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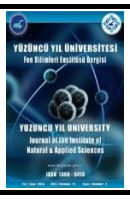

Research Article

Investigation of Complexing Properties with Polyethyleneimine of Some Commercial Lipases

Eda ÖNDÜL KOÇ*[1], Mert YILMAZ[2]

[1]Bolu Abant İzzet Baysal University, Yeniçağa Yaşar Çelik Vocational High School, 14300, Bolu, Türkiye
[2]Van Yuzuncu Yil University, Faculty of Engineering, Department of Food Engineering, 65080, Van, Türkiye
Eda ÖNDÜL KOÇ, ORCID No: 0000-0002-1659-0813, Mert YILMAZ, ORCID No: 0009-0000-1973-9153
*Corresponding author e-mail: edaondulkoc@ibu.edu.tr

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Polyethyleneimine

Abstract: Lipases are enzymes used in various industrial process and are immobilized to increase their applicability as biocatalysts. Ionic polymers such as polyethyleneimine (PEI) make possible the co-precipitation of enzymes. In this study, complexation and aggregation with PEI of enzymes were investigated with commercial enzymes from Novozyme 51032 (*Fusarium solani pisi*), Palatase 20000 L (*Rhizomucor miehei*), Lipolase 100 L (*Thermomyces lanuginosus*), Lipozyme CAL B L (*Candida antarctica* B) and Amano (*Pseudomonas fluorescens*) using PEI as a linker and aggregation agent. The highest percentage of PEI-enzyme agregate was obtained for Novozyme 51032, Palatase 20000 L and Lipolase 100 L at the PEI/enzyme ratio of a 1/20-1/80 range. This study documented that Lipozyme CAL B L and (Amano) *P. fluorescens* enzyme preparations failed to occur precipitates resulting PEI-enzyme aggregates. The some commercial lipase preparations may contain various impurity components that prevent complexation or aggregation with PEI. Complexing with PEI of lipases is based on of basis electrostatic interaction of enzyme with PEI as a cationic polymer resulting in PEI-lipase aggregates.

Bazı Ticari Lipazların Polietilenimin ile Kompleks Oluşturma Özelliklerinin Araştırılması

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Anahtar Kelimeler
Biyokatalizör,
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Öz: Lipazlar, çok sayıda endüstriyel proseste kullanılan enzimlerdir ve biyokatalizör olarak uygulanabilirliklerini artırmak için immobilize edilmektedirler. Bu çalışmada, Novozyme 51032 (*Fusarium solani pisi*), Palatase 20000 L (*Rhizomucor miehei*), Lipolase 100 L (*Thermomyces lanuginosus*), Lipozyme CAL B L (*Candida antarctica* B) ve Amano (*Pseudomonas fluorescens*) kaynaklı ticari enzimlerin, polietilenimin (PEI) ile kompleks ve agregat oluşturması incelenmiştir. Enzimlerin, polietilenimin ile en iyi kompleks oluşturduğu PEI/enzim oranının; Novozyme 51032, Palatase 20000 L ve Lipolase 100 L için 1/20-80 aralığında olduğu görülmüştür. Lipozyme CAL B L ve (Amano) *P. fluorescens*, PEI ile agregat oluşturamamıştır. Bu çalışma; bazı ticari enzimlerin, PEI ile agregat oluşturmasını engelleyen çeşitli safsızlıklar içerebileceğini göstermiştir. Polietileniminin-enzim kompleksi, katyonik bir polimer olan PEI' nin, enzimlerle elektrostatik etkileşimi esasına dayanmaktadır.

1. Introduction

Lipases (triacylglycerol hydrolases E.C. 3.1.1.3) are miscellaneous enzymes that catalyze the hydrolysis or synthesis of wide range of water insoluble esters under natural conditions (Hasan et al., 2009; Widmann et al., 2010; Carvalho & Conte-Junior, 2021; Dash & Banerjee, 2021). Lipases belong to a/β hydrolase fold super-family and have an active site consisting of triad of Ser, Asp (Glu) and His (Javed et al., 2018). Fungal or bacterial origin lipases, such as *Candida rugosa, Candida antarctica, Rhizopus oryzae, Bukholderia cepacia, Aspergillus niger, Thermomyces lanuginosus, Rhizomucor miehei* and *Pseudomonas fluorescens* have a considerable place in industry (Hasan et al., 2006; Wu et al., 2020; Monteiro et al., 2021a). Free or soluble form lipases have some problems related to low operational stability, high cost, difficulties in recovery process and reuse of these biocatalysts (Sharma et al., 2016; Almeida et al., 2021; Zhou et al., 2021). However, when appropriate immobilization techniques used the problems can be solved (Monteiro et al., 2019). Immobilized lipases have been used in a large number of commercial biocatalysis applications as these enzyme protocols have been proved to yield high-quality products at low processing costs and easy recovery of catalyst (Mathesh et al., 2016; Monteiro et al., 2021b; Wang et al., 2021).

