



Optimization of Polyphenol Oxidase (PPO) Extraction from Elephant Grass and Legume Leaves with the Addition of Vitamin C, Vitamin E, and Curcumin to Enhance Enzyme Activity and Stability

Asri Nurul Huda^{1,*}, Hendrawan Soetanto¹, Akhmad Sabarudin², Aprodita Aprodita¹, Najmi A. Hanifah¹, Sayyid M. Ja'far¹, Robby B. Lana¹, Deliana Nurdianthi¹, Dimas Nugroho¹, Alfina Salsabila¹, and Aksauri H. Malis¹

¹ Faculty of Animal Science, Universitas Brawijaya, Malang 65145, Indonesia

² Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang 65145, Indonesia

*nurulasri@ub.ac.id

Abstract. Polyphenol oxidase (PPO) is an enzyme with inhibiting biohydrogenation of lipid properties and found ubiquitously in many plant leaves. The extraction of PPO may require anti-oxidant substances for optimal concentration relevant to industrial application and nutritional enhancements for ruminal-lipid manipulation. This study investigated the enhancing effects of antioxidant addition, that is, vitamin C, vitamin E, and curcumin during PPO extraction of two grasses (*Pennisetum purpureum* and dwarf type-*Pennisetum purpureum*) and two legume leaves (*Leucaena leucocephala* and *Glicidia sepium*) on the PPO activity, yield, and stability. Using a combination of response surface methodology and factorial design, optimal extraction conditions-such as pH, temperature, and solvent type-were determined. The results showed that no single antioxidant under study exhibits enhancement of all intended properties. The addition of vitamin C significantly enhanced PPO activity, while vitamin E and curcumin contributed to improved enzyme stability. In conclusion the findings indicated that optimizing the extraction process of PPO requires the presence of additional multiple antioxidants to enhance enzyme yield and its functional from these plant sources.

Keywords: activity, enzyme, grass, legumes, PPO.

1 Introduction

The production of ruminant-derived food products with improved fatty acid profiles is essential for meeting health standards and mitigating the risks associated with excessive saturated fatty acid (SFA) consumption, such as cardiovascular diseases [1]. Ruminant products, including beef and milk, are often rich in SFA due to the biohydrogenation process in the rumen, where polyunsaturated fatty acids (PUFA) are converted into SFA by rumen microorganisms [2]. This transformation diminishes the

nutritional value of ruminant-derived foods and contributes to health concerns, necessitating strategies to manipulate the biohydrogenation process and preserve PUFA in the rumen.

One promising approach involves the use of bioactive compounds to inhibit biohydrogenation [3-5]. Among these, polyphenol oxidase (PPO)—an enzyme abundant in green forages and legumes—has emerged as a potential candidate. PPO can protect PUFA by forming phenol-protein complexes that shield unsaturated fatty acids from microbial hydrogenation [6,7]. This mechanism offers a natural and sustainable method to enhance the nutritional quality of ruminant products while utilizing available forage resources. Indonesian forages, such as elephant grass (*Pennisetum purpureum*), dwarf elephant grass (*Pennisetum purpureum* cv. Mott), calliandra (*Calliandra calothyrsus*), and leucaena (*Leucaena leucocephala*), are particularly rich in PPO and phenolic compounds, making them promising candidates for this strategy.

Recent studies have explored the use of activators, such as vitamin C, vitamin E, and curcumin, to enhance PPO activity and stability. These activators have shown potential to modulate PPO functionality, but their effects vary depending on factors like source, concentration, and interaction with the enzyme [8-10]. For instance, vitamin C has consistently demonstrated significant activation of PPO enzymes, while curcumin exhibits dual effects, acting as an inhibitor or activator depending on its concentration. This variability underscores the need for optimization to maximize PPO's efficacy in protecting PUFA during rumen biohydrogenation. Additionally, optimizing the extraction process for PPO from forage materials is critical for ensuring its effectiveness in practical applications, such as feed supplementation or forage preservation.

By optimizing PPO extraction and exploring the effects of activators like vitamin C, vitamin E, and curcumin, this study aims to advance strategies for improving the fatty acid profile of ruminant products. Enhancing PPO functionality not only contributes to the production of healthier ruminant-derived foods but also supports sustainable livestock management practices by leveraging locally available forages. This research has the potential to bridge the gap between enzyme functionality and practical applications, paving the way for innovative solutions to improve human health through dietary interventions.

