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Nedim Albayrak: Performed the investigation, conceptualization and visualization of the data and finally supervision, writing and editing final draft.

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Abstract

Using PEI as an aggregating agent with β-galactosidase from Kluyveromyces lactis was investigated with no solid support. PEI, a cationic polymer initiated instant aggregation with the enzyme in solution. The factors affecting the aggregation such as PEI to enzyme ratio, glutar aldehyde for cross-linking and pH were investigated. Aggregation and sedimentation as well as the residual activity of aggregates were effectively dependent on the PEI to enzyme ratios. Easily precipitating aggregates at the ratio of 1/8, PEI enzyme aggregates were able to contain all enzyme in the complexation and displayed 60% of initial lactase activity. The PEI aggregation of enzyme led to enhancements in chemical and physical characteristics compered to free enzyme. The soluble enzyme showed a narrow optimum at about pH 7 while pH optimum of the aggregates extended one pH unit toward the alkaline range. Upon overnight incubation at 40 ºC, aggregated enzyme displayed 30% greater stability on average at all pHs tested. Although the free enzyme showed the highest activity at 40°C, it rapidly lost 50% of its activity at 50°C. In contrast, the aggregated enzyme retained full activity at 50°C and 70% activity at 65°C. With markedly enhanced thermal stability, the half-life of the aggregated enzyme increased from 76 hours to 254 hours at 40ºC. Overall, the simple and rapid aggregation of PEI with the enzyme led to instant and intense clustering, resulting in higher thermal and pH stabilities. This method may potentially offer efficient and cost-effective catalysis in lactase conversion processes.

Keywords: Kluyveromyces lactis, Lactase, Carrier-Free, PEI-Enzyme Aggregation, Stabilization, PEI, GA

INTRODUCTION

Compared to free enzymes, immobilized enzymes are more suitable for industrial applications as they offer ease of separation from the reaction medium and reuse and are more resistant to environmental conditions such as temperature and pH (1–4). Solid supports are generally used in enzyme immobilization for holding the enzyme in a fixed region (5). However, these supports do not provide any catalytic function, despite occupying over 95% of the catalyst volume or mass (6,7). As an alternative approach to immobilize enzymes on solid supports is to turn enzyme mass into macromolecular aggregate such as Cross-Lined Enzyme Aggregate (CLEA). The approach has been evaluated in some applications with beneficial aspects (8,9). However, the method requires to use extensive amount of organic solvent for enzyme precipitation (10) suffers from diffusional resistance (11) due to cross-linking of the precipitated enzyme into a dense structure.

Enzyme aggregation can also be achieved with the assistance of another polymer such as polyethyleneimine (PEI) which tentatively forms electrostatic complexes with negatively charged enzymes while cushioning the space among enzymes and aiding conformational stability (12,13). This approach has the benefits for avoidance of organic solvent thus lowering the cost and suffer relatively limited diffusional resistance due to open structure (14,15). This type of aggregated enzyme exhibits soluble enzyme characteristics during the enzymatic reaction but can be reversibly recovered through physical separation after the reaction and reused (1,16). PEI is an inexpensive and safe polymer with various industrial and biomedical applications (17,18), thus can be easily approved GRAS (Generally Recognized As Safe) in the bioprocessing of food ingredient for contact (18,19).

The general goal of the study was to focus on polymer-assisted aggregation of β-galactosidase (lactase) from Kluyveromyces lactis to effectively increase catalyst density and the operational stability while performing in solution as staying afloat for dairy applications. The enzyme is frequently used in the dairy industry and classified under GRAS substances, making it commercially prevalent (20). The lactase is also notable for its long lifespan at around 35°C, its optimal pH of 6.5-7.5 (21), which is close to the neutral pH of milk and sweet whey, and its relatively low Km value of 20 mM for lactose (22,23), making it one of the most suitable enzymes for lactose hydrolysis at neutral pH (20,22). Additionally, it stands out for its ability to achieve around 30% GOS yield with 70% lactose utilization in GOS production (24,25).

The main goal of the current study is to enhance the performance of the lactase as a catalyst for industrial processes such as lactose hydrolysis and oligosaccharide synthesis. Many issues in enzymatic production arise from inadequate catalyst performance (26). When examining the performance of immobilized enzymes for oligosaccharide production, problems like low activity, short lifespan under production conditions, insufficient stability, and expensive costs are observed (27,28). We have previously immobilized lactase from K. lactis using PEI on the surface of plasma modified cellulose acetate membranes and found that high enzyme loading on the surface did not necessarily resulted in higher rates of catalysis largely due to restriction of enzyme movement (29). To address these issues, the project aims to use a multi-layer system to concentrate the enzyme in solution, thereby improving catalytic performance to meet industrial expectations and reducing the cost for enzyme usage (22,26).

