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Preparation and Characterization of Cross-Linked PEI-Lipase Aggregates with Improved Activity and Stability

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Abstract: Using polyethyleneimine (PEI) as the sole precipitation and aggregation agent, PEI-enzyme complexation was investigated with lipases from *Rhizomucor miehei, Thermomyces lanuginosus and Candida antarctica*. The approach relied on rapid development of PEI-lipase aggregates in a solution and followed by glutaraldehyde cross-linking thus resulting in cross-linked PEI-lipase aggregates. PEI to enzyme mass ratio of a 1/ 20-40 range, alkaline pH and the absence of impurities produced higher coupling yields and activities. The pH affected the precipitatibility and/or relative activity of the aggregates. Impurities in some lipase preparations may prevent the formation or precipitation of the PEI-lipase aggregates. The aggregates attained higher stabilities especially at high pHs and enhanced thermostability with at least a 20-fold at ambient temperatures. By using *p*-nitrophenyl propionate as a soluble substrate, app. *V*max for the immobilized lipase increased by two-fold with only 25% increment in app. *K*m compared with the soluble lipase. Complexation with PEI may have produced favorable interface assisting for conformational change for the lipase activation. Thus, cross-linked PEI-lipase aggregates with ease of recovery and stability can be simple and inexpensive alternative for carrier-free immobilized lipases.

Keywords: Polyethyleneimine, Lipase, Glutaraldehyde, Cross-linked PEI-lipase aggregate

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INTRODUCTION

Since the start of last century (1), the means of enzyme immobilization have been the subject of numerous investigation yielding various methods (2). Apparently, no method fits all enzymes or all applications of a particular enzyme. Immobilized enzymes are expected to enable reuse of enzyme, provide sufficient stability and thus simplified and efficient product formation (3). In majority of enzyme immobilization procedures, the major focus was devoted to the nature of the support for enzyme to be attached (4–7). Enzyme immobilization on support provides the benefits of ease on handling, better separation and mechanical stability but the enzyme density in a given area tends to be low (8,9). Also, highly engineered functional particulate type supports tend to be expensive (7,10,11) which is a great burden for industrial scale applications (12). In order to increase volumetric efficiency of an enzyme reactor, the space devoted to carrier should be limited without sacrificing productivity and stability (13).

Our objective is to prepare high-density, carrier-free lipase catalyst with simple and inexpensive means. In recent years, considerable research is directed towards carrier-free enzyme immobilization approaches so that higher catalyst

densities would be achieved by excluding support such as in the concepts of cross-linked enzyme aggregate (3,14). The preparation involves the physical precipitation of the enzyme with the aid of a solvent or salt and further cross-linking generally with glutaraldehyde (GA) to form insoluble cross-linked enzyme aggregates (14–16). The cross-linked enzyme aggregate method shows a significant achievement in the field providing intense form of biocatalyst and turned into a commercial application (17). However, low catalytic efficiencies were reported in a few of the applications due to close confinement (10,18) and diffusion limitations (19). Recently, e.g., a cross-linked aggregate of *β*-galactosidase from *Aspergillus oryzae* was prepared and used for lactulose/galacto-oligosaccharides (GOS) production (20). It was observed that GOS yield obtained with the immobilized lactase was 25% lower than that of the soluble enzyme. We have previously shown that solo polyethyleneimine (PEI) precipitation of *β*-galactosidase from *Aspergillus oryzae* and GA cross-linking was able to produce carrier-free aggregates (21). The carrier-free lactase aggregate showed over 95% specific enzyme activity and more importantly was able to carry out lactose conversion as good as the soluble enzyme with no reduction in GOS formation, which suggests PEI-enzyme aggregates are flexible and porous to allow the diffusion of large molecules. However, it remains to be seen the applicability of the PEI - enzyme aggregation approach to other enzymes namely industrially relevant lipases.

Lipases carry out various types of reactions, they hold tremendous potential for clean and ecofriendly transformations in energy, oleo-chemistry and the chemical applications (22–24). It was recognized that lipase applicability in industrial processes was not fully explored due to their low stability or short life-times (7,16,25,26) which make immobilized lipase economically attractive. In essence, success in lipase immobilization relies on delicate balance of various parameters including enzyme flexibility to enable conformational change during interfacial activation as well as solubility for all substrates and the enzyme itself (7,27,28). These challenging aspects should be well considered especially with a carrier-free lipase preparation. Simple and inexpensive confinement of a high number of active and adequately stabilized catalyst in an intense state may be an attractive approach to extend the applicability of lipases(28).–

The goal of the study is to investigate cross-linked PEI- enzyme aggregates with industrially relevant lipases as a potential carrier-free enzyme preparation. Our previous experience with PEI-enzyme interaction leading to enzyme immobilization and aggregation (21,29–31) may suggest that PEI unforcefully gathers and steers the enzymes yet practically separates one another by cushioning in between, leading to favorable channeling for mass transfer (32). Therefore, we hypothesize that conveniently avoiding the use of organic solvent for precipitation, solely PEI mediated lipase aggregate may lead a novel type of a dense catalyst with desirable attributes of low cost, rapid preparation and high activity and stability. The key factors affecting PEI-enzyme aggregate formation such as PEI to lipase ratio, pH of the aggregation, extent of the GA cross-linking and impurities in enzyme preparations were investigated. Also, carrier-free immobilized lipase characteristics and relevant stabilities were discussed in this study.

MATERIALS AND METHODS

Enzyme and Reagents

Commercial lipase in liquid forms from *Thermomyces lanuginosus (*Lipozyme TL), and *Rhizomucor miehei* (Novozym 388) were a gift of Novo Nordisk (Bagsvaerd, Denmark). *R. miehei* (ICR-116 Lipase), *Candida antarctica* Cal A (ICR-112) and Cal B (ICR-110) lipases in powder forms were obtained from Codexis (Pasadena, CA, USA). Also, *R. miehei* (Fungal Lipase, 150,000 FIP/g) lipase obtained from Bio-Cat (Troy, VA, USA). *Burkholderia cepacia* (Lipase PS Amano S) was from Amano Inc (Elgin, IL, USA). Branched polyethyleneimine as 50% (w/v) (Number average molecular weight: 60,000; Average molecular weight: 750,000) and glutaraldehyde (GA) as 25% (w/v) aqueous solutions were from Sigma (St. Louis, MO). *para*-Nitrophenyl propionate (*p*-NPP) and *para*-nitrophenol (*p*-NP) (MP Biochemical, France) were used to measure lipase activities. All other reagents were obtained from Merck (Darmstadt, Germany) and they were at least American Chemical Society grade. All solutions used for PEI-lipase aggregate formation including PEI, GA, lipase preparation were made with distilled water and, pH adjustments were made with suitable concentrations of NaOH or HCl.