2 Materials and Methods

The materials used in this study were greens consisting of two grasses (*Pennisetum purpureum* and dwarf type-*Pennisetum purpureum*) and two legume leaves (*Leucaena leucocephala* and *Glicidia sepium*). PPO sources such as grass and legumes were obtained from Junrejo District, Batu City. Aqueous Two-Phase System (ATPS) analysis for the PPO enzyme extraction and purification process and enzyme activity measurement were carried out at the Laboratory of Animal Nutrition, Faculty of Animal Science, Universitas Brawijaya. Enzyme activity was tested at the Extraction of the PPO enzyme from local forages, which requires PBS solution for preservation. Another material needed was ammonium sulfate ((NH₄)₂SO₄). Purification of the

PPO enzyme requires PEG-8000, PBS solution, and distilled water. The steps used in the first stage are extraction and purification. Local greens that are homogenized with liquid nitrogen are carried out by pounding using a mortar and pestle and then soaked in PBS solution. Extraction and purification aim to obtain PPO enzyme in pure form.

The extraction method used centrifugal force and the addition of ammonium sulfate as a material to help enzyme precipitation. This study used purification using the ATPS method or two-phase water extraction. The method was modified according to [11]. This study's modification was adding PEG-8000 to purify PPO, which can show pure PPO results with the best treatment. After purification, the PPO enzyme was added with catechol and ascorbic acid substrates and then incubated at 40°C. The research method used was a basic factorial experiment arranged with a factorial completely randomized design consisting of two treatment factors: the PPO enzyme source from the type of forage and the amount of activator added. The first factor is the source of PPO enzyme of the kind of forages, consisting of Dwarf elephant grass (DEG), Elephant grass (EG), Calliandra (Cal), Leucaena (Leuc). The second factor is the type of activator addition, which includes control, Vitamin E, Vitamin C, and curcumin

3 Results and Discussion

The results of this research are PPO enzyme activity from the extraction of Dwarf elephant grass (DEG), Elephant grass (EG), Calliandra (Cal), Leucaena (Leuc) with the addition of vitamin E, vitamin C and Curcumin activators. Table 1 shows that Leucaena leaves have the highest PPO activity (2.681 U/ml), followed by Calliandra (2.421 U/ml), Elephant grass (2.371 U/ml), and Dwarf elephant grass (2.321 U/ml). Certain legumes, particularly those in the genus *Leucaena*, are known to have naturally higher concentrations of phenolic compounds and the enzymes involved in their oxidation pathway [12,13]. PPO often serves as a defence mechanism against herbivory and disease [14-16]. Legume forages with higher PPO levels may have evolved to deter pests or diseases by oxidizing phenolic substrates to quinones, which can inhibit microbial growth [17-19].

Table 1. Enzyme activity of PPO extracted from different forages.

Forages	Enzyme Activity (U/ml)
DEG	2.321 ^a ±1.257
EG	2.371 ^b ±1.214
Cal	2.421 ^c ±1.313
Leuc	2.681 ^d ±1.736

* Note: DEG: Dwarf elephant grass; EG: Elephant grass; Cal: Calliandra; Leuc: Leucaena.

The study focused on three additives consisting Vitamin E, Vitamin C, and Curcumin and a control (no activator). The activators were chosen for their known antioxidant or biochemical properties, which can modulate enzyme activity in various

biological processes. Curcumin (4.351 U/ml) showed the highest enhancement of PPO activity, Vitamin C (2.996 U/ml) provided moderate enhancement and Vitamin E (1.255 U/ml) conferred minimal impact on PPO activity. Curcumin's superior performance in promoting PPO activity suggests it plays a more robust role in stabilizing or enhancing enzyme function compared to the other two vitamins. Curcumin, the main bioactive compound in turmeric (*Curcuma longa*), exhibits strong antioxidant properties [20,21]. These properties can protect the enzyme's active site from oxidative damage, thereby sustaining or even increasing PPO's catalytic efficiency. Curcumin's chemical structure allows it to bind with proteins and possibly form complexes that maintain enzyme stability, enhancing PPO's overall activity [22,23].