In this study, the aggregation of the lactase enzyme derived from K. lactis using polyethyleneimine (PEI) was investigated without involving any support or solvent for precipitation. Aggregated enzymes offer several advantages, including high catalyst density, minimal or no diffusional resistance, simple and rapid aggregation, and low cost for catalysis (30,31).

The study focused on the aggregation of the Kluyveromyces lactis-derived lactase enzyme with PEI, exploring the optimal PEI/enzyme ratio and characterizing some properties of the aggregated clusters of the enzyme in solution.

MATERIALS AND METHODS

Enzyme and Reagents

β-galactosidase from Kluyveromyces lactis (Lactozym® Pure 2600 L) obtained from Novozymes (Novo Nordisk, Denmark) was used for in immobilization experiments. For enzyme immobilization, polyethyleneimine (PEI) (30% (w/v) aqueous solution, average molecular weight: 50,000-100,000) was from Merck (Darmstadt, Germany), while glutaraldehyde (GA, 50% (w/v)) was obtained from AppliChem (Darmstadt, Germany). All solutions of PEI and GA were prepared using distilled water. Where necessary, pH adjustments were made using HCl or NaOH solutions (Merck). Lactose (D-Lactose monohydrate) and phosphate buffers were from Sigma (USA).

PEI-Enzyme Aggregation

To investigate the effect of different PEI concentrations on the PEI-enzyme-aggregation, PEI solutions of varying concentrations were prepared by diluting a 1% PEI stock solution with distilled water. 1 mL volumes of these PEI solutions were mixed with 0.5 mL of enzyme solution in microcentrifuge tubes. The mixtures were briefly vortexed, and the formation of a milky turbidity was observed. To assess whether the resulting PEI-enzyme aggregates were strong or dense enough to precipitate, the mixtures were centrifuged at 10,000 rpm for 2 minutes. The formation of a precipitate before and after centrifugation of the PEI-enzyme suspensions was observed (Figure 1). Enzyme activity was analyzed using samples taken from the free enzyme solution, the turbid PEI-lactase aggregation solution formed after mixing the PEI and enzyme solutions, and the supernatant remaining after centrifugation.

Glutaraldehyde Cross-Linking

To crosslink the PEI-lactase-aggregates formed in solution, 0.1 mL of GA solutions containing 0.01% to 0.20% GA were added and incubated at ambient temperature for 5 to 10 minutes. The GA solutions were prepared in distilled water and applied following the formation of the PEI-enzyme aggregates.

Enzyme Assay

Enzyme activity was determined using a spectrophotometric assay comprising o-nitrophenyl-beta-D-galactopyranoside (o-NPG, Sigma) as the substrate. The reaction was initiated by adding 0.1 mL of enzyme solution to a mixture containing 1.5 mL of 0.1 M sodium phosphate reaction buffer (pH 7) and 0.2 mL of 2 mM o-NPG. The reaction mixture was incubated at 37°C for 5 minutes and the reaction was stopped by adding 0.5 mL of 1 M sodium carbonate (Merck) solution. The absorbance was then measured at 420 nm using a spectrophotometer (Shimadzu UV-1800, Japan). As a blank, 0.1 mL of 0.1 M sodium phosphate buffer (pH 7) was used instead of the enzyme extract. One unit of beta-galactosidase activity was defined as the amount of enzyme that releases 1 µmol of o-nitrophenol per minute.

Temperature and pH Optimum

The effects of temperature and pH on the activities of free enzyme and PEI-enzyme aggregate in solution were investigated. Relevant pH values ranging from 4.5 to 8.0 were achieved using 50 mM phosphate buffers to determine the effect of pH on enzyme activities at 37°C. The effects of various temperatures ranging from 30 to 65°C on enzyme activities were determined according to the enzyme assay. The activities obtained under the varying pH or temperatures were indicated as relative activities for comparisons among the pH levels or temperatures to observe the variation between the soluble and aggregated lactase.