Aggregate Preparation

The procedure for formation of cross-linked PEI-lipase aggregates is composed of two main steps as complexation between PEI and the enzyme, and cross-linking of the complexes with GA. PEI-lipase aggregate was prepared by mixing PEI and lipase solutions. After vortexing the turbid

mixture, a GA solution was added to the PEI-lipase slurry and incubated for 5 minutes for cross-linking. PEI solution (0.1 mL) was mixed with a designated lipase solution (0.9 mL) in microcentrifuge tubes. Turbid slurry of PEI-enzyme mixture was centrifuged at 10,000 rpm for 1-2 min (Sigma 1-14, Germany). To evaluate the exclusive effect of PEI on the lipase activity and complex formation, initial lipase activities of PEI-lipase slurry and the supernatant after the centrifugation were determined. The relative lipase activities were compared with respect to free enzyme (containing neither PEI nor GA). The morphology of PEI-enzyme aggregates in cloudy turbid slurry was analyzed with a phase-contrast microscopy (LEICA DM 2500, Meyer, Texas, USA) (Figure 1). Prior to the GA cross-linking, the effects of PEI to enzyme ratio (1/2.5 - 1/250), pH (6 to 11), mixing (50-200 rpm) and duration (5 to 20 minutes) for effective PEI-lipase complexation were studied. The differences in the activities of the complex solutions and the supernatants can be considered as a degree of the enzyme incorporation into the complexes.

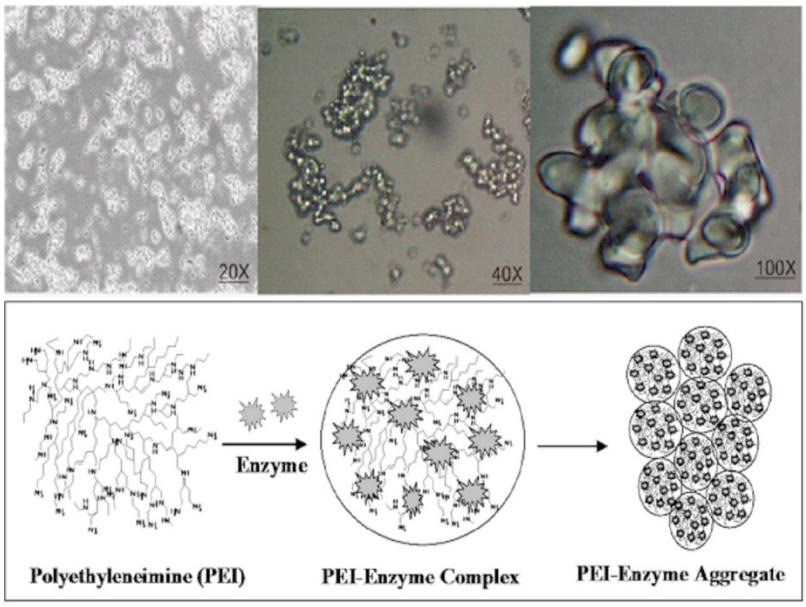


Figure 1: Light microscopy images of PEI-lipase aggregates at 20, 40 and 100 magnifications along with the projected mechanism from PEI-enzyme complexation towards aggregation.

Finally, under the optimized conditions for PEI-lipase complexation, the GA solution (0.1 mL) was added to the PEI-lipase mixture and incubated for about 5 min. The effects of the GA concentrations (0.1-10 mg/mL) and the duration (5 to 30 min) for cross-linking were studied. The factors were evaluated according to the outcome of initial lipase activities of the GA cross-linked PEI-lipase complex and the activity remaining in the supernatant. Stability of cross-linked aggregate obtained under the optimized condition was monitored by washing thoroughly with distilled water and buffer (50 mM phosphate buffer pH 7.0). Unless otherwise noted, all the experiments were carried out at room temperature. The experiments were performed in triplicate and the standard deviations were shown in the figures as vertical bars.

Characterization

Variations in the characteristics of cross-linked PEI-lipase aggregates were investigated by using the *Rhizomucor miehei* lipase (RML) from Novozyme to exemplify the effect of the lipase immobilization procedure obtained under the optimized conditions for the immobilization. After preparation, the precipitates of cross-linked PEI-lipase aggregates in tubes were resuspended to the original volume of the enzyme with 50 mM phosphate buffer (at pH 7) after being washed two times with distilled water. The resuspended aggregates were refrigerated until use and employed for all of the characterization studies including thermal and storage stabilities and the determinations of apparent kinetic constants. For determinations of pH optimum and stability against change in pH, the aggregates resuspended in distilled water were used. All characterization studies for the resuspended aggregates were carried out side-by-side with

free lipase solutions. Examination of the optimum temperature for free and the immobilized enzyme was avoided because of greater interference of autohydrolysis of *p*NPP substrate at higher temperatures (33).

Thermal and Storage Stabilities
For thermal stability, cross-linked PEI-lipase aggregates were held in a constant-temperature water bath between 35 and 75°C for two hours and then the lipase activities were measured and compared with free enzyme incubated under the same conditions. To determine the storage stability, free and the resuspended aggregates in 50 mM phosphate buffer at pH 7 were kept in refrigeration or at room temperature for nearly one month, and lipase activities were measured from the aliquots taken from samples during incubation. The storage stability of a dry form of cross-linked PEI-lipase aggregates was also included in the study. The aggregate preparation resuspended in distilled water in a glass dish was left to dry and then kept at room temperature for two weeks. After the incubation, the dried aggregates were resuspended in distilled water and rehydrated for 30 min before the measurement of the lipase activities at the end of incubation.

pH Optimum and Stability
In order to study the effect of pH on the activities of cross-linked PEI-lipase aggregate and soluble lipase, 50 mM acetate (pH 4.0–6.0), phosphate (pH 6.0–7.0), phosphate/carbonate (pH 8.0-10.0) buffers were employed to dissolve or resuspend the enzyme preparation, and also the same buffers were used for diluting the substrate for lipase assays. For the determinations of stability against pH, free lipase and the aggregate preparations dissolved in the same buffer at corresponding pHs were incubated at 25°C for 18 hours. After the incubation, residual lipase activities were measured.

Kinetic Constants
In order to determine the effect of *p*-nitrophenyl propionate (*p*-NPP) substrate concentrations on the reaction rates for soluble and the aggregated lipases, the concentrations of 0.512-25 mM range were used. The apparent kinetic constants were determined from the data linearized with Lineweaver-Burk plots for approximation of the Michaelis constant (*K*m) and *V*max.

Analytical Methods

Enzyme Assay
A lipase assay based on the hydrolytic activity of the enzyme from p-nitrophenyl propionate (p-NPP) substrate was used. The absorbance of *p*-nitrophenol (*p*-NP) product concentration at 404 nm wavelength was measured spectrophotometrically (Shimadzu UV-1700, Japan). The reaction mixture contained 3 mL of 50 mM phosphate buffer (pH 7.0) and 0.1 mL *p*-NPP solution in acetonitrile (4.1 mM). A 30 μL volume of free or immobilized enzyme preparations (resuspensions of PEI-lipase aggregates) was added to the substrate solution and incubated for 5 to 10 minutes at 30ºC in a water bath without shaking. The blank composed of the same composition of the reaction mixture but with no enzyme addition and incubated at the same conditions to exclude minor amounts of free or autohydrolzed *p*-NP product from the amounts catalyzed by the enzyme (33). One International Unit (IU) of lipase activity was defined as the amount (mg) of lipase protein (expressed as Bovine Serum Albumin (BSA)) that liberates one μmol *p*-NP from *p*-NPP under the conditions indicated above. Molar absorptivity of *p*-NP product was experimentally determined (ε=8736 L/M.cm) and used to indicate activity as IU (33).

Protein Assay
The Coomassie brilliant blue G-250 dye-binding technique was used to determine the protein amounts of the enzyme solutions (34). Relative concentrations of enzyme solutions were indicated as BSA (Acros Organics, Belgium).

