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Measurement of temperature, pH and substrate levels on the activity of the Galactose-1-Phosphate Uridyltransferase enzyme

Sisca, Edy Parwanto, Monica Dwi Hartanti, David Tjahyadi
Universitas Trisakti, Jakarta, Indonesia
Email : sisca@trisakti.ac.id, edy.parwanto@gmail.com, mdhartanti@trisakti.ac.id, davesaboch@trisakti.ac.id
Correspondence: sisca@trisakti.ac.id*

KEYWORD

Temperature; pH; enzyme; Galactose-1-Phosphate Uridyltransferase

ABSTRACT

Galactose-1-phosphate uridyltransferase (GALT) is an important enzyme involved in galactose metabolism. Understanding the factors influencing GALT activity is critical to elucidate its physiological role and potential therapeutic implications in galactosemia. In developing new drugs, chicken intestine/liver powder can be used as an enzyme source, GALT, to treat galactosemia. Therefore, it is necessary to research the characterization of the GALT enzyme in chicken intestine and liver powder. In this study, we investigated the influence of temperature, pH, and substrate level on GALT enzyme activity using an experimental approach *in vitro.* The optimum pH extraction results show that the optimum pH for the extraction of the chicken intestine and liver GALT is pH 7, with activity values of 0.47 units/mL and 0.3953 units/mL, respectively. The optimum temperature for the extraction of chicken intestine and liver GALT is 37°C with substrate hydrolysis capabilities of 0.48 U/mL and 0.57 U/mL, respectively. Meanwhile, the optimum substrate content is 400x. These insights provide a valuable foundation for further research aimed at comprehensively understanding GALT function, developing targeted interventions for disorders of galactose metabolism, and possible application in the development of new drugs for galactosemia.

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1. Introduction

Galactosemia is an inherited metabolic disorder of carbohydrate metabolism. Although rare, cases that occur are accompanied by multi-organ involvement. These cases are potentially fatal if not diagnosed in time. Galactosemia is an autosomal recessive genetic disease that can be diagnosed at birth, even without symptoms. Newborn screening can be done by assessing galactose levels and the activity of the enzyme galactose-1-phosphate uridylyltransferase (GALT). Genetic defects in the GALT gene are the most common cause of galactosemia (Succoio et al., 2022).

Based on its constituent molecules, GALT is a homodimeric enzyme with 2 active parts. The two active sites are located proximal to the intersubunit interface (Verdino et al., 2021). The

absence of this enzyme results in classic galactosemia in humans and is fatal in newborns if lactose is not eliminated from the diet (Stettner et al., 2023).

GALT catalyzes the second step of the Leloir pathway in galactose metabolism, namely the conversion of UDP-glucose + galactose-1-phosphate to glucose-1-phosphate + UDP-galactose (Haskovic et al., 2020). This enzyme functions to transfer the uridyl group from UDP-glucose to galactose-1-phosphate to produce UDP-galactose and glucose-1-phosphate which are then metabolized in the glycolytic pathway (Brophy et al., 2021).

To date, galactosemia cannot be cured, but is only treated with a diet low in galactose and lactose. It is reported that only 2/3 of these cases receive intervention (Fridovich et al., 2019). The incidence rate of galactosemia in the population varies widely, namely 1 case per 30,000-40,000 people in Europe (Welling et al., 2017), 1 case per 80,000 people in Japan and 1 case per 10,000 people in Africa respectively (Iwasawa et al., 2014). The results of a study of 34 Korean patients showed that the most frequent variation was the Duarte variant (c.940A > G, 35.3%), followed by c.507G > C (p.Gln169His, 9.6%) (Choi et al., 2019).The enzymatic activity of GALT can be determined using a spectrophotometer to detect NADH or NADPH (Succoio, 2022; Brophy, 2021).

