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The Effect of Substrate Concentration and Incubation Time on The Activity of The Uricase Enzyme From Goat Liver

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Abstract: Uricase is an oxidoreductase enzyme that catalyzes the degradation of uric acid into allantoin, hydrogen peroxide, and carbon dioxide. Allantoin, the primary product of uric acid degradation, exhibits 5-10 times greater solubility in water compared to uric acid. This property underscores the importance of uricase in managing hyperuricemia, a condition characterized by elevated uric acid levels in the blood. Hyperuricemia is associated with diseases such as gout, kidney dysfunction, and hypertension. While humans and primates lack the uricase enzyme, it is naturally present in the liver of non-primate mammals, including goats. This study investigated the activity of uricase extracted from goat liver, focusing on the optimum concentration of uric acid as the substrate and incubation time necessary for achieving maximum enzymatic activity. Goat liver samples were processed using borate buffer (pH 8.5) ammonium sulfate fractionation and dialysis to isolate uricase. The enzymatic activity was evaluated at uric acid concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 mM and incubation times of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 hours. The results revealed that the optimum substrate concentration for uricase was 2 mM, yielding total enzyme activity of 0.6704 U/mL and specific activity of 0.0443 U/mg. Additionally, the optimum incubation time was determined to be 5 hours, resulting in total enzyme activity of 0.8421 U/mL and specific activity of 0.0556 U/mg. These findings provide valuable insights into enhancing uricase activity and optimizing its application in therapeutic strategies for hyperuricemia management. Further research is recommended to explore the potential of uricase in clinical and pharmaceutical contexts.

Keywords: Goat liver, incubation time, substrate concentration, uricase enzyme.

INTRODUCTION

Hyperuricemia, a metabolic disorder characterized by excessive uric acid levels in the blood, is a significant precursor to chronic conditions such as gout, hypertension, kidney diseases, and diabetes [1, 2]. Uric acid, a nitrogenous wasteIC ACID product, is derived from the catabolism of purines, which are crucial components of nucleotides [3]. Unlike most mammals, humans and primates lack the enzyme uricase, which catalyzes the oxidation of uric acid into more soluble products, leading to the accumulation of uric acid in tissues and fluids [4, 5]. This evolutionary adaptation, likely influenced by selective pressures, has increased susceptibility to hyperuricemia-related complications in humans [6, 7].

In mammals other than primates, uricase is predominantly found in the peroxisomes of liver cells. This oxidoreductase enzyme catalyzes the conversion of uric acid into allantoin, hydrogen peroxide, and carbon dioxide, a process crucial for maintaining homeostasis [8, 9]. Allantoin, being 5–10 times more soluble than uric acid, is readily excreted by the kidneys, thereby mitigating the risk of uric acid crystallization and associated pathological conditions [10]. The absence of this metabolic pathway in humans underscores the potential therapeutic relevance of uricase in managing hyperuricemia and related disorders [5, 11].

Recent advancements in enzymology have focused on exploiting uricase for therapeutic purposes, including the treatment of gout and tumor lysis syndrome [12]. Optimizing the catalytic activity of uricase, such as determining the ideal substrate concentration and incubation conditions, has become a critical area of research. The substrate concentration significantly affects the rate of this enzymatic reaction, following the Michaelis-Menten kinetics model [13, 14]. At low substrate concentrations, the reaction rate is directly proportional to the substrate availability. However, as the substrate concentration increases, the enzyme approaches its maximum catalytic capacity (Vmax), beyond which additional substrate does not further enhance activity [14]. For uricase, determining the optimal substrate concentration is crucial for efficient degradation of uric acid. Also, investigating this parameter ensures that uricase functions efficiently in therapeutic or industrial settings.

The duration of enzyme-substrate interaction significantly influences catalytic efficiency. Prolonged incubation can lead to enzyme denaturation or reduced activity due to byproduct accumulation, while shorter times may result in incomplete substrate conversion [15]. Determining the ideal time frame helps prevent overexposure to potentially destabilizing conditions, ensuring maximum activity. Studies have also investigated microbial sources of uricase, such as *Bacillus subtilis* and *Pseudomonas aeruginosa*, which exhibit robust activity and are amenable to biotechnological applications [16, 17].

This study investigates the enzymatic properties of uricase extracted from goat liver, a mammalian source with known enzymatic activity. The novelty of this study lies in the source of the uricase enzyme, as goat liver is a rarely utilized mammalian source within the Bovidae family, offering potential as an alternative to the extensively studied beef liver. The enzyme was purified through ammonium sulfate fractionation and dialysis, and its catalytic efficiency was characterized by determining the optimum uric acid concentration and incubation time. These findings not only enhance the understanding of uricase biochemistry but also contribute to its potential application as a therapeutic agent for hyperuricemia management.