Membrane Dialysis
Regenerated cellulose membranes (Cellu Sep H1, nominal MWCO: 15 kDa) (Membrane Filtration Products, Inc., Texas, USA) were used to dialyze the lipase solutions. Prior to dialysis, lipase solution (0.1 g/mL) in distilled water was centrifuged at 5000 rpm for 5 min. The membranes (holding 0.32 mL/cm) were filled with the centrifuged lipase solution and dialyzed against 1 L phosphate buffer (pH 7, 50 mM). By changing the buffer solution every 12 h, dialysis continued for 36 h at +4 ºC under constant stirring.

RESULTS AND DISCUSSION

Cross-Linked PEI-Lipase Aggregates

When a PEI solution was simply mixed with a lipase solution, an instant development of turbid slurry was observed indicating a favorable interaction between molecules of PEI and the enzyme leading to complex formation and aggregation. The colloidal aggregates in a solution were then cross-linked with the GA for permanent fixation. Upon the centrifugation, the aggregates were collected at the bottom leaving clear supernatant solution. Figure 2 shows the

degrees of precipitates obtained at varying PEI concentrations.

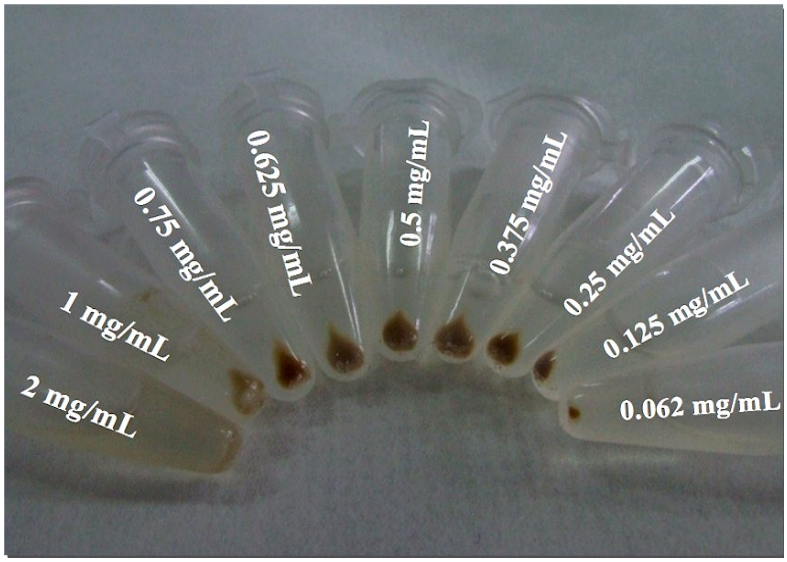
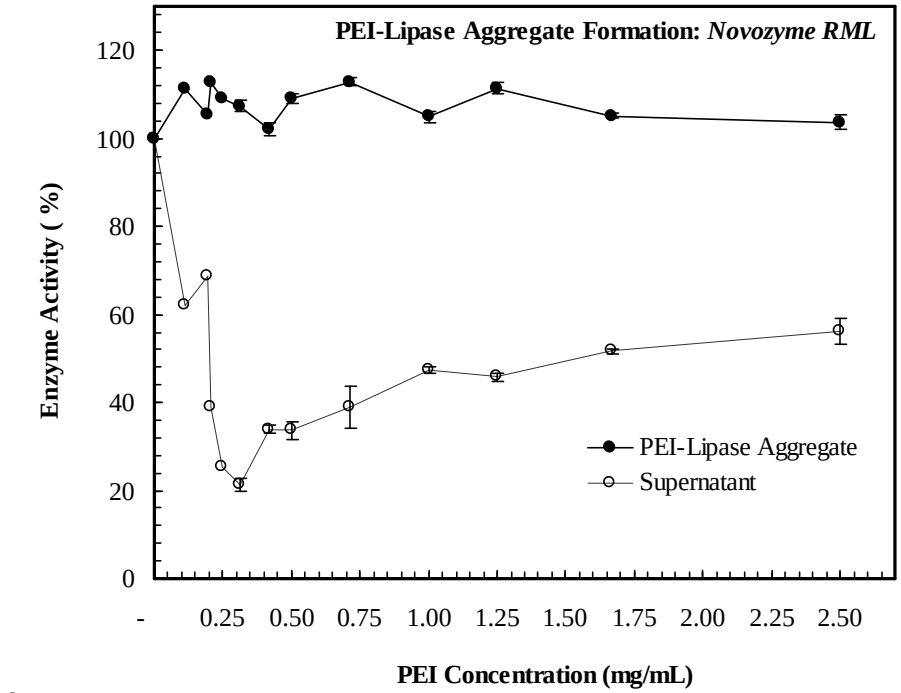


Figure 2: Degrees of PEI-lipase precipitates obtained at varying PEI concentrations (different PEI to lipase mass ratio) after centrifugation at 10,000 rpm for 2 min.

PEI to Enzyme Ratio

PEI-enzyme complex formation with *Rhizomucor miehei* commercial lipase (RML) preparations from Novozyme or Codexis was investigated at varying PEI to lipase ratios. As seen in Figure 3, increasing concentrations of PEI in the lipase solutions resulted in 110 - 115% lipase activity with Novozyme RML compared to the original free lipase solution with no PEI. Increasing concentrations of PEI lead to a gradual decline in the activity of Codexis RML. As the PEI concentration increased up to 0.3 or 2.5 mg/mL for Novozyme or Codexis RML, the residual lipase activities in supernatant sharply decreased to approximately 15-20% of its initial activity. Further increase in the PEI concentration resulted in a gradual increase in supernatant activities with both sources of RML. Thus, there appeared to be an optimum PEI to enzyme ratio (or range) associated with not only a highest PEI-lipase aggregate activity in the solution but also a lowest remaining activity in supernatant. The optimal complexation with Novozyme RML or Codexis was attained at the PEI to lipase mass ratio of 1/85 or 1/16 resulting in immobilization yields of 90 or 85%, respectively, based on the lipase activity. Similar to RMLs, as we have previously reported, an optimal PEI-lipase complex formation with lipase A from *C. antarctica* and *T. lanuginosus* (Lipozyme TL) was obtained at a range of PEI to lipase ratio of 1/20 and 1/50 with similar immobilization yields (31).



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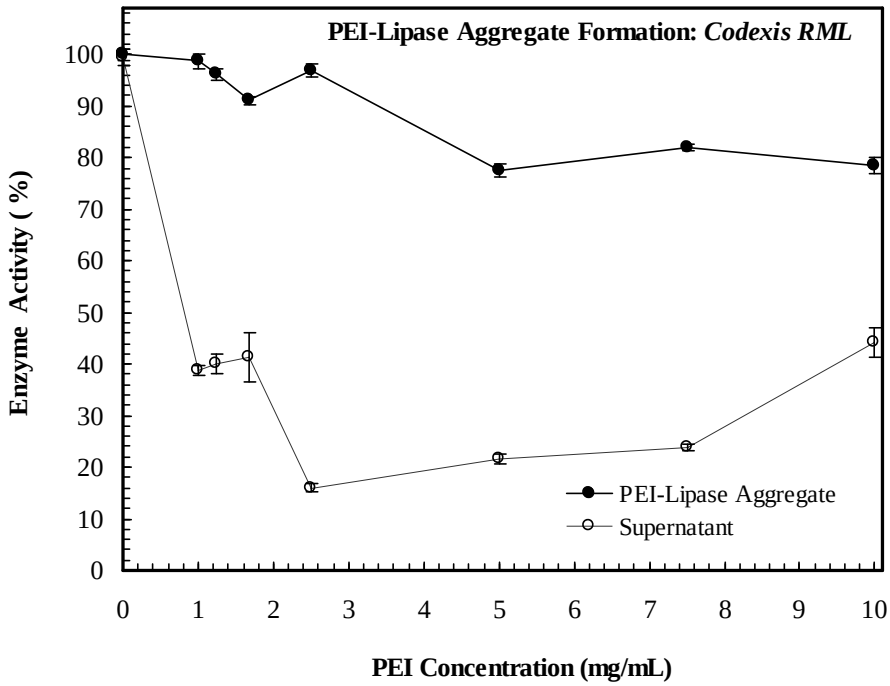

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Figure 3: The effect of PEI concentration on the activity of PEI-lipase complexes in a solution and the remaining activity in supernatant after centrifugation at 10,000 rpm for 2 min by using the *Rhizomucor miehei* commercial lipase preparations from Novozyme and Codexis.