Previous research on the activity of the Galactose-1-Phosphate Uridyltransferase (GALT) enzyme has primarily focused on genetic mutations and their impact on enzyme function, particularly in the context of disorders such as classic galactosemia. These studies have extensively explored the genetic and molecular basis of GALT deficiency, detailing the specific mutations that lead to reduced or absent enzyme activity. However, there has been less emphasis on how external factors, such as environmental conditions, influence GALT activity. The current study differs from this previous research by systematically measuring the effects of temperature, pH, and substrate levels on GALT activity. This approach provides a more comprehensive understanding of the enzyme's functionality under varying physiological conditions, which could have implications for optimizing therapeutic interventions and improving metabolic stability in affected individuals (Iwasawa, 2019).

Despite its clinical significance, the factors influencing GALT activity are still not completely understood. Temperature, pH, and substrate levels are known to influence enzyme function in general, but their specific effects on GALT activity have not been fully elucidated. In this study, we aimed to systematically investigate the impact of these parameters on GALT enzyme activity, providing insight into its regulation and potential implications for disorders of galactose metabolism. Exploratory studies on the characterization of GALT enzyme genes based on the molecular weight contained in chicken intestines and liver are very limited. Therefore, the aim of this research was to characterize proteins in chicken intestines and liver based on molecular weight. It is hoped that chicken intestines and liver assources of GALT can be developed natural medicine.

2. Materials and Methods

The research employs a combination of controlled laboratory experiments, enzyme assays, and data analysis to understand how temperature, pH, and substrate levels influence the activity of the Galactose-1-Phosphate Uridyltransferase enzyme. This approach allows researchers to determine the optimal conditions for enzyme activity and understand its kinetic properties.

Chickens were obtained from Bekasi, West Java, Indonesia. Fresh chicken is cleaned and the chicken liver/intestines are separated, then washed with running water. Cleaned chicken liver/intestines are stored in the refrigerator -40^{0}C for 12 hours. A 200 gram sample of chicken liver/intestine was ground. The process of grinding the chicken intestines/livers is carried out until they become fine particles. The fine particles were then added with distilled water to a volume of 1000 mL. The solution was centrifuged at 10,000 rpm for 2 minutes. The pellet resulting from centrifugation was discarded, while the supernatant obtained was used to measure GALT enzyme activity.

GALT activity with respect to temperature was carried out starting with 5 clean micro tubes prepared first, the five tubes were placed in different vessels (temperature variations 0, 25, 37, 60, 100 ^{0}C). The tube was incubated for 5 minutes, pipette 1ml of substrate buffer solution (p-NPP substrate in citrate buffer pH 4.8 and carbonate-bicarbonate buffer pH 9.6) into the reaction tube, add 50 microliters of enzyme (enzyme at time t= 0), mixed and incubated for 15 minutes at 45 ^{0}C, add 0.1M Na OH solution 4 mL read the absorption at a wavelength of 410nm (this was done in a substrate buffer of pH 4.8 and pH 9.6 which was reacted with the enzyme isolated from chicken liver and intestines).

GALT activity on pH is made by preparing 5 clean micro tubes, these 5 tubes are placed in water bath 45℃. Leave it for 5 minutes, pipette 1ml of p-NPP substrate solution in substrate buffer at various pH (3, 7, 9, 11, and 13) into a tube, add 50 microliters of enzyme, mix and incubate for 15 minutes at a temperature of 45℃, add 4 mL of 0.1M NaOH solution, the absorbance is read at a wavelength of 410nm (pH variations of the substrate buffer are reacted with enzymes isolated from chicken liver and intestines). The control is made by preparing 5 clean micro tubes, placing the 5 tubes in water bath 45℃. Leave ita for 5 minutes, pipette 1ml of p-NPP substrate in substrate buffer at various pH (3, 7, 9, 11 and 13) into the tube, the tube is incubated for 15 minutes at 45℃, add the solution NaOH 0.1M 4 mL, absorbance was read at a wavelength of 410nm (pH variations of the substrate buffer were reacted with enzymes isolated from chicken liver and intestines).

