HRP; 90% (v/v) acetone, 1 mM GA, retention time 0.34 min

Batch reaction

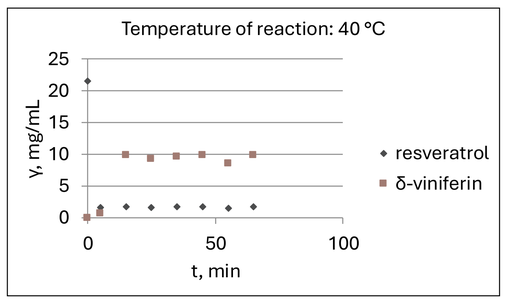

Figure 8: Concentrations of resveratrol and δ-viniferin at 40 °C, initial mass of resveratrol: 100 mg

The yield of the reaction performed in a batch process was 45.7% after 15 min (**Figure 8**).

CONCLUSIONS

The highest recovered activity of 99% was achieved at a residence time of 0.34 min with acetone and glutaraldehyde concentrations of 90% (v/v) and 1 mM, respectively. The resulting CLEA-HRP exhibited an average particle radius of 150 nm.

The reaction was successfully performed with a yield of 45.7% after 15 min.

ACKNOWLEDGEMENT

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REFERENCES

(1) Shang, Y.; Li, X.; Sun, T.; Zhou, J.; Zhou, H.; Chen, K. Comparative theoretical researches on the anti-oxidant activity of δ-viniferin and ε-viniferin. Journal of Molecular Structure **2021**, 1245, 131062. https://doi.org/10.1016/j.molstruc.2021.131062.

(2) Zwygart, A. C.; Medaglia, C.; Huber, R.; Poli, R.; Marcourt, L.; Schnée, S.; Michellod, E.; Mazel-Sanchez, B.; Constant, S.; Huang, S.; Bekliz, M.; Clément, S.; Gindro, K.; Queiroz, E. F.; Tapparel, C. Antiviral properties of trans-δ-viniferin derivatives against enveloped viruses. Biomedicine & Pharmacotherapy **2023**, 163, 114825. https://doi.org/10.1016/j.biopha.2023.114825.

(3) Menegatti, T.; Lavrič, Ž.; Žnidaršič-Plazl, P. Microfluidics-based preparation of cross-linked enzyme aggregates. WO2023175002A1.

(4) https://www.sigmaaldrich.com/HR/en/technical-documents/protocol/protein-biology/enzyme-activity-assay-of-peroxidase-abts-as-substrate

(5) Mattio, L. M.; Dallavalle, S.; Musso, L.; Filardi, R.; Franzetti, L.; Pellegrino, L.; D'Incecco, P.; Mora, D.; Pinto, A.; Arioli, S. Antimicrobial Activity of Resveratrol-Derived Monomers and Dimers against Foodborne Pathogens. Sci. Rep. **2019**, 9 (1), 1–13.