Enzyme immobilization technology is used both in industrial processes (food, detergent etc.) and for medical diagnosis, therapy, bioenergy and biomaterial detection (Nguyen & Kim, 2017). Enzyme immobilization can be described as reduction or loss of mobility of free or soluble enzymes. So that the free enzymes have attached to several types of support and prevented their catalytic activities (Nguyen & Kim, 2017; Ismail et al., 2021). A great number of immobilization procedures have been demonstrated in the literature (Soares et al., 2011). Immobilization of enzyme on solid supports among immobilization methods has been extensively investigated (Nguyen & Kim, 2017). Immobilized enzymes present higher resistance to harsh environmental conditions and have commonly used in industrial field (Mittersteiner et al., 2017; Bilal et al., 2021). The enzymatic activity and stability depend on both the choice of the support and the method of immobilization (Tocco et al., 2021). In this way, enzyme immobilization has become a very basis step in the design of an industrial enzyme biocatalys (Rodrigues et al., 2019). Immobilized enzymes permit their reused and recovery as well as reducing operational costs in industrial processes (Mokhtar et al., 2021; Guimarães et al., 2022).

Polyethylenimine (PEI), is an abundantly branched cationic polymer, has many applications in biochemistry that has been widely used to immobilize nucleotides, cells and enzymes due to its attraction negatively charged species via electrostatic interaction (Albayrak & Yang, 2002; Karimpil et al., 2012). Branched PEI (BPEI), is a highly viscous and has electron-rich nitrogen atoms. It has widely used due to its several unique features such as low toxicity, ease of separation, recycling and odorless in numerous applications (Sun et al., 2015; Virgen-Ortíz et al., 2017). The goal of this study is to investigate PEI-lipase aggregate formation as a result of electrostatic interaction with some commercial lipases in aqueous solution. The aggregation of PEI and lipase has industrially a critical importance as a potantial support material-free enzyme preparation. In our previous work, we experienced PEI-enzyme attraction leading to enzyme aggregation and precipitation with other some commercial lipases (Ondul et al., 2012).

2. Materials and Methods

2.1. Materials

Commercial lipases in liquid forms from Novozyme 51032 (15 KLU/g), Palatase 20000 L (20000 LU/g), Lipolase 100 L (100 KLU/g), Lipozyme CAL B L (5000 LU/g) were kindly donated by Novozymes (Novo Nordisk, Denmark). Commercial lipase in powder form from Amano *Pseudomonas fluorescens* (20000 LU/g) was purchased from Sigma-Aldrich (Darmstadt, Germany). Branched polyethyleneimine (BPEI) as 50 % (w/v) (it has an average molecular weight 750.000) was obtained from Sigma (USA). p-Nitrophenyl palmitate (p-NPP) was used to determine catalytic activity obtained from Sigma Aldrich (USA). p-Nitrophenol (p-NP) for a standard graph was obtained from MP Biomedicals (France). PEI and enzyme solutions were dissolved with distilled water. The protein amount was determined by Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as the reference stardard (Sigma, USA).

2.2. PEI-lipase complex preparation

PEI-lipase aggregates were prepared by mixing a certain of PEI and lipase solutions prepared with distilled water. In order to form PEI-lipase aggregate in solution, 0.1 mL solutions containing varying portions of PEI 0.125-5 mg in 1 mL of solution) were added to the 900 µL of enzyme solutions in microcentrifuge tubes. PEI-lipase mixture after vortexing was highly a cloudy appearance. The cloudy mixture containing PEI and lipase aggregates were centrifuged at 10.000 rpm for 2 min. Finally, initial enzyme activities both in cloudy mixture including the PEI-enzyme and in supernatant after centrifugation were measured to determine effect of PEI concentration on lipase activity and compared with free enzyme activity no including PEI. The microscopic image analysis of PEI-enzyme aggregates in cloudy turbid mixture was assessed with a light microscopy (Seiler SXS 820, USA) (Figure 1). Furthermore, Bradford method was used to evaluate content of protein both in the turbid mixture containing PEI-lipase complex and in supernatant after centrifugation.