GALT enzyme activity on substrate levels is carried out by preparing 5 clean micro tubes and placing the 5 tubes in a water bath at 45℃. Leaveit for 5 minutes, pipette 1ml of p-nitophenylphosphate (p-NPP) substrate solution with a concentration of 100x; 200x; 300x; 400x; 500x into a test tube, add 50 microliters of enzyme, mix, and incubate for 15 minutes at 45℃, add 4mL of 0.1M Na OH solution, read the absorption at a wavelength of 410nm (the substrate buffer used is varied by diluting it first and then reacting with phosphatase enzymes isolated from chicken liver and intestines). This research has been approved by the Research Ethics Committee, Faculty of Medicine, Universitas Trisakti.

3. Results and Discussions

GALT Enzyme Activity on Temperature of Chicken Intestines and Livers

Enzymes are biological catalysts and consist of proteins. Enzymes enhance chemical reactions that occur within living cells without the enzyme itself undergoing a change in form. Enzyme reactants have specific characteristics that work on certain substrates to produce certain products (Voet et al., 2016).

The research results showed that crude chicken intestine extracts have GALT enzyme activity at different temperatures. Based on the graph below, temperature affects GALT enzyme activity. GALT enzyme activity from chicken intestine extract incubated at varying temperatures of 0, 25, 37, 60, and 100 OC, respectively, was 0.30 U/mL, 0.37 U/mL, 0.48 U/ mL, 0.34 U/mL, and 0.24 U/mL. At 37^{0}C, the GALT enzyme reaches the optimum temperature to hydrolyze the substrate with an activity value of 0.48 U/mL.

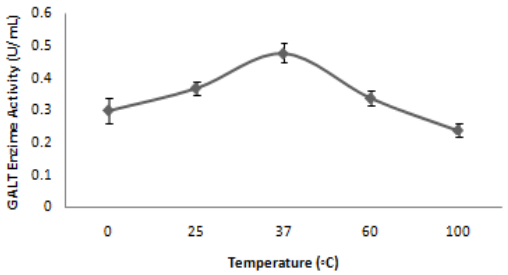

Figure 1 Chicken intestinal GALT enzyme activity against temperature (°C)

GALT enzyme activity from chicken liver extract incubated at temperature variations of 0, 25, 37, 60, and 100 ^{0}C, respectively, was 0.36 U/mL, 0.46 U/mL, 0.57 U/mL,0 .40 U/mL, and 0.35 U/mL. At 37 OC, the GALT enzyme reaches the optimum temperature to hydrolyze the substrate, with an activity value reaching 0.57 U/mL.

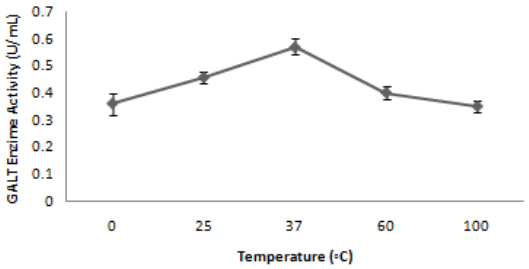

Figure 2 Chicken liver GALT enzyme activity against temperature (˚C)

Effect of pH on GALT enzyme activity in chicken intestines

GALT has activity at pH 3, 5, 7, 9, and 11, respectively 0.09 units/mL, 0.22 units/mL, 0.47 units/mL, 0.31 units/mL, and 0.21 units/mL (Figure 3), GALT enzyme activity is optimum at pH 7 with enzyme activity of 0.47 units/mL.

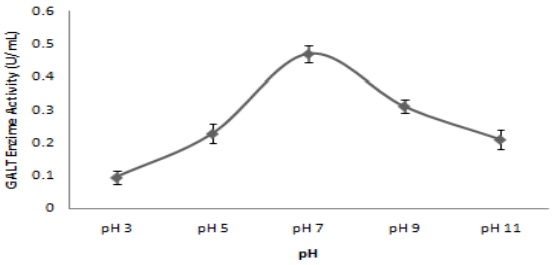

Figure 3 Chicken intestinal GALT enzyme activity towards pH

Effect of pH on GALT enzyme activity in chicken liver

Chicken liver GALT enzyme activities at pH 3, 5, 7, 9, and 11 were respectively 0.1136 units/mL, 0.2383 units/mL, 0.3953 units/mL, 0.2012 units/mL, and 0.0847 units/mL (Figure 4), optimum GALT activity at pH 7 is 0.3953 units/mL.