Table 1 compares the levels of protein and associated specific activities of RMLs from Codexis and Novozyme. The Codexis RML in powder form contained nearly 20 times more

protein (expressed as BSA), but only half of the specific activity compared with those of liquid lipase preparation from Novozyme. Apparently, the majority of the protein content of the Codexis RML preparation was proteins without lipase activity. The variation in protein contents of the RMLs could probably be the main reason for the observed difference regarding PEI to lipase mass ratio. Moreover, the gradual decline in the activity of the PEI-lipase aggregates in Codexis RML may be attributed to the precipitation of a large protein content along with active lipase protein. Nevertheless, lipase contained in the aggregate at the optimum complexation ratio functions as good as free enzyme with a coupling yield of nearly 90% of its initial lipase activity, which may imply porous and the partitioning nature of the PEI complexation.

Table 1: *Comparisons* of protein contents and specific activities for *Rhizomucor miehei* lipases expressed as BSA.

RML Source	RML Protein Conc. [(a)]	Specific Lipase Activity (IU/mg) [(b)]	PEI / Lipase (w/w) [(c)]
Codexis (powder)	43.0% (w/w)	145	1 / 16
Novozyme (liquid)	1.8% (w/v)	299	1 / 85

[(a)] Protein concentration in the enzyme source was expressed as BSA (determined by Bradford method).
[(b)] Experimentally determined specific enzyme activity of the enzyme source based their protein content (expressed as BSA).
[(c)] PEI to lipase mass ratio where the optimal complexation were obtained (see Fig 3).

The Effect of pH

The effect of pH was investigated for *Thermomyces lanuginosus* (TL) lipase by adjusting the pH of the PEI solution and, the results are displayed in Figure 4. At pH 7, the activity of the PEI-lipase aggregate in solution increased up to 170% (at PEI/lipase ratio 1/50) or even 200% (at PEI - lipase ratio 1/80) and leaving about 20% lipase activity in supernatant, which means that more activity could be precipitated than initially present. At higher pHs between 8 and 10, the PEI-lipase aggregates showed relative activities of 100-120%.

PEI-protein interaction is usually referred to as electrostatic attraction. Considering a high pH of the PEI solution (pH ~ 11.5) used, PEI bearing little or no charge at a dense conformation (35) apparently favors interaction with highly anionic lipases (*p*I 3.0-5.5) (36,37). Therefore, the probable mechanism for the PEI-enzyme interaction should be dominated by polymer bridging and to lesser extent electrostatic attraction (38). During the previous PEI-lipase complexation experiments, complexation was obtained at natural pH (~ 11.5) set by PEI with no pH adjustment. With the TL lipase, it appeared that more of an electrostatic interaction between pH 7 and 8 might bring about overactivation. Among the lipases studies, TL obviously favored electrostatic interaction toward increased catalytic efficiency.

Dialyzable Impurities

Unlike the other RMLs investigated, with a RML commercial preparation from Bio-Cat, adding PEI to lipase solution failed to produce a turbid mixture or any precipitation after the centrifugation despite variations in the PEI to enzyme ratio. As shown in Figure 5, increasing the PEI concentration was accompanied with a gradual decrease up to 60-70% in the lipase activity of regarding the PEI- lipase mixture or supernatant. When a membrane dialysis (MWCO 15 kDa at 4°C for 36 hours) was performed to remove some of the impurities likely to interfere with enzyme complexation including salts and organic acids, the dialysis had no effect on the protein concentration or lipase activity, but led to a decline in solution pH from 8 to 7 (data was not shown). Also, unlike the effect of PEI on nondialyzed lipase, increasing the PEI concentration did lead to an apparent increase in relative lipase activity of the PEI-lipase mixture up to 120% (Fig 5). However, the interaction of the PEI with dialyzed lipase toward complexation failed to occur again as apparent from either the absence of turbidity or almost no precipitation after centrifugation. Thus, a decrease in the enzyme activity upon PEI-enzyme mixture should not be directly related to sole effect of PEI on enzyme. Similarly, the PEI mixture with lipases from *Candida antarctica* lipase B (Cal B) and *Pseudomonas cepacia* failed to produce precipitatable aggregates (data was not shown), although the PEI-lipase aggregates showed a highly turbid slurry and over 100% activity. The Commercial lipase preparation (Cal B) from

Novozyme was reported to contain a substantial amount of sodium benzoate and potassium sorbate and/or nucleic acids (39) and, these are all inhibitory to the PEI enzyme aggregation (21). Assuming that the commercial RMLs had a similar lipase profile, disengagement between the PEI and the lipase could be attributed to other nonproteinaceous constituents such as salts and other anionic compounds (39) which are found in commercial lipase preparations (36). It was determined that a commercial lipase preparation showed 22% protein but of which only 3% corresponded to lipase (40). There is no question that nonenzymatic proteins and even nonproteinaceous compounds encountered in commercial enzyme powders or solutions interfere with the success of most methods of enzyme immobilization. Similarly in the cross-linked enzyme aggregate formation with lipases, nitrogen containing constituents was found to interfere with the method as well (41). To rule out these factors, a rational strategy to eliminate nonproteinaceous components of a commercial extract can be followed (39) in the case of unsatisfactory precipitation with PEI.