Table 2. Enzyme activity of PPO from different activators.

Activators	Enzyme Activity (U/ml)
Control	1.193 ^a ±0.021
Vit E	1.255 ^b ±0.049
Vit C	2.996 ^c ±0.129
Curc	4.351 ^d ±0.562

Note: Control: no activator; Vit E: Vitamin E addition; Vit C: Vitamin C addition; Curc: Curcumin addition.

Table 3 presents data on PPO enzyme activity, an interaction between the type of green fodder and the activator. Combined, *Leucaena* and Curcumin reached the highest recorded PPO activity (5.264 U/ml). This notable synergy likely arises from *Leucaena*'s high innate PPO levels, further amplified by Curcumin's enzyme-stabilizing effects. *Leucaena* consistently exhibits higher PPO activity among the studied forages. Curcumin outperforms Vitamin C and Vitamin E in enhancing PPO. The *Leucaena*–Curcumin pairing offers optimal PPO activity, suggesting it could be integral to developing high-efficiency ruminant diets. *Leucaena*'s baseline PPO content and Curcumin's ability to promote enzyme activity can facilitate more extensive protein–phenolic complex formation.

The findings from this study highlight the significant impact of forage type and the addition of activators (Vitamin C, Vitamin E, and Curcumin) on PPO activity. Among the forages, *Leucaena* leaves consistently exhibited the highest PPO activity, a result attributable to their rich phenolic content and high natural PPO levels. This aligns with previous studies suggesting legumes, particularly *Leucaena*, are inherently better sources of phenolic compounds and PPO due to their evolved defense mechanisms against herbivory and pathogens. Curcumin, as an activator, demonstrated superior performance compared to Vitamin C and Vitamin E in enhancing PPO activity. Its antioxidant properties and ability to form stable protein–enzyme complexes likely contributed to this outcome. This synergy between *Leucaena* and Curcumin offers practical implications for optimizing forage-based diets for ruminants. The combination could serve as a strategic approach to protect PUFA from biohydrogenation in the

rumen, potentially improving the fatty acid profile of ruminant-derived products like milk and meat.

Table 3. Enzyme activity of PPO extracted from different forages added by different activators.

Activators	Forages	Enzyme Activity (U/ml)
Control	EG	1.178 ^a ±0.006
	Leuc	1.255 ^a ±0.008
	DEG	2.996 ^a ±0.024
	Cal	1.215 ^{ab} ±0.026
Vit E	DEG	1.232 ^{bc} ±0.090
	Cal	1.243 ^{bc} ±0.043
	Leuc	1.257 ^c ±0.005
	EG	1.288 ^d ±0.020
Vit C	DEG	2.950 ^c ±0.199
	EG	2.965 ^c ±0.208
	Leuc	3.019 ^f ±0.006
	Cal	3.048 ^f ±0.003
Cur	DEG	3.908 ^g ±0.082
	EG	4.051 ^h ±0.026
	Cal	4.180 ⁱ ±0.065
	Leuc	5.264 ^j ±0.020

From a practical perspective, integrating Leucaena-Curcumin formulations into ruminant diets could reduce the reliance on synthetic additives for improving nutritional quality. This approach leverages locally available forage resources, aligning with sustainable livestock management practices. Furthermore, the ability of Curcumin to stabilize PPO under various conditions could extend its application in feed preservation, reducing nutrient losses during storage. Despite these promising results, certain limitations warrant further investigation. For instance, while Curcumin enhanced PPO activity significantly, its cost and scalability in large-scale applications remain concerns. Future studies should explore cost-effective methods for Curcumin production or identify alternative natural compounds with similar efficacy.

Additionally, the long-term effects of these formulations on animal health, productivity, and the sensory qualities of ruminant-derived products need evaluation. Research could also focus on optimizing the dosage and delivery methods for Curcumin and other activators to maximize PPO stability and functionality in real-world settings. Exploring the molecular interactions between PPO, Curcumin, and phenolic compounds at the biochemical level could provide deeper insights into their synergistic effects. This knowledge could guide the development of tailored formulations for specific forage types and dietary goals.