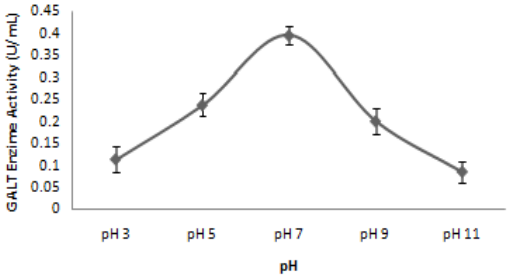

Figure 4 Chicken liver GALT enzyme activity toward pH

Effect of substrate levels on GALT enzyme activity in chicken intestines

Chicken intestinal GALT enzyme activity at substrate levels of 100x, 200x, 300x, 400x, and 500x, respectively, namely 0.0088 units/mL, 0.1581 units/mL, 0.245 units/mL, 0.441 units/mL, and 0, 32 units/mL (Figure 5), optimum GALT activity at a substrate level of 400x is 0.44 units/mL.

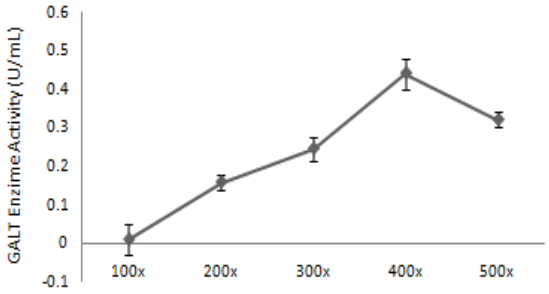

Figure 5 Chicken intestinal GALT enzyme activity on substrate levels

Effect of substrate levels on GALT enzyme activity in chicken liver

Chicken liver GALT enzyme activity at substrate levels of 100x, 200x, 300x, 400x, and 500x, respectively, were 0.0087 units/mL, 0.156 units/mL, 0.273 units/mL, 0.438 units/mL, and 0.325 units/mL (Figure 6), the optimum GALT activity at a substrate level of 400x is 0.438 units/mL

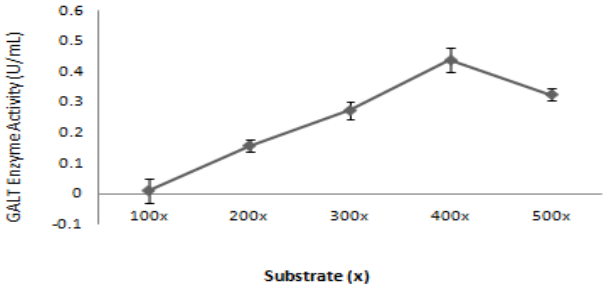

Figure 6 Chicken liver GALT enzyme activity on substrate levels

Discussion

Galactose-1-phosphate uridylyltransferase (or GALT) is the enzyme responsible for changing galactose into glucose. GALT expression is influenced by the FOXO3 gene. The absence of this enzyme results in classic galactosemia in humans and can be fatal in newborns if lactose is introduced into the baby's diet (Berry, 2014). The pathophysiology of galactosemia has not been clearly defined. The three-dimensional structure at 180 pm resolution (x-ray crystallography) of GALT was discovered by Wedekind, Frey, and Rayment, and analyzing the structure discovered the main amino acids important for GALT function. Among these are Leu4, Phe75, Asn77, Asp78, Phe79, and Val108, which are consistent with residues that have been implicated both in point mutation studies and in clinical screens for a role in human galactosemia (Wedekind et al., 1995).