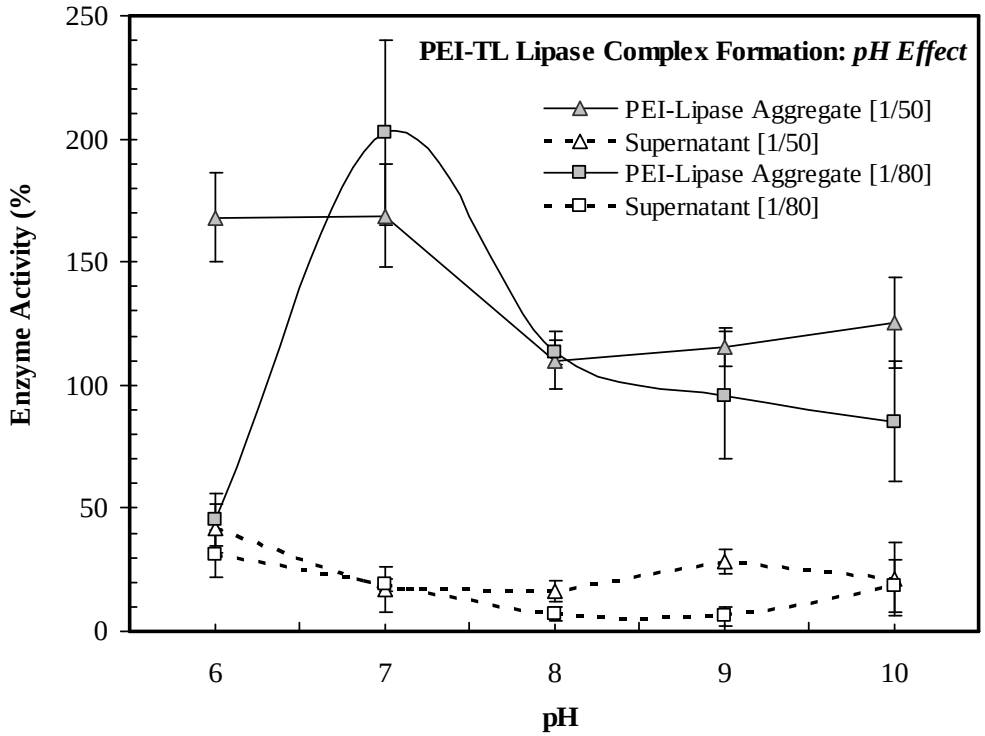

Figure 4: The effect of pH on PEI-enzyme aggregate formation with lipase from *Thermomyces lanuginosus* at or near the optimal PEI to lipase mass ratio of 1/50 and 1/80. Lipase activities of the aggregates in solution and the remaining activities in supernatant after centrifugation at 10,000 rpm for 2 min. HCl was used to adjust the pH. The experiments were performed in triplicate and the standard deviation shown as vertical bars.

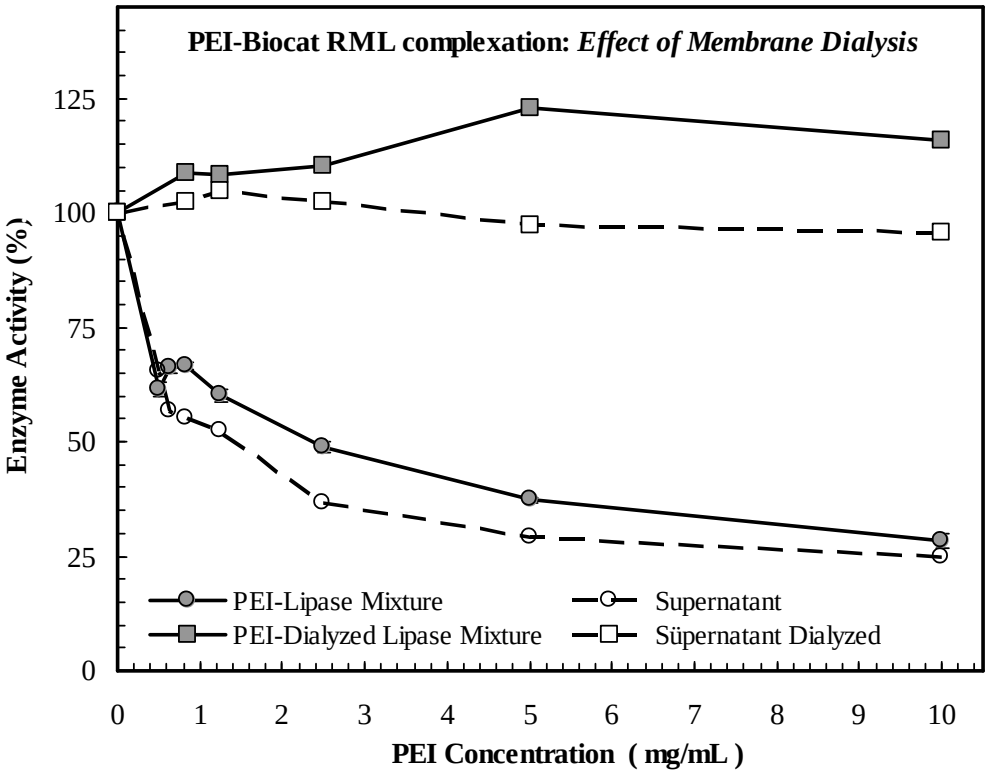

Figure 5: The effect of membrane dialysis (with a 15 kDa nominal MWCO regenerated cellulose membrane at 4°C for 36 h) of the lipase solution (0.1 g/mL in distilled water) (dashed lines) compared with normal lipase (straight lines) preparation on the activity of the PEI-lipase complex and precipitability after centrifugation at 10,000 rpm for 2 min by using *the Rhizomucor miehei* commercial lipase preparations from Bio-Cat.

GA Cross-linking

The aggregates formed by PEI-enzyme interactions are considered as reversible and can be disintegrated to some extent by small anionic species leading to enzyme leach. When the PEI-enzyme aggregates are permanently fixed with a chemical cross-linking, the desired chemical stability against disintegration as well as general physical stability are achieved (42). Since both PEI and enzymes are rich in ammonia, glutaraldehyde (GA) was conveniently employed for permanent fixation of the aggregates. The effect of increasing the GA concentration on the activity of the PEI -lipase aggregate in solution as well as the efficiency of immobilization was tested for all the lipases at previously optimized PEI to lipase ratios. Figure 6 illustrated the GA effects on RMLs. In general, compared to the absence of GA cross-linking shown in previous experiments, GA cross-linking within a certain range of GA concentration did not change the desirable attributes of the PEI-lipase aggregate formation in all of the lipases studied. It is known that the increase in cross-linking commonly assist greater stabilization against denaturing conditions while often accompanying a reduction in the activity of immobilized enzyme (43).

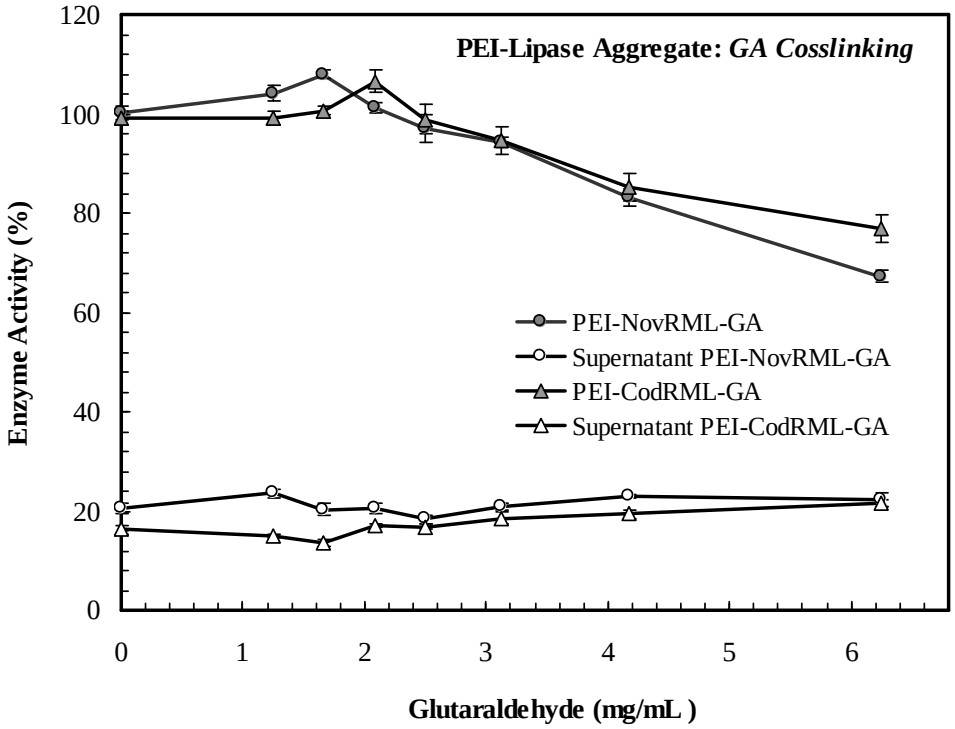

Figure 6: The effect of increasing the glutaraldehyde concentration (for 5 min at 20 ºC) on the activities of the PEI- *Rhizomucor miehei* lipase aggregates from Novozyme or Codexis in a solution at the PEI to lipase mass ratio of 1/85 or 1/16 and on the levels precipitation after centrifugation (at 10,000 rpm for 2 min), respectively.