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METHODS

Material and Equipment

The Materials used were Fresh Goat Liver (sourced from a local slaughterhouse), Uric Acid (Sigma-Aldrich, USA), Distilled Water, Deionized Water, Boric Acid (H_3BO_3) (Merck, Germany), Hydrogen Peroxide (H_2O_2) (Merck, Germany), Bovine Serum Albumin (Vivantis, Malaysia), Sodium Carbonate (Na_2CO_3) (Merck, Germany), Ammonium Sulfate (Merck, Germany), 95% Ethanol (Merck, Germany), Coomassie Brilliant Blue G-250 (Fluka, Germany), Phenol (Merck, Germany), Whatman No. 1 Filter Paper (Cytiva, UK), Horseradish Peroxidase (Merck, Germany), and 4-Aminoantipyrine (Merck, Germany). The instruments used were UV/Vis Spectrophotometer (Genesys 10s, Thermo Scientific, Singapore), pH Meter (Eutech Instruments, Singapore), Analytical Balance (Shimadzu, Japan), Magnetic Stirrer and Stirring Bars (IKA, Germany).

Extraction of Uricase from Goat Liver

The goat liver was weighed and finely ground using a mortar and pestle. The homogenate was prepared by mixing the liver tissue with borate buffer at pH 8.5. The mixture was then centrifuged at 13,000 rpm for 30 minutes at 4°C to separate the supernatant. The resulting supernatant, serving as the crude extract, was subjected to protein concentration analysis and enzymatic activity assays.

Purification of Uricase Extract

The supernatant was fractionated using ammonium sulfate in the 0–20% saturation range. This process was carried out on an ice bath at 4°C with slow stirring. The homogenate was left undisturbed for 16 hours, followed by centrifugation at 13,000 rpm for 30 minutes at 4°C. The resulting pellet was analyzed for enzymatic activity and protein concentration and then subjected to dialysis. The pellet was dissolved in a buffer solution and placed into a cellulose membrane dialysis bag during the dialysis process. The bag was immersed in 100 mL of 0.1 M borate buffer at pH 8.5. Dialysis was conducted for 16 hours at 4°C using a magnetic stirrer at 125 rpm, with the buffer solution replaced every 4 hours to ensure optimal purification.

Determination of Total Activity of Uricase

The uricase enzyme's total activity is determined using a Trinder-based method by measuring the absorbance of H_2O_2 and establishing a standard curve for its concentration. A total of 0.1 mL of the enzyme solution was mixed with 0.6 mL of 2 mM uric acid prepared in sodium borate buffer (pH 8.5), 0.15 mL of 30 mM 4-aminoantipyrine, 0.05 mL of 15 U/mL peroxidase, and 0.1 mL of 1.5% (v/v) phenol. The mixture was incubated for 4 hours at 37°C. To terminate the enzymatic reaction, 1 mL of ethanol was added. The absorbance of the resulting solution was measured at 540 nm using a UV-Vis spectrophotometer. An inactive enzyme served as a control to ensure the specificity of the enzymatic reaction. The activity of uricase can be determined using Equation 1.

$$Total\ Activity\ \left(\frac{U}{ml}\right) = \frac{([s]-[k]\times f_p\times(\frac{Vtotal}{Venzyme})}{t\times MW\ H_2O_2} \quad \text{..... (1)}$$

Where:

[S] = Concentration of H_2O_2 from the substrate (mg/mL)
[K] = Concentration of H_2O_2 from the control (mg/mL)
MW = Molecular weight of H_2O_2 (mol/g)
fp = Dilution factor
t = Incubation time (minutes)

Determination of Protein Content and Specific Activity of Uricase

The protein content of the crude enzyme extract was evaluated using the Bradford method, with bovine serum albumin serving as the standard. Each experiment was performed in triplicate under identical conditions, and the mean values and standard deviations were calculated. The crude uricase extract was mixed with Bradford reagent and then incubated at room temperature for 5 minutes. A blank sample containing only the Bradford reagent was also prepared. Absorbance measurements were conducted using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Singapore) at a wavelength of 595 nm. Equations 2 and 3 were used to determine the sample's protein content and specific uricase activity, respectively.

$$Protein\ content\ \left(\frac{mg}{ml}\right) = \frac{Abs \pm C \times f_p}{m} \text{..... (2)}$$

$$Specific\ Activity\ \left(\frac{U}{mg}\right) = \frac{Total\ Activity\ of\ enzyme}{Protein\ Content} \text{.... (3)}$$

Where:

Abs = Absorbance
C = Intercept of the curve
fp = Dilution factor
m = Gradient of the curve

RESULT AND DISCUSSION

Uricase Activity and Protein Levels in Goat Liver Purification

The properties of uricase derived from goat liver, spanning the purification stages from crude enzyme extract, the 0-20% ammonium sulfate fractionation, and the final dialyzed form, are comprehensively summarized in Table 1.