Temperature and pH Stability

The changes in the activities of free enzyme and PEI enzyme aggregates were measured at room temperature for 18 hours in buffer solutions with a pH range of 5.0-8.0. The residual activities were determined at the end of incubation period. Thermal deactivation is the best indication for the loss of activity under process conditions in free or aggregated enzymes. Thus, the half-lives of PEI enzyme aggregates and free enzyme were determined from the incubation at three different temperatures (30, 40, and 50°C) in buffer solutions (pH 7) During the storage period for 60 minutes, samples were taken at specific intervals to determine the remaining activities of the enzymes, and the changes in activity were comparatively examined. From the residual activity levels for each temperature, the temperature stability of a soluble enzyme and PEI-enzyme aggregate was determined by plotting the natural logarithm of the rate constants against the reciprocal of temperatures [lnk vs 1/T (K)]. The slope of the fitted straight line, deactivation constants k_d (h^{-1}) and also t 1/2 (h), were determined (Table 1.).

Table 1 Comparison of temperature stabilities of soluble lactase and PEI-lactase aggregate at PEI to enzyme ratio of 1/8 during 60 minutes of incubations at pH 7.0 .

(°C)	Soluble Enzyme $t_{1/2}$(h)	PEI-Enzyme Aggregate $t_{1/2}$(h)
30	224	1358
40	76	254
50	1	72

Storage stability at ambient temperature for three weeks was also determined for the soluble enzyme and PEI-lactase aggregate at PEI to enzyme ratio of 1/8 in buffer (pH 7). Residual enzyme activities were determined and expressed as relative activity compared with the initial activity.

RESULTS AND DISCUSSION

PEI-Lactase Aggregation

The aggregating characteristics of lactase enzyme from K. lactis within PEI polymer matrix through electrostatic interactions was first studied. The goal of the approach was to create concentrated enzyme clusters as aggregates while performing as good as soluble enzymes. A successful formation of the colloidal electrostatic complexes between PEI and the enzyme considered is a key to achieving effective and stable enzyme clustering. Therefore, several success criteria were tentatively set to maximize the yield efficiency of the PEI enzyme aggregation. First, PEI and enzyme associates lead to milky complex formation. Second, a complete PEI-enzyme complexation can be achieved at a certain PEI to enzyme ratio, where PEI and enzyme aggregation gather most of the enzyme activity/protein in turn diminishing the unbound enzyme activity/protein in solution. Third, PEI-enzyme aggregates catalytically function as good as soluble enzymes while easily and reversibly separated from the medium by means of simple filtration or centrifugation. Finally, the aggregation leads to greater stability for a longer operational lifespan.

Figure 1 shows the schematic representation of PEI-enzyme monolog complex formation and aggregation, along with photographs of the enzyme clusters. PEI, with a molecular weight range of 100,000 to 750,000, spreads into the solution as a cationic polar polymer. In contrast, the enzyme, with a molecular weight range of 100,000 to 200,000 and an isoelectric point of 4-6, is smaller and anionic compared to PEI. This results in the formation of several smaller enzyme particles that aggregate with PEI into beads, which then attract each other to form clusters. The colloidal milky appearance of the PEI-enzyme solution is attributed to this mechanism.

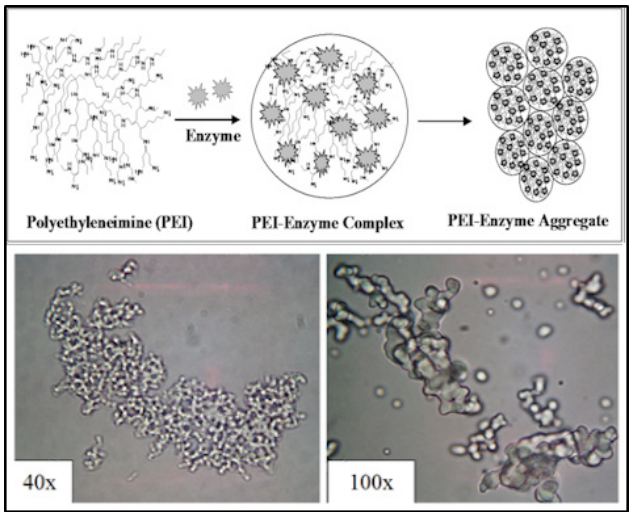

Figure 1 The proposed mechanism of aggregation of PEI and free enzyme in solution, and photographs of the resulting aggregate taken under a light microscope at 40x and 100x magnification.