The duration for GA cross-linking was found to be an insignificant factor in the activity of aggregates nor the efficiency of precipitation beyond 5 to 10 min of incubation (data not shown). Considering the results for the GA cross-linking, about 2mg/mL GA concentration for 5 to 10 min at room temperature appeared optimum and kept at this level for the rest of the study. It is important to note that the GA cross-linking at this degree provided sufficient stability against leaching of enzyme form the aggregates due to the addition of a salt solution or presence of buffer.

Thermal Stability

Thermal stability of the GA cross-linked PEI-lipase aggregates from Novozyme RML lipase were shown in Figure 7. The soluble lipase gradually decreased its activity upon increasing temperatures above 35°C and lost 86% at 65°C while the cross-linked aggregates kept full activity up to 55°C and only lost 18% at 65°C. Thus, compared to free lipase, thermal stability of the aggregates in a solution increased substantially.

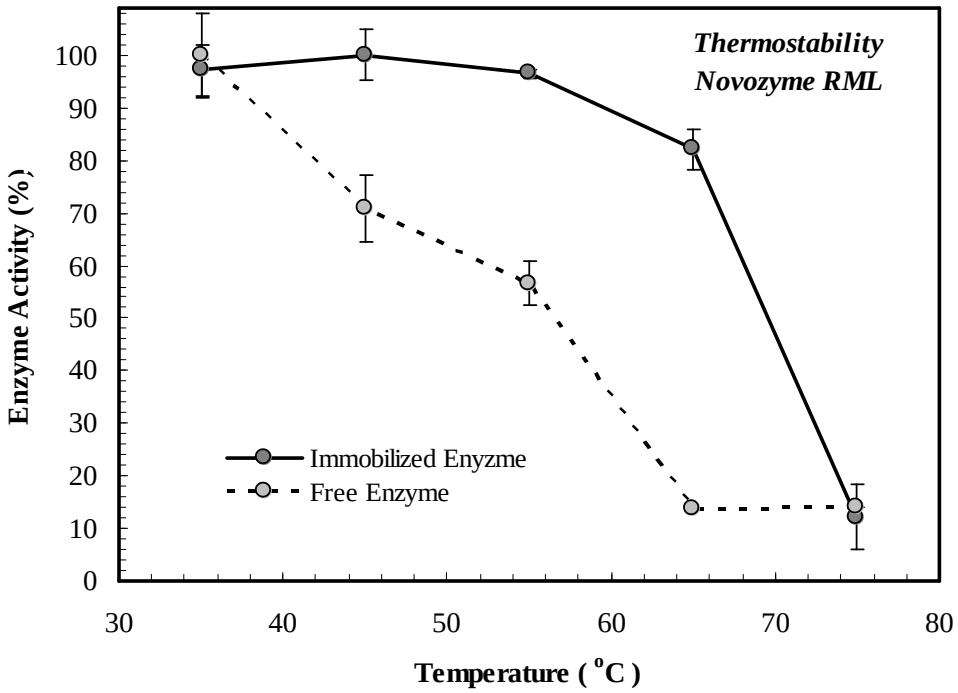

Figure 7: Thermal stabilities soluble and GA cross-linked PEI-lipase aggregates (*Rhizomucor miehei* from Novozyme) suspended in 50 mM phosphate buffer at pH 7 in a constant-temperature water bath between 35-75 °C. Lipase activities were measured after the incubation for two hours, and the experiments were carried out three parallels at each temperature.

In another set of experiments, the storage stability was evaluated (Figure 8). The soluble RM lipase and the cross-linked aggregates resuspended in phosphate buffer were kept at various conditions for nearly a month. The free enzyme in buffer either refrigerated or stored at room temperature lost nearly 80 or 90 % of its initial activity within three weeks while the aggregates retained nearly full activity. The aggregates had an estimated half-life of 220 at 4°C or 250 days 20°C, which was at least 20-times more stable than that of the soluble enzyme (Table 2). It is interesting to note that the aggregates stored at 20°C was slightly more stable than that of 4°C while the opposite was observed with the soluble enzyme. Furthermore, the dried form of the aggregates stored at room temperature retained nearly as much activity as the one stored in a buffer. In conclusion, similar to increase in thermal stability, it is clear that the carrier-free preparation approach greatly improved the stability for storage. It was generally observed that PEI is good at preserving native structure at ambient temperatures due to depressing protein denaturation and oxidation during long-term storage (44–46).

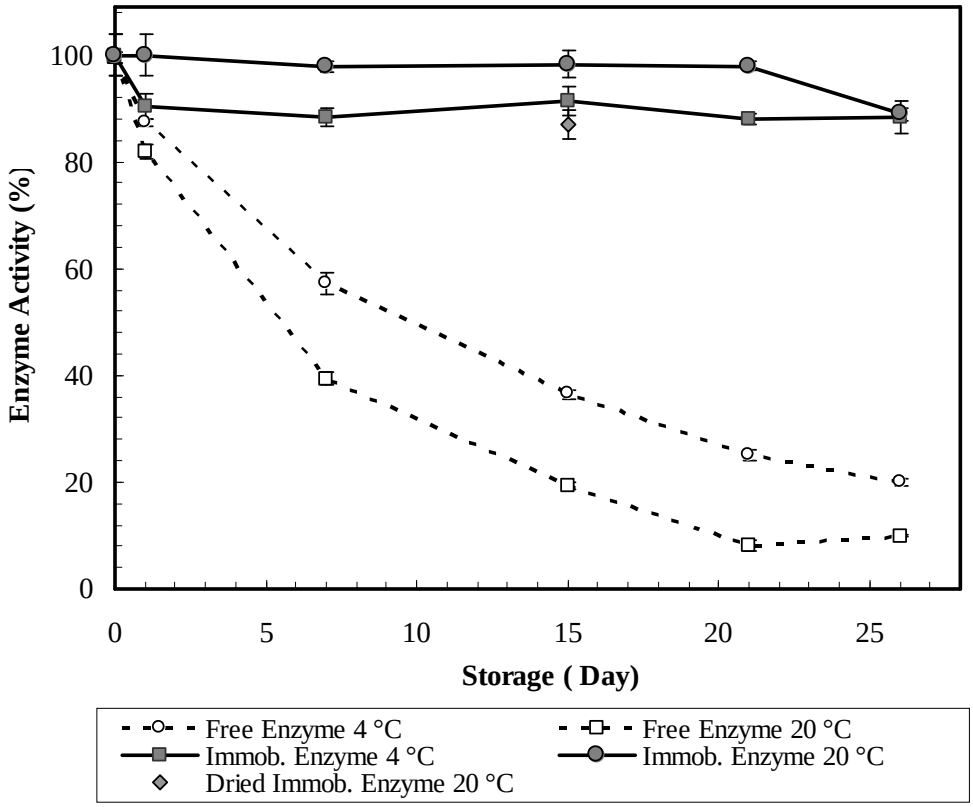

Figure 8: Storage stabilities for free and GA cross-linked PEI-lipase aggregates (*Rhizomucor miehei* from Novozyme) suspended in 0.1 M phosphate buffer (at pH 7) incubated at 4 or 20 °C for extended period of time. The dried form of immobilized lipase was stored at room temperature for only two weeks. Lipase activities were measured from the aliquots taken from the samples during incubation, and the experiments were carried out three parallels at each temperature.