Table 1. Uricase Activity and Protein Concentration across Purification Stages of Goat Liver

Uricase State	Samples	Total Activity (U/mL)	Protein Content (mg/mL)	Specific Activity (U/mg)
Crude Extract	S-1	0.0199 ± 0.07	27.0552 ± 0.31	0.0007 ± 0.001
Fractionation with Ammonium Sulfate 0-20%	$F_{20}t_{16}$	0.0351 ± 0.06	15.1911 ± 0.48	0.0023 ± 0.006
After Dialysis	$D_{8,5}t_{16}$	0.6673 ± 0.02	15.1416 ± 0.28	0.0441 ± 0.004

The crude extract of uricase showed a high protein concentration but low enzymatic activity, which can likely be attributed to non-uricase proteins and impurities interfering with the enzyme's function. This is a common issue observed in enzyme preparations, where contaminants diminish the specific activity of the target enzyme. It is well-documented that impurities in crude extracts reduce the overall enzymatic efficacy, underscoring the importance of further purification steps to isolate the active enzyme. As noted in previous studies, techniques such as ammonium sulfate fractionation can help selectively enrich the target enzyme by removing some of these impurities [18, 19].

The dialysis process yielded a sample with a total activity of 0.6673 U/mL, a protein concentration of 15.1416 mg/mL, and a specific uricase activity of 0.0441 U/mg. The total activity and specific activity obtained after dialysis were significantly higher than those achieved during the fractionation stage. This increase in total enzymatic activity indicates that the enzyme was more concentrated following dialysis, likely facilitating a more optimal enzymatic reaction. These findings highlight the efficacy of dialysis in purifying and concentrating the enzyme by removing more minor impurities and excess salts, ultimately enhancing its functional performance [20].

Effect of Substrate Concentration on The Activity of Uricase

The uric acid concentration as the substrate is pivotal in determining uricase activity, as it directly influences the enzyme's catalytic efficiency and reaction kinetics. Optimal substrate levels are essential to ensure maximum enzymatic turnover without causing substrate inhibition, which could compromise the overall reaction rate [21]. The results in this research indicate that both the total enzymatic activity and specific activity increased with uric acid concentrations of 1.0 mM, 1.5 mM, and 2.0 mM and then stabilized from 2.0 mM to 3.0 mM, as shown in Figure 1. The increase in enzymatic activity is influenced by substrate concentration, where higher substrate levels allow more enzyme molecules to bind to the substrate, producing more product. The total and specific enzymatic activities remained constant at substrate concentrations of 2.0, 2.5, and 3.0 mM, likely because the enzyme had fully interacted with the available substrate, achieving maximum product formation.

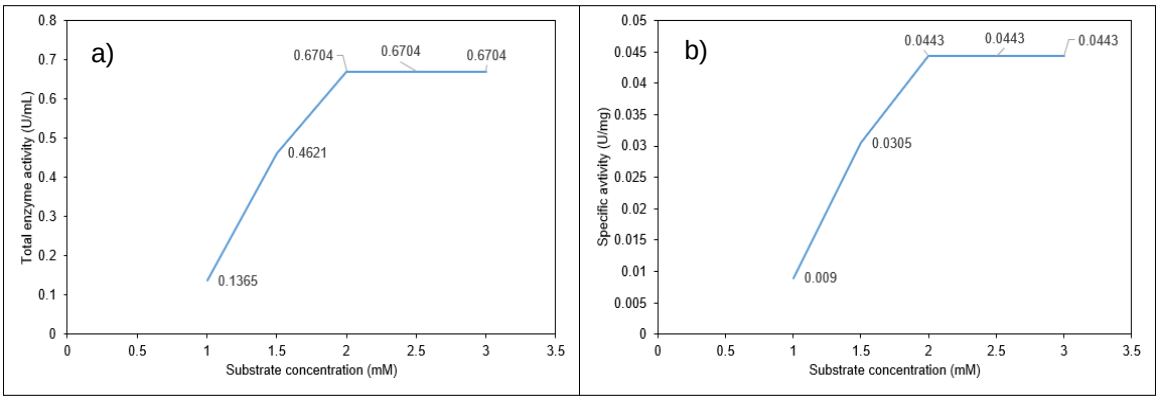

Figure 1. (a) Curve of total activity across varying substrate concentrations, (b) Curve of specific activity across varying substrate concentrations