In a specific ratio of PEI to enzyme, the clustering of all free enzymes within PEI matrices gently floating in the solution was anticipated and targeted. The resulting aggregation was evaluated by precipitating through centrifugation, serving as an indicator of the density or the strength of the interaction between PEI and the enzyme. Subsequently, the aim was to minimize enzyme activity remaining in the supernatant after centrifugation, as enzyme activity was only expected from the PEI-lactase aggregates. Therefore, an experiment was carried out to determine the optimal PEI concentration (i.e., PEI to enzyme ratio) that formed a complex with the enzyme, resulting in a milky structure with minimal decline in the enzyme activity associated with sedimenting aggregates. The measurements of enzyme activity without PEI were taken as control parameters for the evaluation of the activities in the PEI enzyme aggregates prior to centrifugation and the supernatant at 10,000 rpm for 2 minutes. The effects of varying conditions were evaluated based on enzyme activity. Higher enzyme activity in the aggregate or lower activity in the supernatant were considered more effective for aggregation, indicating the optimal PEI to enzyme ratio.

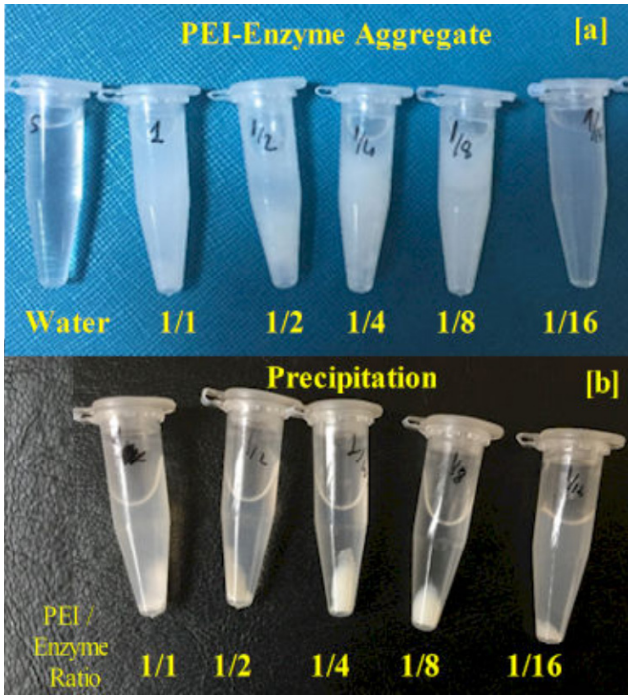

Figure 2 Images of PEI-enzyme aggregates with different concentrations of PEI from highest (1/1) to lowest ratio (1/16) and K. lactis lactase Lactozyme 2600L enzyme samples (1/300 dilution) before (a) and after (b) centrifugation (10,000 rpm for 2 minutes).

Figure 2 provides visual evidence of the aggregation of K. lactis lactase with different PEI concentrations before (a) and after (b) centrifugation. When PEI solutions prepared with distilled water (ranging from 1/1 to 1/16) were added to the diluted enzyme solutions in microcentrifuge tubes, a significant turbidity was observed, indicating that PEI caused the enzyme samples to aggregate. The visual appearance of

the turbidity and intensity varied in the 1/1 toward 1/8 mixtures but less intense in the 1/16, indicating a direct relationship between turbidity and PEI concentration, and notably the turbidity moved with the mixing of the aggregate solution. In the 1/1 and 1/2 samples with the highest PEI concentrations, some PEI-enzyme aggregates did not precipitate and remained suspended in the solution.

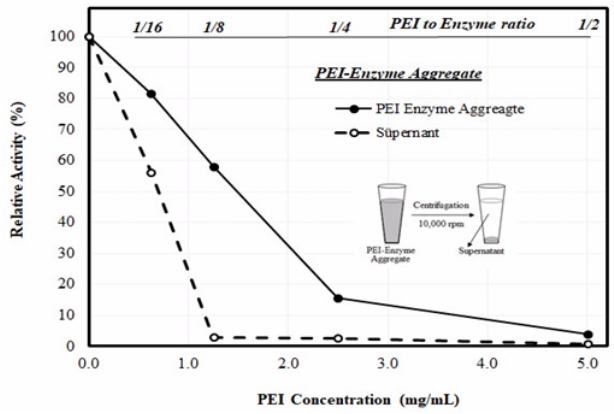

Figure 3 The enzyme activities of PEI-enzyme aggregates and the supernatant thereafter. The effect of PEI concentration from highest (1/1) to lowest ratio (1/16) on the aggregation of PEI and K. lactis lactase (Lactozyme 2600L) enzyme.