According to studies, Dari Ning et al. (2000), who researched the metabolism of rat galactose, found that the GALT enzyme was found in the liver, kidney, and brain of rats, and the highest levels were found in the liver (Ning et al., 2000). According to Tang et al (2012), the GALT enzyme has been intensively researched using bacterial purification Escherichia coli, where the identification of the GALT protein in humans and E.coli has 46% similarity. Until now, research on chickens has not been carried out, so with this research, it is hoped that chicken intestines/livers can be used as a source of enzymes (Tang et al., 2012)

At low temperatures, GALT enzyme activity is not optimal because the energy absorbed by the enzyme is not enough to hydrolyze the substrate, so the enzyme activity value is low. Meanwhile, when the temperature is too high, the enzyme will experience denaturation. That is, the active part of the enzyme will be disrupted so that the reaction speed will decrease (Robinson, 2015). According to Masson (2022), the tertiary structure of an enzyme which consists of hydrophobic

bonds, if it absorbs high energy, will break and result in the opening of the tertiary structure so that the conformation of the enzyme changes and causes its activity to decrease (Masson, 2022).

The decrease in enzyme activity after the optimum temperature occurs because at temperatures higher than the optimum temperature, the protein can be denatured. Besides that, the substrate can also undergo conformational changes so that it does not enter the active site as freely as at the optimum temperature and causes enzyme activity to decrease (Almeida et al., 2019).

The GALT enzyme in the liver may be more stable at certain temperatures because the liver's role in metabolism is more extensive and intensive than the intestine. The liver is the main organ in galactose metabolism, so the GALT enzyme in the liver may have better adaptation to temperature variations to ensure optimal metabolic function.

In experimental studies, GALT enzyme activity can be measured at various temperatures using the substrate galactose-1-phosphate and monitoring the reaction product uridine diphosphate-galactose (Teixeira et al., 2024). Activity can be compared between enzyme extracts from the intestine and liver at various temperatures and 37C is the optimal temperature and thermal stability of each source. At a physiological temperature of 37°C, GALT enzyme activity from chicken intestine and liver showed significant variations. Chicken intestines, as the main part of the digestive system, tend to have higher enzyme activity to maximize the conversion of galactose from the diet into glucose that can be used by the body. In contrast, chicken liver, which functions as a major organ in carbohydrate detoxification and metabolism, also shows significant GALT activity, but with more focus on the regulation of blood sugar levels and energy storage. Enzyme activity in these two organs at 37°C reflects tissue-specific adaptation to their respective metabolic functions, which is important for the overall efficiency of galactose metabolism in chickens (Teixeira et al., 2024).

Increasing the temperature before reaching the optimum temperature will increase the rate of enzyme catalytic reactions due to increasing the kinetic energy of the reacting molecules. On the other hand, the temperature is increased after the optimum temperature of the enzyme-substrate complex exceeds which the activation energy is too large, thus breaking the secondary bonds in the enzyme conformation and its active site. This results in the enzyme being denatured and losing its catalytic properties (Peterson et al., 2007).

pH plays an important role in determining the rate of enzyme catalytic reactions. Enzymes are proteins that have a very characteristic three-dimensional structure. This structure allows the enzyme to interact with its substrate and facilitate the desired chemical reaction. However, this three-dimensional structure is very sensitive to changes in environmental pH (Khan, 2023).

Changes in pH can affect the charge and conformation of enzymes, which in turn can affect their catalytic activity. This is caused by changes in the ionization groups of the amino acids that make up the enzyme. For example, changes in pH can cause ionization of amino acid groups on glutamic or aspartic acid residues or on basic lysine or arginine residues. These changes in ionic charge can influence enzyme-substrate interactions and, consequently, can influence the rate of the enzyme's catalytic reaction (Robinson, 2015.)