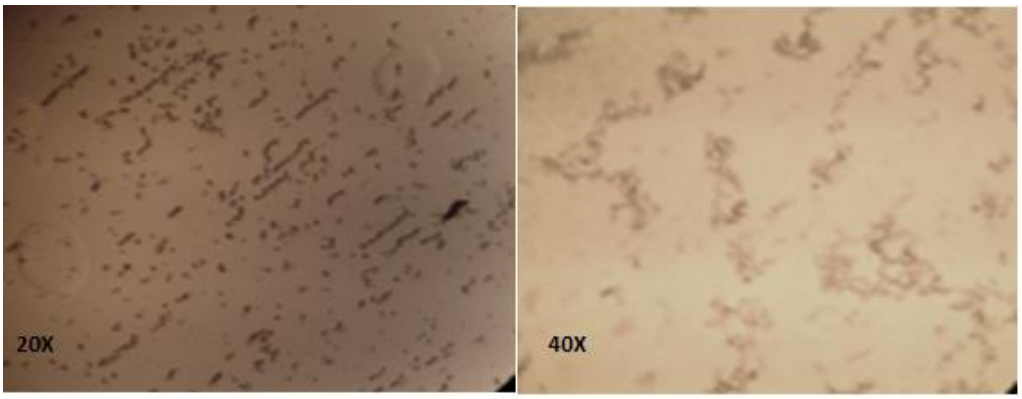

Figure 1. The images of PEI-lipase aggregates in cloudy turbid mixture at 20X and 40X magnifications under light microscopy.

2.3. Analytical methods

2.3.1. Lipase activity assay

Lipase activity assay was assessed by spectrophotometric method using p-nitrophenyl palmitate (p-NPP) as substrate by modifying method reported by Costa et al. (2003). p-NPP (a sample of 10 mg) was dissolved in 5 mL acetonitrile and was adjusted to 4.1 mM with acetonitrile. Reaction medium was consisted of 30 µL of soluble enzyme solution diluted with distilled water or PEI-lipase aggregates resuspended by vortexing, 3 mL of 50 mM potassium phosphate buffer (pH 7.0), and 100 µL of p-NPP solution prepared in acetonitrile. The hydrolysis reaction was directly generated inside of the spectrophotometer cuvette at 30 ^{o}C in a incubator. Measuements of reaction carried out with the blank prepared with reaction mixture no including enzyme. The absorbance was measured at the end of five minutes incubation time at 404 nm. The yellow coloured p-nitrophenol (pKa 7.15) occuring after hydrolysis reaction has an absorbance at 405 or 410 nm in spectrophotometer (Hasan et al., 2009). The calibration curve was used to determine lipase activity by linear equation (pNP) (y=12.306x-0,0372, R^2 =0.9985) (Figure 2). One international unit of activity was defined as the amount of enzyme that hydrolyzes 1 µmol of p-NPP per minute and liberates 1 µmol p-NP in defined conditions.

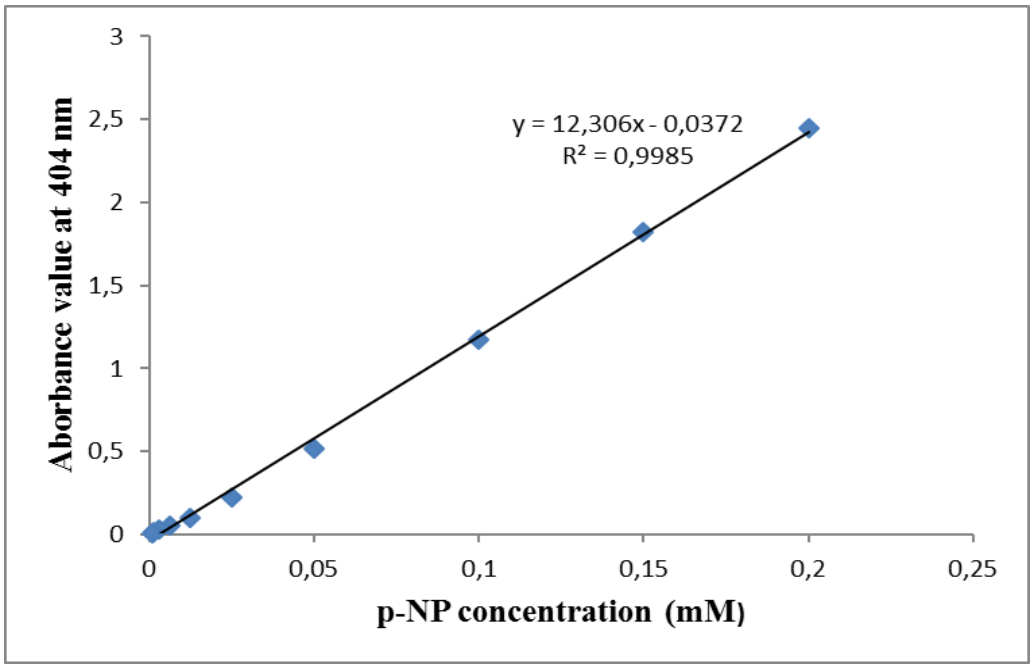

Figure 2. The calibration curve of p-NP standard determined by measured absorbance value at 404 nm.