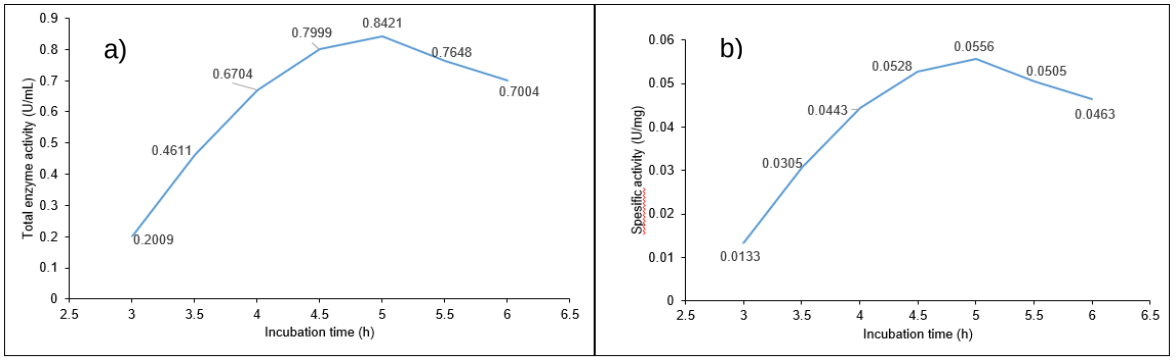

Figure 2. (a) Curve of total activity across varying incubation time, (b) Curve of specific activity across varying incubation time.

Enzymes typically reach a saturation point with their substrate, leading to an enzymatic activity curve that initially increases and then plateaus. This study's findings differ from previous research conducted on *Gliomastix gueg* [14, 22], which identified an optimal substrate concentration of 10 µg, and on *Lactobacillus plantarum*, which showed optimal total activity at a substrate concentration of 0.005 mM [23]. The variations in optimal substrate concentration are likely influenced by differences in the samples used and the purification methods employed. The specific activity of the enzyme in this study demonstrated an optimal substrate concentration of 2.0 mM, with a specific activity of 0.0443 U/mg.

Effect of Incubation Time on The Activity of Uricase

The incubation time is crucial in determining the efficiency of enzymatic reactions, as it directly influences the interaction duration between the enzyme and substrate. Prolonged incubation can enhance product formation up to an optimal point, beyond which enzyme denaturation or substrate depletion may occur, reducing activity. Understanding this parameter is

essential for optimizing reaction conditions and achieving maximum enzymatic efficiency [15]. According to this research, the results showed that both total enzymatic activity and specific activity increased with incubation times from 3.0 to 5.0 hours, followed by a decline at incubation times between 5.5 and 6.0 hours, as depicted in Figure 2.

CONCLUSION

The study identified the optimal conditions for uricase activity in goat liver dialysate extract. The optimal substrate concentration was 2.0 mM, yielding a total enzymatic activity of 0.6704 U/mL and a specific activity of 0.0443 U/mg. Similarly, the optimal incubation time was determined to be 5.0 hours, resulting in a total activity of 0.8421 U/mL and a specific activity of 0.0556 U/mg, reflecting maximal enzyme-substrate interactions. These results highlight the significant influence of substrate concentration and incubation time on uricase activity, providing valuable insights for optimizing enzymatic performance in purification and catalytic applications.

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REFERENCES

[1] J. Maiuolo, F. Oppedisano, S. Gratteri, C. Muscoli, and V. Mollace, "Regulation of uric acid metabolism and excretion," *International Journal of Cardiology*, vol. 213, pp. 8-14, Jun. 2016.

[2] S. V. Pawar and V. K. Rathod, "Ultrasound assisted process intensification of uricase and alkaline protease enzyme co-production in *Bacillus licheniformis*," *Ultrasonics Sonochemistry*, vol. 45, pp. 173-179, Jul. 2018.

[3] A. Abou-Elela, "Epidemiology, pathophysiology, and management of uric acid urolithiasis: A narrative review," *J Adv Res*, vol. 8, no. 5, pp. 513-527, Sep. 2017.

[4] D. Pasalic, N. Marinkovic, and L. Feher-Turkovic, "Uric acid as one of the important factors in multifactorial disorders – facts and controversies," *Biochem Med (Zagreb)*, vol. 22, no. 1, pp. 63-75, Feb. 2012.

[5] S. K. Abdullah and M. T.Flayyih, "Production, Purification and Characterization of Uricase Produced by *Pseudomonas aeruginosa*," *Iraqi Journal of Science*, vol. 56, no. 3B, Art. no. 3B, 2015.