4 Conclusion

This study highlights the significant influence of forage type and activator choice on PPO enzyme activity. When combined with curcumin, *Leucaena* leaves offer a highly effective solution for optimizing forage utilization and enhancing ruminant nutrition. Future research could explore scaling these findings for improving the fatty acid profile of ruminant-derived products like milk and meat.

Disclosure of Interests. The authors have no competing interests to declare that are relevant to the content of this article.

References

1. S. Bronzato, and A. Durante, *International Journal of Preventive Medicine*, 8,1-7 (2017).
2. J. Djordjevic, T. Ledina, M.Z. Baltic. D. Trbovic, M. Babic, and S. Bulajic, *The 60th International Meat Industry Conference*, 333 : 1 – 6 (2019).
3. F. Gadeyne G. V. Ranst, B. Vlaeminck, E. Vossen, P. V. Der Meeren, and V. Fievez, *Food Chemistry*, 171: 241–250 (2015).
4. L. Dewanckele, B. Vlaeminck, E. H. Sanabria, A. R. González, S. Debruyne, J. Jeyanathan, and V. Fievez, *Frontiers in Microbiology*, 9, 1–14 (2018).
5. N. D. Neve, B. Vlaeminck, F. Gadeyne, E. Claeys, P. V. Der Meeren, and V. Fievez, *Animal*, 12, 2539–2550 (2018).
6. F. Gadeyne, N. D. Neve, B. Vlaeminck, E. Claeys, P. V. Der Meeren, and V. Fievez, *Journal of Agricultural and Food Chemistry*, 64, 3749-3759. (2016).
7. Gadeyne, Frederik, N. De Neve, B. Vlaeminck, and V. Fievez, *European Journal of Lipid Science and Technology*, 119, 1–22 (2017).
8. X. Yin, K. Chen, H. Cheng, X. Chen, S. Feng, Y. Lagu, and L. Liang, *Antioxidants*, 11, 2-20.(2021).
9. M. Aksoy, *Protein Expression and Purification*, 171 (2020).
10. L. Zhang, Z. Liu., Y. Sun., X. Wang, and L. Li, *Food Packaging and Shelf Life*, 24, 100470 (2020).
11. B. K. Vaidya, H. K. Suthar, S. Kasture, and S. Nene, *Biochemical Engineering Journal*, 28, 161-166 (2006).
12. N. M. Abdelhady, and G. M. Abdallah, *European Journal of Medicinal Plants*, 17, 1-9 (2016).
13. Z. Zarina, C. M. R. Ghazali, and S. T. Sam, In *AIP Conference Proceedings*, 1885 (2017).
14. D. Seram, *Think India Journal*, 22, 306-317 (2019).
15. K. Prasannath, *Agricast Journal of Agricultural*. 11, 38-48 (2017).
16. S. Singh, I. Kaur, and R. Kariyat, *International Journal of Molecular Sciences*, 22, 1442 (2021).
17. S. Ito, M. Sugumaran, and K. Wakamatsu, *International Journal of Molecular Sciences*, 21, 6080 (2020).
18. A. Tilley, M. P. McHenry, J. A. McHenry, V. Solah, and K. Bayliss, *Current Research in Food Science*, 7, 100623 (2023).
19. A. I. Kalogianni, T. Lazou, I. Bossis, and A. I. Gelasakis, *Foods*, 9, 794 (2020).
20. Z. Hussain, H. E. Thu, M. W. Amjad, F. Hussain, T. A. Ahmed, and S. Khan, *Materials Science and Engineering: C*, 77, 1316-1326 (2017).

21. L. H. Chen, T. Chen, R. N. Zhao, D. Wu, Y. N. Du, and J. N. Hu, *Food Chemistry*, 460, 140449 (2024).
22. C. H. Tang, *Food Hydrocolloids*, 109, 106106 (2020).
23. M. E. A. El-Hack, M. T. El-Saadony, A. A. Swelum, M. Arif, M. M. A. Ghanima, M. Shukry, A. Noreldin, A. E. Taha, and K. A. El-Tarabily, *Journal of the Science of Food and Agriculture*, 101, 5747-5762 (2021)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits any noncommercial use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