By observing the visual images of samples in Figure 2a and 2b and corresponding the levels of the enzyme activities for the PEI-lactase aggregates in Figure 3 before and after centrifugation, one can evaluate the effectiveness of PEI in promoting enzyme aggregation with minimal enzyme loss and optimize the PEI concentration to achieve maximal activity. Increasing PEI to enzyme ratio resulted in a gradual decline in enzyme activity for PEI-enzyme aggregate. At the lowest PEI to enzyme ratio (1/16), the aggregates showed 80% of initial activity; however, 60% remained in the supernatant upon spinning. At the ratio of 1/8, the PEI-enzyme aggregates showed 60% enzyme activity whereas no enzyme activity was detected in the supernatant, which coincides well with the visual clarity of the supernatant. At higher ratios (1/4), less than 20% of the enzyme activity associated with the aggregates while no activity was detected in the supernatant.

GA cross-linking is the final step for permanent fixation of the PEI-enzyme aggregates, which can be disrupted by negatively charged molecules (30). When using a solution GA ranging from 0.01% to 0.20% applied for 5-10 minutes showed no significant difference in either the PEI-lactase clustering or turbidity, nor in enzyme activity. Therefore, 0.1% GA solution treatment for 10 min was used to cross-link the aggregates for further studies (data was not shown). Rapid cross-linking effects were obtained regarding the effects of GA on the enzyme activity and aggregation characteristics with PEI for lipase enzymes (32).

The success in immobilization of an enzyme usually relies on projected favorable chemical interactions. Enzyme aggregation with PEI is an example of such molecular interactions (33). As seen in Figure 3, increase in PEI to enzyme ratio regrettably resulted in greater decline in the activity of the PEI enzyme aggregates. Although the activity yield of 60% is the best result currently obtained, probable causes could be attributed to steric hindrance and rendered flexibility of the PEI aggregated enzyme. Interaction of enzymes with PEI was often expected to result in aggregation, but the activity of the aggregates depends on a number of factors that are difficult to predict, including pH, presence of impurities, surface chemistry, the chemical characteristics of the active site, etc (34). To explore these unknowns requires comprehensive analytical studies. Nevertheless, some of the reasons can be attributed to the complex properties of lactase from K.lactis. It was reported in several studies that the enzyme was nearly 240 kDa in size, composed of two nonidentical monomers (35) and largely dominated with beta-strands secondary structure (21) with a need of space for motility. The stability of lactase from K.lactis is increased by phosphate ions and glycerol (23,36); therefore, the enzyme samples may likely contain some amount of phosphates and glycerol. Exclusion of these stabilizers from the enzyme conformation could have reduced the activity of the aggregated enzyme.

Characterizations for PEI-Lactase Aggregate

Considering the fact that most efficient PEI-enzyme aggregation and precipitation outcomes were obtained at PEI to enzyme ratio of 1/8, the ratio and the same aggregation conditions were used for the characterization studies.

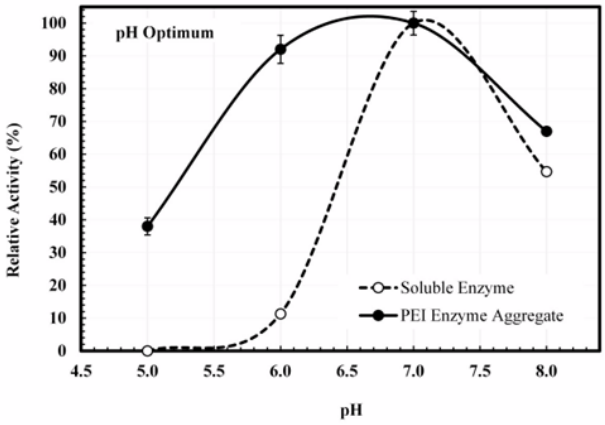

Figure 4 The effects of pH on the activities of soluble lactase and PEI-lactase aggregate at PEI to enzyme ratio of 1/8 with Lactozyme 2600L (enzyme activities were measured at 40°C).