2.3.2. Determination of total protein by Bradford method

Bradford method was used to quantify total poroteins of enzyme solutions at 595 nm in a spectrophotometer (UV-Mini 1240 UV-VIS, Shimadzu, Kyoto, Japan) (Bradford, 1976). Figure 3 indicated that calibration curve was prepared by mesured the optical density absorbance values at 280 nm against the concentration of bovine serum albumin. The equation obtained from this calibration curve was expressed as y =0.6355x + 0.0015.(y and x indicated absorbance value at 280 nm and protein concentration in mg/mL, respectively).

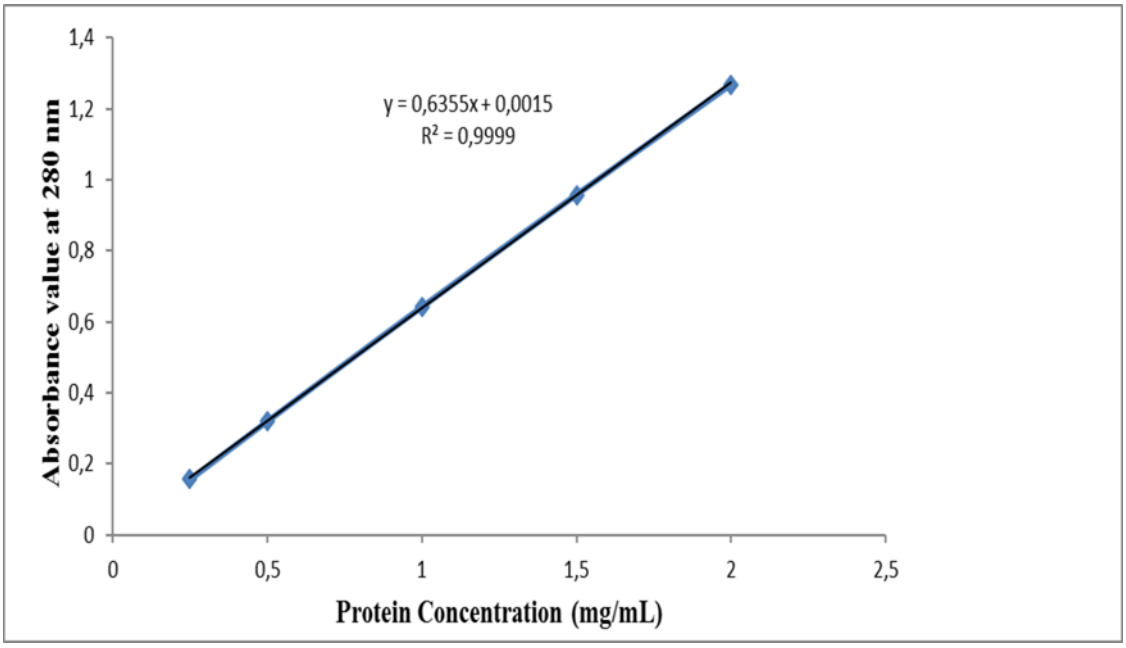

Figure 3. The calibration curve of protein standard as expressed bovine serum albumin.

3. Results and Discussion

3.1. PEI/Enzyme aggregate formation

Polyethyleneimine and commercial liquid and powder lipase preparations were mixed to occur aggregates that have exhibited an complexed structure. As seen in Figure 4, when PEI solution (original pH 11.0) and Novozyme 51032 liquid enzyme solution are mixed in microcentrifuge tubes, a milky cloudy structure is formed and precipitated after centrifugation at 10.000 rpm for 2 min. When Palatase 20000 L liquid enzyme preparation solution was mixed with PEI solution in microcenrifuge tubes, PEI-lipase aggregates appeared as highly cloudy mixture at 0.625 mg/mL concentration of PEI before centrifugation. PEI-lipase precipitates occured in all microcentifuge tubes after centrifugation at 10.000 rpm for 2 min. PEI-Lipolase 100 L lipase aggregate was prepared by mixing at varying concentrations of PEI and lipase solution. After mixing, PEI-lipase slurry appeared as milky or cloudy solution. The highest turbidity and the goodest precipitate was showed at 2.5 and 5.0 mg/mL concentration of PEI. When Lipozyme CAL B L was mixed with PEI solution, neither a cloudy turbid structure nor PEI-enzyme precipitates were shown after centrifugation. Similarly, *P.fluorescens* lipase and PEI mixture was occured no cloudy turbid solution and too little precipitation after centrifugation (figure was not given).