Table 2: Comparison of storage stabilities of free and immobilized lipase from *Rhizomucor miehei* from Novozyme.

Temperature	Free Lipase (RML)		Cross-linked PEI-Lipase Aggregate	
(°C)	k_d (h^{-1})	half life (h)	k_d (h^{-1})	half life (h)
4	0.0633	11	0.0028	248
20	0.0967	7	0.0032	217

pH

The effects of pH on the activities of the free and cross-linked PEI-lipase aggregates were presented in Figure 9. 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. A change in the optimum pH depends on the charge of the support and/or enzyme. PEI-enzyme aggregation involves bridging between the negatively charged enzyme and the PEI. Thus, the optimum pH of enzyme is likely to change. It was reported that the pH optima of lipase (47) adsorbed on methacrylate membranes modified with PEI extended towards an alkaline range.

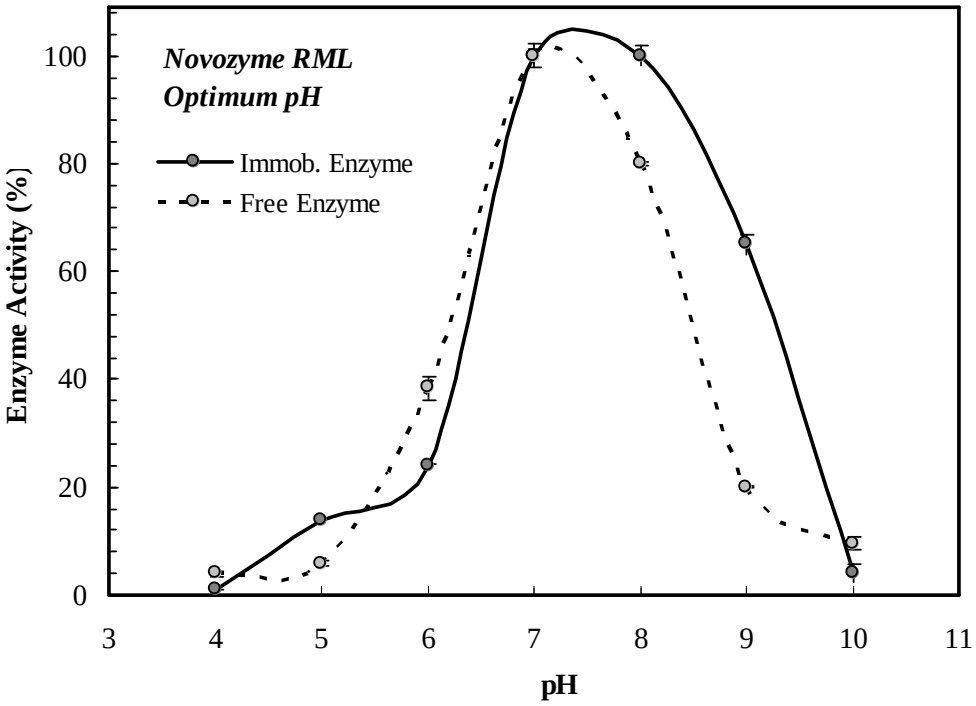

Figure 9: The effect of pH on the activities of free and GA cross-linked PEI-lipase aggregates (*Rhizomucor miehei* from Novozyme) suspended in the corresponding buffers of 0.1 M.

The ability to withstand change in the medium pH is an important indication of enzyme stability especially for the immobilization technique initiated with favorable charge interaction. If the bonds between the enzyme and PEI are not sufficiently strong and/or the GA cross-linking was unable to tie the aggregates, then they may disassociate thereby show little or no resistance against the weakening effect of the medium pH. As presented in Figure 10, free lipase kept full activity at its optimum pH 7 and lost nearly 10% of its initial activity at pH 6 or 8. It seems soluble enzyme was more resistant towards the acidic range where it nearly lost 30% of its activity at pH 5 but lost 50% at pH 9. However, the cross-linked aggregates showed no loss within pH 6 and 9. Also, the aggregates retained complete activity at the pH 9 yet free enzyme lost 50% of its initial activity. Similar to our observations, Cao et al (8) reported that PEI-cross-linked lipase from *Candida rugose* on the surface of magnetic microspheres showed increased alkaline pH and thermal stabilities.

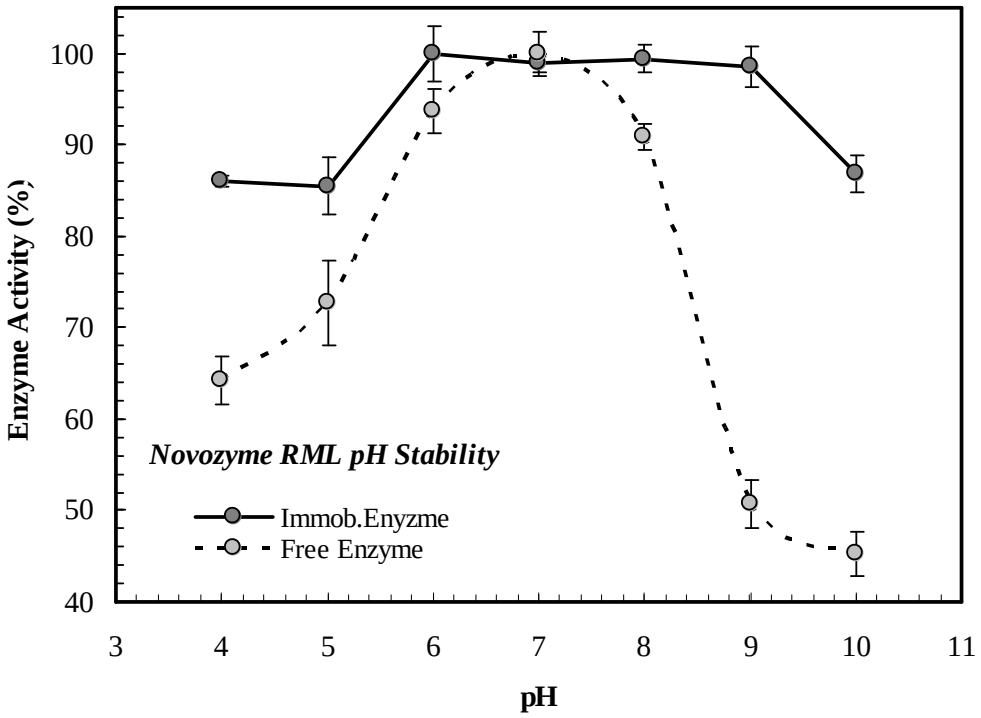

Figure 10: pH stabilities of free and GA cross-linked PEI-lipase aggregates (*Rhizomucor miehei* from Novozyme) suspended corresponding buffers. The enzymes were incubated 0.1 M acetate and phosphate buffers at 25 °C for 18 h.