Optimal pH

Compared with the soluble enzyme, the effects of pH and temperature on the enzyme activities for the PEI-lactase aggregates were determined and results were displayed in Figure 4. While the optimum pH value for the free enzyme was 7.0 and rather quickly lost 90% of activity at pH 6, exhibiting

a narrower optimal pH range. In contrast, the aggregated enzyme retained over 90% of its activity between pH 6.0 and 7.0 revealing broader optimal pH and noticeably shifted optimal activity towards acidic pH of 6.5-7.0. There was a minimal increase in activity at pH 8. Our results in terms of soluble enzyme very well agreed with the findings of various studies (21,37) where the structure-activity relationship as a function of the pH was investigated and they showed that the highest activity conveying the highest secondary structure content was found at pH 7.0. Enzyme exhibited structural change from pH 7.0 to 6.5, which was accompanied by a decline in the activity significantly with probable variations in critical residues (21). We can also deduce from the study that PEI-enzyme aggregation was able to withstand the structural change due to preserving nearly full activity between pH 6.0 and 7.0. Owing to the eminent electrostatic interaction between enzyme and PEI, changes in optimal pH and extension toward acidic (38) or alkaline ranges (32) were often observed depending on the enzyme. The change in optimal pH can be attributed to alterations in the charged residues of PEI molecules in the local vicinity surrounding the enzyme.

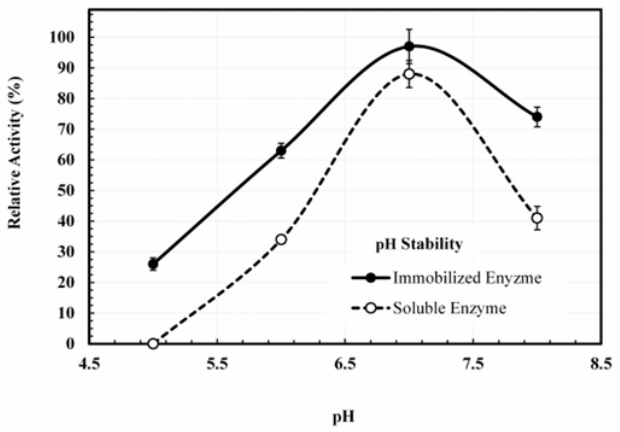

Figure 5 The effects of pH on the stabilities of soluble lactase and PEI-lactase aggregate at PEI to enzyme ratio of 1/8 (the enzyme activities were measured at 40°C after 18h of incubation).

pH stability

For evaluation of pH stability, Figure 5 compares the residual activities of the soluble lactase and PEI-lactase aggregates stored at various pHs from 5.0 to 8.0 for 18 hours of incubation at 40°C. The PEI-lactase aggregate exhibits about 30% greater stability across a range of pH values compared to the soluble lactase. Although the activities of both enzymes are maximized at pH 7.0, the PEI-lactase aggregate show a broader optimal pH range or higher stability within the same range compared to the soluble enzyme. PEI may act as an intermediary for chemical interactions among the local charges, stabilizing their positions and mitigating disruptive forces (17,39). Therefore, it may be more tolerant to pH changes during catalysis (31,39). Enhanced pH stability of the PEI-lactase aggregate means it is more versatile for various industrial processes over a wider range of conditions thus potentially deliver more efficient and cost-effective applications(40,41).

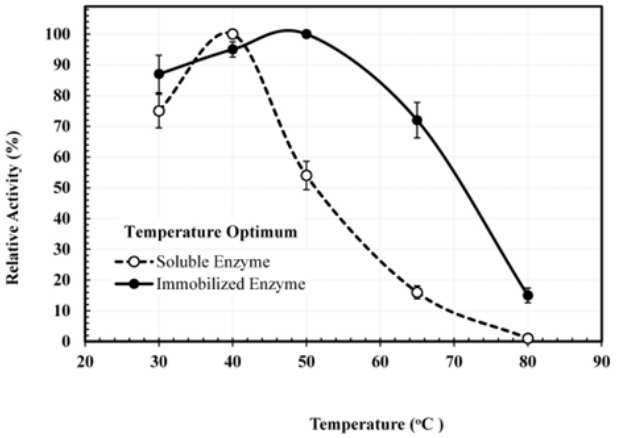

Figure 6 The effects of temperature on the activities of soluble lactase and PEI-lactase aggregate at PEI to enzyme ratio of 1/8 (pH 7).