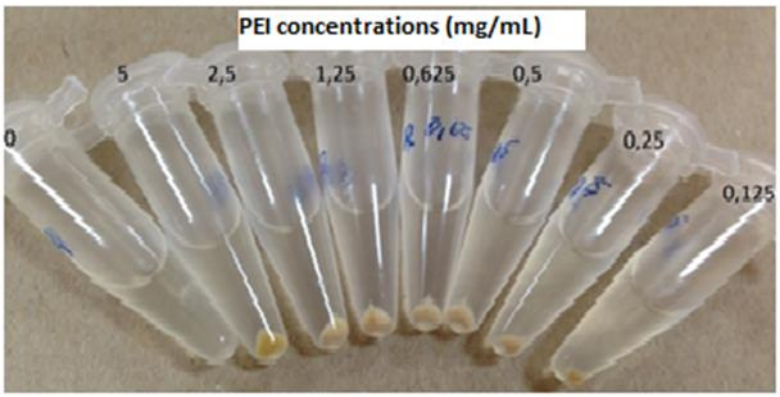

Figure 4. Images of PEI-lipase precipitates at PEI concentrations from 0.125 to 5.0 mg/mL after centrifugation for Novozyme 51032 liquid enzyme preparation at 10.000 rpm for 2 min.

3.2. PEI/Enzyme ratio

PEI-enzyme complex formation with lipase preparation from Novozyme 51032 was investigated at concentrations of PEI from 0.125 to 5.0 mg/mL ratios. As seen Figure 5, at the all PEI concentrations, lipase activity in PEI-lipase slurry have prevented its initial activity before centrifugation. At 5 mg/mL concentrations of PEI have seen the highest precipitation after centrifugation resulting approximately % 71 residual lipase activity in supernatant.

PEI-enzyme complex formation with lipase preparation from Palatase 20000 L was investigated at varying PEI concentrations. As seen Figure 6, the optimum complex formation with Palatese 20000 L was obtained at the 0.625 mg/mL concentration of PEI resulting in complexation yield of 62 %. PEI have abundant primary and secondary amine groups in its structure and have complexed with enzymes via electrostatic interaction (Liu et al., 2020). When the PEI concentration in lipase solutions was increased, PEI-enzyme comlex activity in slurry showed a gradual increase. Also, the residual lipase activity in supernatant inceased. Figure 6 showed that Palatase 20000 L was significantly improved the stability with increasing PEI concentrations. The physical intermolecular crosslinking with PEI reduces enzyme leakage and it is a important tool in some co-immobilization strategies (Arana-Peña et al., 2019). PEI has a important role in increasing the stability of enzymes as well as the ability to increase the rate of enzyme-catalyzed reactions (Andersson & Hatti-Kaul, 1999). At the same time, the use of PEI as a linker may also cause some problems such a enzyme inactivation due to interaction with inside pocket of the protein (Virgen-Ortíz et al., 2017).

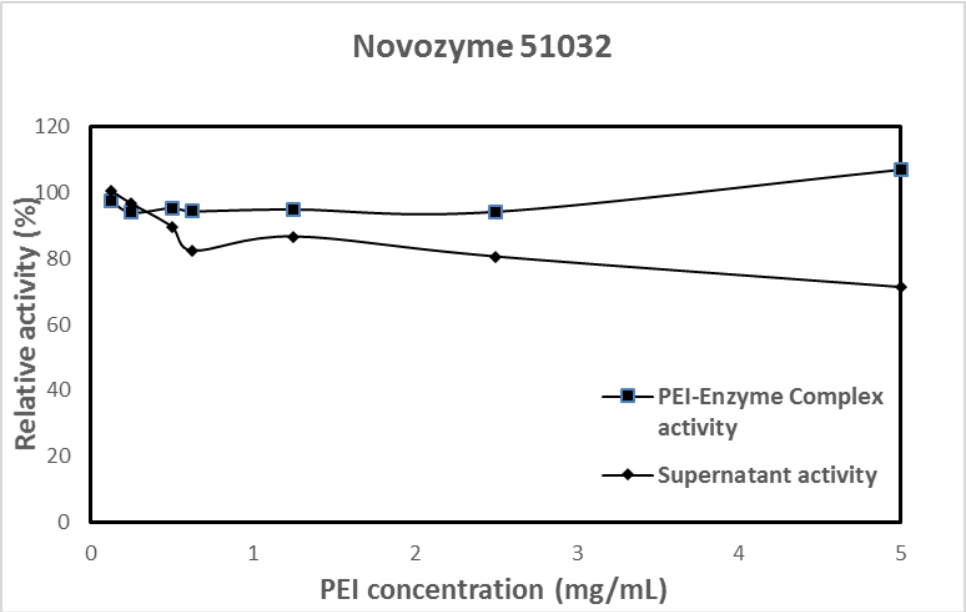

Figure 5. The effect of PEI concentrations on PEI–enzyme aggregate formation and precipitation in cloudy mixture and in supernatant after centrifugation at10.000 rpm for 2 min.