[6] P. Pfrimer *et al.*, "Cloning, Purification, and Partial Characterization of *Bacillus subtilis* Urate Oxidase Expressed in Escherichia coli," *J Biomed Biotechnol*, vol. 2010, p. 674908, 2010.

[7] L. Du *et al.*, "Hyperuricemia and its related diseases: mechanisms and advances in therapy," *Signal Transduct Target Ther*, vol. 9, p. 212, Aug. 2024.

[8] W. Handayani, L. N. Brigiyanti, Sudarko, and A. A. I. Ratnadewi, "Optimum pH Buffer of Phosphate and Carbonate on The Crude Extraction of Uricase Enzyme from Goat Liver: Uricase," *Indonesian Chimica Letters*, vol. 2, no. 1, Jun. 2023.

[9] N. Otani *et al.*, "Dysuricemia-A New Concept Encompassing Hyperuricemia and Hypouricemia," *Biomedicines*, vol. 11, no. 5, p. 1255, Apr. 2023.

[10] P. Szczurek *et al.*, "Oral uricase eliminates blood uric acid in the hyperuricemic pig model," *PLoS One*, vol. 12, no. 6, p. e0179195, Jun. 2017.

[11] Y. M. Roman, "The Role of Uric Acid in Human Health: Insights from the Uricase Gene," *Journal of Personalized Medicine*, vol. 13, no. 9, Sep. 2023.

[12] N. Schlesinger and D. Kaufmann, "Uricases: reflections on recent developments in the management of challenging gout patients," *Explore Musculoskeletal Dis.*, vol. 2, no. 6, Oct. 2024.

[13] P. K. Robinson, "Enzymes: principles and biotechnological applications," *Essays Biochem*, vol. 59, pp. 1-41, Nov. 2015.

[14] C. Mbira, "Influence of Substrate Concentration on Enzyme Activity in Bio Catalysis," *Journal of Chemistry*, vol. 3, no. 1, Apr. 2024.

[15] P. Rajagopalan, H. C. Chandramoorthy, and A. A. Elbessoumy, "Pilot scale production and characterization of uricase from *Penicillium purpurescens*" *IJBT Vol.16(4) [October 2017]*, Oct. 2017.

[16] M. I. Shaaban, E. Abdelmegeed, and Y. M. Ali, "Cloning, expression, and purification of recombinant uricase enzyme from pseudomonas aeruginosa Ps43 using *escherichia coli*," *J Microbiol Biotechnol*, vol. 25, no. 6, pp. 887-892, Jun. 2015.

[17] B. Pugin, S. Plüss, D. Mujezinovic, R. C. Nielsen, and C. Lacroix, "Optimized UV-Spectrophotometric assay to screen bacterial uricase activity using whole cell suspension," *Front. Microbiol.*, vol. 13, Apr. 2022.

[18] A. H. Westphal and W. J. H. van Berkel, "Techniques for enzyme purification," in *Biocatalysis for Practitioners*, John Wiley & Sons, Ltd, 2021.

[19] W. Handayani, E. Utarti, M. K. A. Joenata, and A. A. I. Ratnadewi, "Fractionation of goat hepatic uricase using ammonium sulphate with variations in concentration and precipitation time," *AIP Conference Proceedings*, vol. 2818, no. 1, p. 030007, Aug. 2023.

[20] K. Elbanna, A. Alshareef, L. A. Neyaz, M. Z. El-Readi, and H. H. Abulreesh, "Microbial uricase and its unique potential applications," *Advancements of Microbiology*, vol. 63, no. 2, pp. 81-91, Jul. 2024.

[21] T. Phuadraksa, J. Chittrakanwong, K. Tullayaprayouch, N. Onsirisakul, S. Wichit, and S. Yainoy, "Engineering of bifunctional enzymes with uricase and peroxidase activities for simple and rapid quantification of uric acid in biological samples," *Catalysts*, vol. 10, no. 4, Apr. 2020.

[22] Atalla, M. M., Farag, M. M., Eman, R. H., Abd-El-Lataif, M. S., and Nehad, E. A., "Optimum conditions for uricase enzyme production by Gliomastix gueg," *MJM*, Jun. 2009.

[23] D. Iswantini, R. Rachmatia, N. R. Diana, N. Nurhidayat, Akhiruddin, and D. Saprudin, "Activity and stability of biofilm uricase of lactobacillus plantarum for uric acid biosensor," *IOP Conf. Ser.: Earth Environ. Sci.*, vol. 31, no. 1, p. 012028, Jan. 2016.