In general, each enzyme has an optimum pH at which its activity is highest. Around this optimum pH, the enzyme will reach its maximum catalytic activity. However, outside this optimum pH range, enzyme activity will decrease because the conformation and charge of the enzyme change, which disrupts enzyme-substrate interactions. Therefore, to understand how pH influences the catalytic reaction rate of enzymes, it is important to evaluate enzyme activity at various pH values and map the pH activity profile. In this way, we can determine the optimum pH for each enzyme and understand how environmental factors such as pH affect enzyme function in biological systems (Bowman et al., 2020).

The activity of the enzyme galactose-1-phosphate uridyltransferase (GALT) at pH 7 in chicken intestine and liver indicates an important role for this enzyme in galactose metabolism. At neutral

pH conditions, such as pH 7, the GALT enzyme tends to have optimal activity because this pH supports a stable and active protein conformation. In the intestine, GALT functions in the conversion of galactose obtained from food into glucose-1-phosphate, which can then be further metabolized to produce energy. Meanwhile, in the liver, GALT plays a crucial role in regulating blood galactose levels, preventing the toxic accumulation of galactose-1-phosphate, and supporting glucose homeostasis (Coelho et al., 2017). Study of GALT activity at pH 7 from these two organs provides important insights into physiological adaptations and tissue-specific differences in carbohydrate metabolism in chickens.

Substrate levels also play an important role in determining the rate of enzyme catalytic reactions. The substrate concentration influences the enzyme reaction rate because the enzyme can only interact with the available substrate. There are two main models that explain the relationship between substrate levels and enzyme reaction rates: the Michaelis-Menten model and the Lineweaver-Burk model. In both models, as the substrate concentration increases, the reaction rate (V) will increase until it reaches a saturation point or maximum, which is determined by *Vmax*. However, if the substrate concentration is very high, the reaction rate will remain constant at a value *of Vmax because all active enzymes are saturated with the* substrate (Leskovac, 2020).

The activity of the Galactose-1-phosphate uridyltransferase (GALT) enzyme towards substrates from chicken intestine and liver shows significant differences related to the physiological function of each organ. The GALT enzyme plays an important role in galactose metabolism, where it catalyzes the conversion of galactose-1-phosphate to UDP-galactose, which can then be used in various biosynthetic pathways. In the chicken intestine, GALT activity tends to be higher to support the digestive process and absorption of galactose from food. Meanwhile, in the liver, this enzyme plays a role in regulating blood sugar homeostasis and energy storage, with activity that is also quite significant considering the liver's role in systemic metabolism. These differences reflect organ-specific enzymatic adaptations to optimize metabolic function according to the physiological needs of each organ in chickens (Anika et al., 2022).

Our results demonstrate the importance of temperature, pH, and substrate levels in modulating GALT activity. The observed temperature and pH optima provide insight into the physiological conditions of the enzyme for optimal function. In addition, the substrate concentration dependence highlights the complex interaction between substrate availability and enzyme kinetics (Berezhkovskii, 2017). These findings increase our understanding of GALT regulation and its relevance to disorders of galactose metabolism. Future research may explore additional factors influencing GALT activity and therapeutic strategies targeting this enzyme for the management of galactosemia.

4. Conclusion

The GALT enzyme activity of chicken intestines and liver is optimal at pH 7 and a temperature of 37°C. Substrate content 400x. This study elucidates the influence of temperature, pH, and substrate levels on GALT enzyme activity, providing valuable insight into its regulation and potential implications for disorders of galactose metabolism. The identified optimal conditions and substrate dependencies form the basis for further research aimed at elucidating the molecular mechanisms underlying GALT function. By identifying optimal conditions for GALT activity, the implication of this research can develop targeted therapies to enhance enzyme function in individuals with partial GALT activity. This can lead to improved treatments for galactosemia patients.

The limitations of this research are that this research mainly uses in vitro experimental methods to assess GALT activity. Although this approach provides valuable insight into enzyme kinetics, it may not fully recapitulate the complex regulatory mechanisms that exist in vivo. Therefore, the findings from this study should be interpreted with caution when extrapolating to physiological conditions.

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