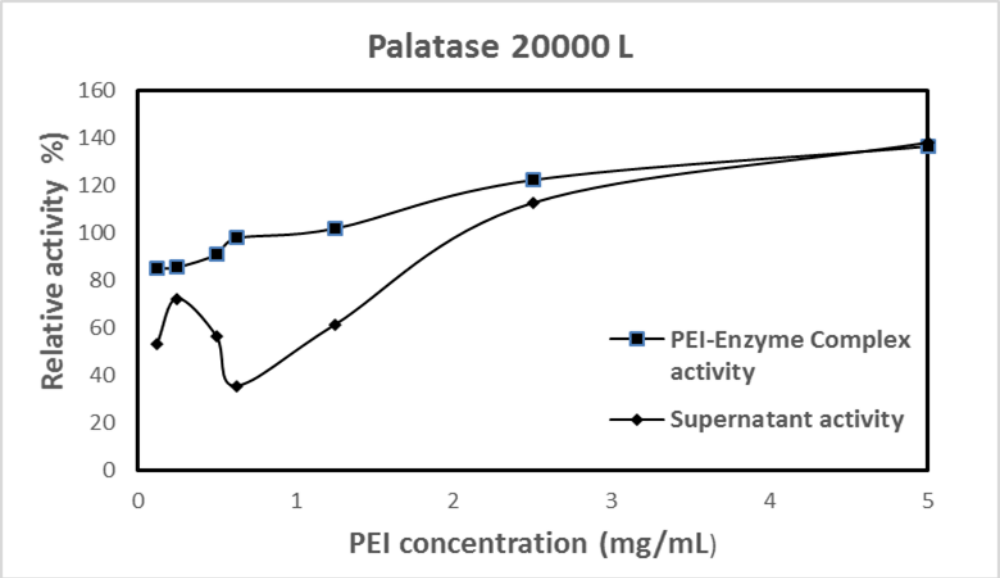

Figure 6. The effect of PEI concentration on enzyme activities of PEI–enzyme aggregates in solution and in supernatant after centrifugation at 10.000 rpm for 2 min.

PEI-enzyme complex formation with lipase preparation from Lipolase 100 L was investigated at varying PEI to lipase ratios. As seen Figure 7, increasing concentrations of PEI in slurry nearly resulted in 100 % lipase activity with Lipolase 100 L compared to the lipase solution with no PEI. Increasing concentrations of PEI did not affect the activity of Lipolase 100 L. Polyethyleneimine is a hyrophilic cationic macromolecule has abundantly amine groups on its (Jiang et al., 2019). This polycations act to bind with negatively charged molecules as powerful adsorbents via strong electrostatic interactions (Chen et al., 2020). It should be consulted that PEI has proved to have many positive effects on enzyme features (Arana-Peña et al., 2020). As the PEI concentration increased up to for 0.625-5.0 mg/mL for Lipolase 100 L, the residual lipase activity in supernatant decreased to 42%. The optimal PEI-enzyme complex formation was obtained at concentration of 5.0 mg/mL PEI resulting in immobilization yield of 61%. PEI, a polymer composed of the amine groups, plays an important role between of the a wide range of polycations used as a versatile polymer in biocatalysts design (Chen et al., 2020; Sampaio et al., 2022; Wu et al., 2023).

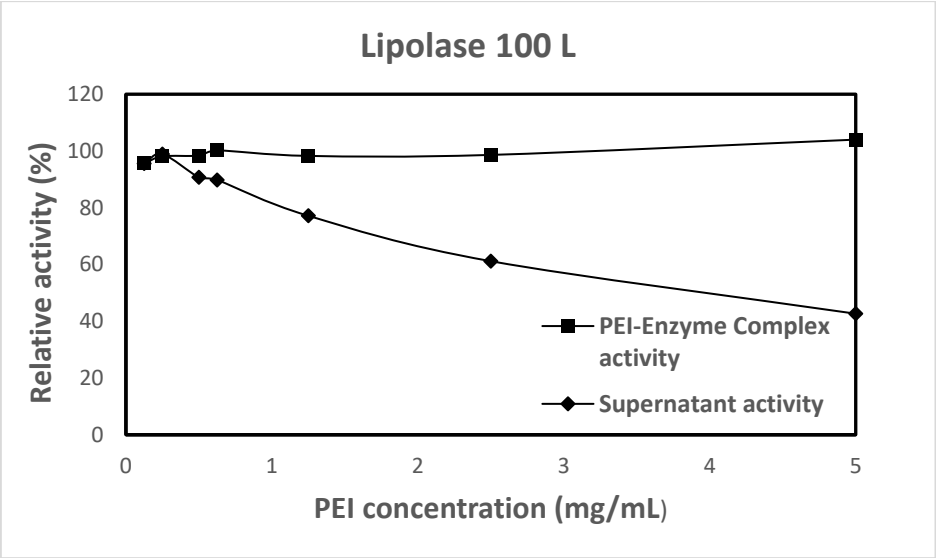

Figure 7. Effect of PEI concentration on activities of PEI–enzyme aggregates in solution before centrifugation and in supernatant after centrifugation at 10.000 rpm for 2 min.