Optimal Temperature

The effects of temperature on the enzyme activities of PEI-lactase aggregates were compared to those of the free enzyme at different temperatures in Figure 6. The free enzyme showed the highest activity at 40ºC, but it rapidly diminished its activity at increasing temperatures e.g., 50% residual activity remained at 50ºC. In contrast, the PEI-aggregated enzyme is able to preserve greater activity at higher temperatures e.g., retained full activity at 50ºC and 70% at 65ºC. The PEI-lactase aggregate clearly exhibited a broader optimal temperature range including 50ºC underlining higher stability at elevated temperatures. Similar to our results, it was reported that the enzyme optimum temperature at nearby 30 - 35oC (37) but quickly deactivated at 50oC (23).

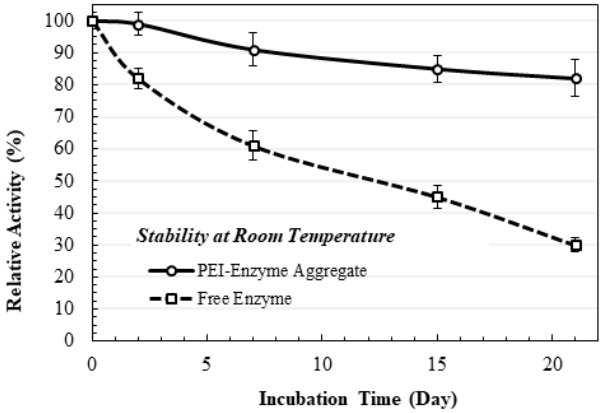

Figure 7 The effects of storage stability at room temperature for the soluble lactase and PEI-lactase aggregate at PEI to enzyme ratio of 1/8 (pH 7).

Figure 7 compared the storage stabilities of soluble lactase and PEI-lactase aggregates at room temperature for their

extended storage. The PEI-lactase aggregate showed significantly higher stability, retaining a higher percentage of its initial activity over the same period compared to the soluble lactase. The figure demonstrates that the PEI-lactase aggregate has superior storage stability at room temperature compared to the soluble lactase. This increased stability makes the PEI-aggregated enzyme more effective for long-term use in industrial applications. It was reported that the enzyme is stable at room temperature for several days (23). However, our results showed that soluble enzyme lost 50% of initial activity in 10 days.

Storage and Temperature Stabilities

Table 1 summarized the temperature stabilities of the soluble and the aggregated enzyme in buffers (pH 7.0) incubated at varying temperatures for 60 minutes. The thermal stability of the PEI-aggregated lactase increased significantly compared to the soluble enzyme. At 40°C, the half-life ($t_1/_2$) of the soluble enzyme increased from 76 to 254 hours with PEI aggregation. Additionally, the deactivation energy for the soluble enzyme decreased from 210 kJ/mol to 105 kJ/mol for the PEI-aggregated enzyme, indicating a substantial increase in enzyme stability (from the Arrhenius plot, plot lnk vs 1/T (K)). The thermal stability and kinetic behavior of enzymes, particularly when aggregated or immobilized with agents like polyethyleneimine (PEI), are crucial for optimizing industrial applications (42). Studies have shown that PEI aggregation can significantly enhance the stability of enzymes, such as lactase (30). For example, similar research on other enzymes like amyloglucosidase and lipases has demonstrated notable improvements in thermal stability and deactivation energy with immobilization techniques. High temperatures are beneficial for enzyme-catalyzed reactions not only to get higher reaction rates but also to avoid microbial contamination (43).

CONCLUSIONS

Using only PEI as an aggregating polymer, large clusters of PEI-enzyme particles were formed with β-galactosidase from Kluyveromyces lactis. PEI likely formed multiple polar ionic bonds with the enzyme molecules, causing them to link together into large, visible aggregates. At an optimal PEI to enzyme ratio, precipitating PEI-enzyme aggregates encapsulated all the enzyme during complexation and retained the majority of its initial activity. This study demonstrated that PEI, as a safe and inexpensive cationic polymer, could be used to form large clusters of PEI-lactase aggregates that preserve most of the initial enzyme activity and significantly enhance thermal and pH stability, thereby greatly extending the operational lifespan. PEI may act as intermediary shield for chemical interactions and physical forces among the groups within and among the enzyme by keeping the groups in place. The approach offers simple, rapid, and low-cost means to concentrate and stabilize the enzyme activity. In order to derive information regarding the type of interaction and mode of association with PEI and enzyme, further experiments could be conducted to explore the exact nature of the thermal stability and activity differences, possibly incorporating the analytical techniques and optimization strategies along with varying characteristics of enzymes and polymers.

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