It is important to note that not only PEI interaction but also GA cross-linking of PEI-enzyme aggregate greatly affected overall stability of the immobilized aggregates against pH and heat/duration, indicating minimal distortion of protein structure while retaining catalytic activity (42).

***K*m and *V*max**

In the formation of the macroscopic aggregates between the PEI and the enzyme, a reduction in the enzyme activity due to external and internal diffusion limitation (of substrate/products) imposed by the complexation may be expected. For enzyme immobilization procedures involving intensification of catalyst, it is very much common to observe substantial reduction in $V\text{max}_{app}$ while increase in $K\text{m}_{app}$ due to chemical modification of enzyme as well as steric hindrance (48). Our preparation of PEI-Lipase aggregates containing over 98% enzyme protein can be reversibly precipitated or dispersed in solution yet they display the reaction rates as good as free enzyme with improved stabilities.

Figure 11 presents comparison of the reaction rates for soluble and the aggregated RM lipases on *p*-NPP substrate concentrations within the range of 0.512-25 mM. The apparent kinetic constants were determined from the data linearized with Lineweaver-Burk plots for approximation of the Michaelis constant (*K*m) and *V*max (48), which are presented in Table 3.

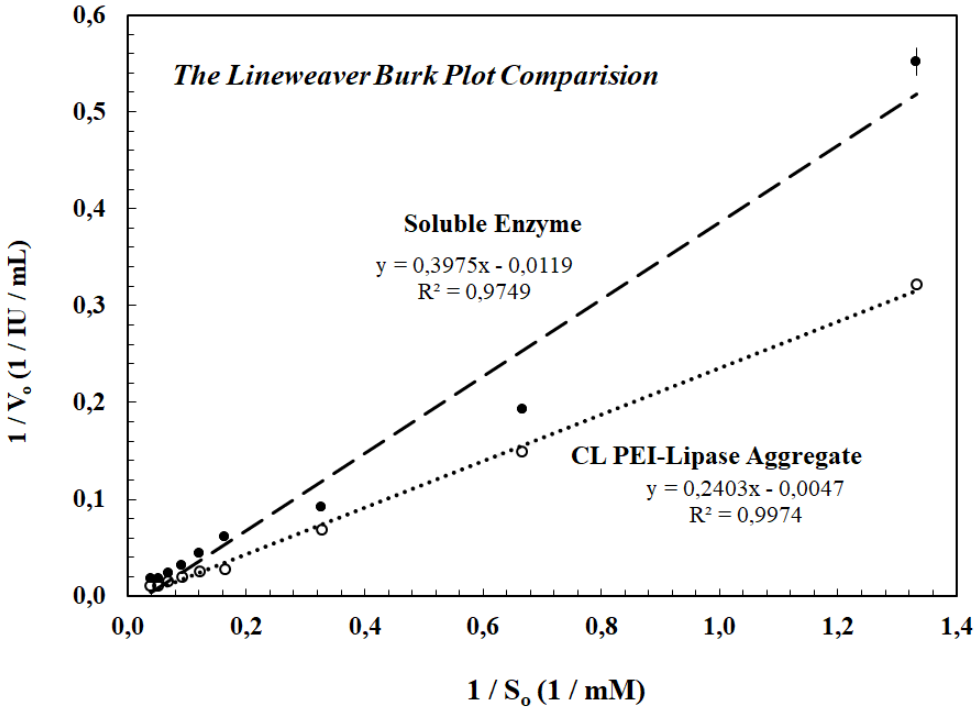

Figure 11: The effect of *p*-nitrophenyl propionate (*p*-NPP) substrate concentrations on the reaction rates for soluble and GA cross-linked PEI-lipase aggregates (*Rhizomucor miehei* from Novozyme) suspended corresponding buffers. Both reactions were carried out in a water bath without shaking. The apparent kinetic constants were determined from the data linearized with Lineweaver-Burk plots for approximation of the Michaelis constant (*K*m) and *V*max.

Table 3: Apparent kinetic constants for free and immobilized *Rhizomucor miehei* lipase from Novozyme.

Parameters (*)	Soluble Lipase RML	Cross-linked PEI-Lipase Aggregate
V_{max} (IU/mL)	84	172
K_m (mM)	33	42

(*) Based on hydrolysis of *p*-nitrophenyl propionate substrate in the range 0.512 - 25 mM in a water bath without shaking.

The approximation derived from Lineweaver-Burk linearized plots in Fig 11.

Compared with the soluble enzyme, the $V\text{max}_{app}$ for PEI-lipase aggregates increased two-fold while the $K\text{m}_{app}$ increased 25%. Representing the highest possible rate when the enzyme is saturated with a substrate, $V\text{max}_{app}$ reveals the intrinsic characteristics of the immobilized enzyme. It was found that the activation step of the lipases (RML or TLL) requires large concerted motions involving the displacement of the helical lid which begins with electrostatic interaction between the lid surface and the adjacent loop resulting in a rollover and the exposure of hydrophobic residues in the active site (49–52). A slightly hydrophobic backbone of the PEI (44) may have stabilized active conformation. Parallel to our observation, nearly two-fold (180%) increase in relative lipase activity in oppositely charged two polyelectrolyte complex was reported (53). In contrast, compared with free enzyme, 25% increase in *K*m and 40% decrease in *V*max was also reported with PEI-cross-linked lipase from *Candida rugose* on the surface of magnetic microspheres (8). In fact, the aqueous phase which perfectly solubilizes the lipase but energetically puts the enzyme in a difficult state in terms of energy need for conformational change required for lid opening prior to the catalysis (51,52). An increase in $K\text{m}_{app}$ simply means that it takes a little more substrate to saturate the enzyme, which may usually suggest an involvement of diffusion limitations but it may also reflect a weaker bond with the substrate

thus a reflection of the partially open lid of the active site. Thus, better performance of PEI-lipase complex under the unfavorable conditions for reaching the substrate may suggest that the association of lipase with PEI may have produced a favorable interface assisting for conformational change for lipase activation (dislocation of helical lid) through electrostatic interactions resulting in higher rates of catalysis. It is important to note that our assertions about the reaction rates of the PEI-lipase aggregates as well as the values for the kinetic constants were based on *p*-nitrophenyl propionate substrate, which is soluble in water. The characteristics and performance of the immobilized enzyme may vary in various applications with other substrates or in two-phase systems. Nevertheless the present carrier-free lipase aggregates with fully dispersible micron sized particles in aqueous solution as well as organic solvents showing great potential in variety of biocatalytic applications in microemulsion, suspension, dispersion type systems (7,22).

In conclusion, the study presents the preparation and characterization of a carrier-free PEI-enzyme aggregate with several commercially important lipases. The approach relies on PEI as a single aggregating agent. Considering PEI to enzyme mass ratio of 1/20-1/40 for optimal complexation, the PEI-lipase aggregates was mainly composed of enzyme mass with only a few percent of PEI. The cross-linked aggregates at low GA sufficiently provided stability without affecting the aggregate activity. As a simple and inexpensive carrier-free immobilized enzyme, the approach brings about not only enhancing stability and ease of separation but also concentrating the valuable catalyst in volumes or on surfaces without much diffusion resistance.

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