Similar to Lipolase 100 L, increasing concentrations of PEI did not adversely affect the activity of Lipozyme CAL B L as seen Figure 8. Electrostatic interaction of the enzyme with PEI has a more significant effect on the stability of the enzyme. Also, polyethylenimine has been used to keep the activated form of lipases (Peirce et al., 2016). Lipozyme CAL B L preparation with PEI were not complexed by forming aggregate at the varying PEI concentrations. Lipozyme CAL B L commercial preparation contains a high amount of coumpounds such a glycerol, sorbitol, sodium benzoate and potassium sorbate according to a technical notes provided by Novozymes (Llerena-Suster et al., 2014). This compounds have a negative effect on formation of PEI-lipase aggregation. Because PEI is a polycationic polymer and can bind small negatively ions. According to a research paper related to glycerol-crosslinked PEI, use of glyserol to modify the buffering capacity of PEI is possible to see in the literature (Singh et al., 2015).

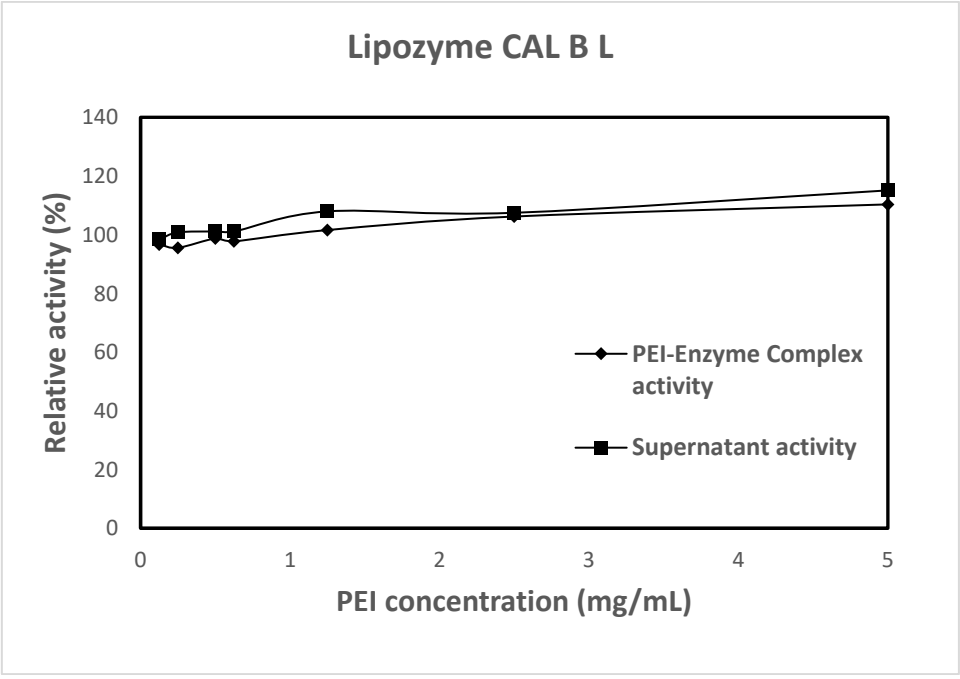

Figure 8. Effect of PEI concentration on activities of PEI–enzyme aggregatesin solution before centrifugation and in supernatant after centrifugation at 10.000 rpm for 2 min.

PEI-enzyme agregate formation with lipase from Amano *P. fluorescens* was invesitigated at varying PEI concentrations. Similarly, lipase preparation from *P. fluorescens* did not produce aggregates and precipitates at all the concentrations of PEI. PEI-lipase comlex activity showed 100 % activity but PEI-enzyme slurry was no turbid and precipited after centrifugation.

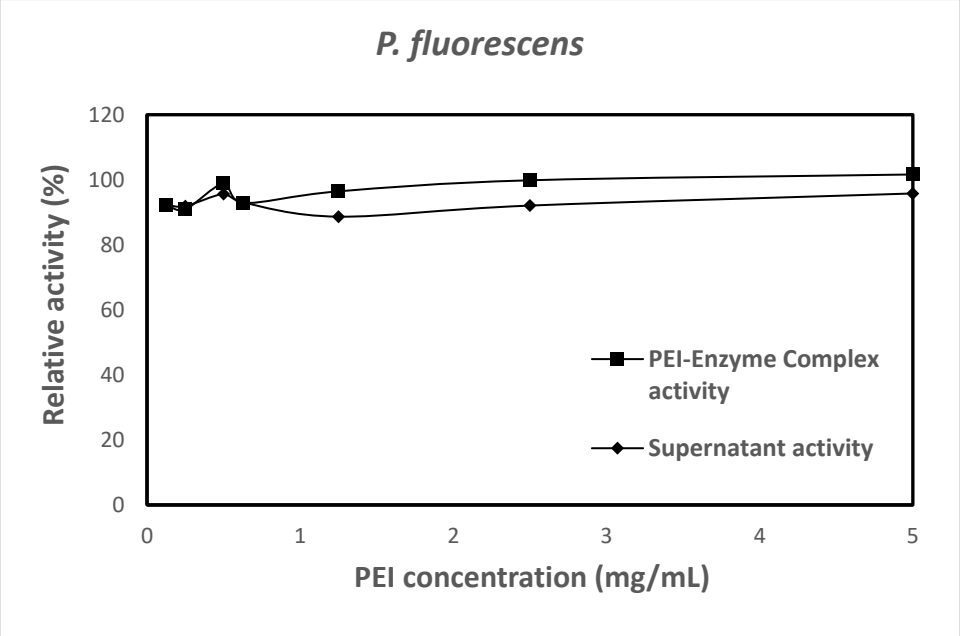

Figure 9. Effect of PEI concentration on activities of PEI–enzyme aggregates in solution before centrifugation and in supernatant after centrifugation at 10.000 rpm for 2 min.

Table 1 indicates the amount of protein of liquid and powder form the commecial enzymes. Lipolase 100 L preparation have the highest protein content compared to those of the other liquid and powder enzymes preparations. High level of protein of Lipolase 100 L could be caused to PEI-lipase precipitaion and agregation. Polyethyleneimine has successfully used to occur multilayers enzyme biocatalysts as a linker agent between lipase layers. This polymer can be adsorbed on enzyme molecules through a strong attraction. In addition, the enzyme adsorbed with PEI can also attach other enzyme molecules via ion exchange. PEI is an important the polycation reported to have a favourable effect on enzyme activity and stability (Peirce et al., 2016). It has also used to keep the activated form of lipases in biocatalyst design (Andersson & Hatti-Kaul, 1999). The protein content of *P.fluorescens* was the lowest. This powder preparate failed to occur aggregate with polyethyleneimine. Lipozyme CAL B L couldn't showed precipitation although it has high level protein amount.

Table 1. Protein contents, specific activities and pH of commercial liquid and powder enzyme preparations

Enzyme Source	**Protein Conc.**[(1)]	**pH**[(2)]	**PEI/Enzyme (w/w)**[(3)]
Novozyme 51032 (liquid)	94.0 % (w/v)	7.66	1/20
Palatase 20000 L (liquid)	50.0 %(w/v)	4.62	1/80
Lipolase 100 L (liquid)	156.0 % (w/v)	6.20	1/33
Lipozyme CALB L (liquid)	74.0 %(w/v)	4.15	-
Amano *P. fluorescens* (powder)	5.0 %(w/w)	6.39	-

(1) Bradford method was used to quantify protein amount of enzyme sources in liquid and powder form.
(2) pH was measured by the Hanna pH meter.
(3) PEI/enzim mass ratio that the optimum precipitation was obtained (Figure 5,6,7)

4. Conclusion

Among the commercial lipases studied, Novozyme 51032, Palatase 20000 L and Lipolase 100 L aggregated and precipitated with PEI via electrostatic interaction. Lipozyme CAL B L and Amano *P. fluorescens* enzyme preparations failed to occur PEI-enzyme aggregates due to lack of strong ionic interactions. This study shows could make be possible of preparation of support material free PEI-enzyme aggregates with the some commercial lipase preparations. Use of the appropriate concentration of PEI has explained to able be a proper concentration to produce PEI- lipases agregates. Lipozyme CAL B L liquid form enzyme preparation and Amano *P. fluorescens* powder form enzyme preparation failed to occur PEI-enzyme aggregates due to some the impurities including glycerol, sorbitol, sodium benzoate and potassium sorbate. Previous studies have reported that PEI can easily attract with negatively charged metal ions and competes with different substrates compared to enzymes. Some components such as salts, glycerol and other anionic compounds can interact electrostatically with PEI through strong ionic interactions.

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