Improved Catalytic Performance of Carrier-Free Immobilized Lipase by Advanced Cross-Linked Enzyme Aggregates Technology

Xia Jiaojiao

Jiangsu University of Science and Technology: Jiangsu University

Yan Yan

Jiangsu University

Bin Zou (✉ binzou2009@ujs.edu.cn)

Jiangsu University <https://orcid.org/0000-0003-3816-8776>

Adesanya Idowu Onyinye

Jiangsu University

Research Article

Keywords: lipase, carrier-free, immobilization, ionic liquid, stability

Posted Date: May 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-505612/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Bioprocess and Biosystems Engineering on October 5th, 2021. See the published version at <https://doi.org/10.1007/s00449-021-02648-x>.

Abstract

The cross-linked enzyme aggregates (CLEAs) are one of the technologies that quickly immobilize the enzyme without a carrier. This carrier-free immobilization method has the advantages of simple operation, high reusability and low cost. In this study, ionic liquid with amino group (1-aminopropyl-3-methylimidazole bromide [IL]) was used as the novel functional surface molecule to modify industrialized lipase (*Candida rugosa* lipase, CRL). The enzymatic properties of the prepared CRL-FIL-CLEAs were investigated. The activity of CRL-FIL-CLEAs (5.51 U/mg protein) was 1.9 times higher than that of CRL-CLEAs without surface modification (2.86 U/mg protein). After incubation at 60°C for 50 min, CRL-FIL-CLEAs still maintained 61% of its initial activity, while the value for CRL-CLEAs was only 22%. After repeated use for five times, compared with the 22% residual activity of CRL-CLEAs, the value of CRL-FIL-CLEAs was 51%. Further kinetic analysis indicated that the K_m values for CRL-FIL-CLEAs and CRL-CLEAs were 4.80 mM and 8.06 mM, respectively, which was inferred that the affinity to substrate was increased after modification. Based on the above results, it was indicated that this method provided a new idea for the effective synthesis of immobilized enzyme.

Highlights

- Novel immobilized lipase was prepared with carrier-free immobilization method.
- The aggregates of ionic liquid and lipase proved to be an efficient catalyst.
- The activity and stability of carrier-free immobilized lipase were improved.
- The activity of the novel aggregates reached 1.9 times of conventional lipase.

1. Introduction

Lipase (*EC 3.1.1.3*) is a very unique biocatalyst with interface activation mechanism^[1]. Its active center at the hydrophobic end is a catalytic triad composed of serine, histidine and aspartic acid. Usually, the active center is covered by a polypeptide chain lid. When the lipase is at hydrophobic interface, the lid opens and the active center exhibits catalytic activities^[2]. Among them, *Candida rugosa* lipase (CRL) is widely applied in bioengineering industries, such as the production of fatty acids and the synthesis of various esters. However, the low stability limited the application of free CRL in catalytic reactions. Immobilization technique is an effective way to solve these problems. After immobilization, the enzymatic properties regarding to the storage stability, reusability and resistance to organic solvents are significantly improved^[3]. Unfortunately, common immobilization methods usually require the assistance of external carriers. Inert carriers often hinder the contact between the lipase and the substrate, thereby affecting the reaction rate and reducing production capacity. In addition, additional immobilization carrier also increased the cost of immobilized lipase.

In recent years, carrier-free immobilization technology has been rapidly developed. Lipase can be self-immobilized through aggregation and cross-linking to form water-insoluble aggregates under mild

conditions^[4]. Carrier-free immobilized lipase do not require expensive carriers, which greatly reduces the cost. The lipase molecules are connected by cross-linking agent, the reusability could be improved. At the same time, the particles of carrier-free immobilized lipase are generally smaller, and the dispersion in the solvent is more uniform. Carrier-free immobilization technologies mainly include cross-linked enzyme crystals (CLECs) technology^[5] and cross-linked enzyme aggregates (CLEAs) technology^[6]. Although CLECs have excellent operational stability, the high purity requirements of crystallase lead to enhanced costs^[7]. CLEAs do not require high-purity lipases, the preliminary purification could be integrated with immobilization process simultaneously. Therefore, CLEAs have more broad application prospects.

Many different enzymes such as esterase^[8], lipase^[9], cellulase^[10] and xylanase^[11] have been immobilized by CLEAs technology and used in various biocatalytic applications. However, when there are fewer amino groups on the surface of the lipase protein, the cross-linking efficiency may decrease. At the same time, prolonged exposure to cross-linking agent will damage the lipase activity. By adding more functional molecules with amino groups (for example: bovine serum albumin) in lipase cross-linking process, the loss of lipase activity can be reduced. Simultaneously, using functional molecules with amino groups to modify the surface of the lipase could affect the opening degree of the "lid" and speed up the substrate to enter the active center. Razib et al. studied the effect of adding bovine serum albumin (BSA) in CLEAs process^[12]. The results showed that the recovery activity of CLEA-elastase-SB after adding BSA was 80%, while the recovery activity of CLEA-elastase was only 60%. The CLEA immobilization of elastase enhanced the stability of the enzyme at high temperature and at a broader pH.

In this work, the industrialized lipase (*Candida rugosa* lipase, CRL) was selected as the research object. Based on excellent biocompatibility of ionic liquid solvent to lipase^[13, 14], a novel ionic liquid with amino groups (1-aminoethyl-3-methylimidazole bromide) was chosen as a modifier. Surface chemical modification of the lipase was carried out simultaneously with CLEAs preparation process (Fig. 1). Compared to CRL-CLEAs without modify agent, the effects of reported modifier BSA and new ionic liquids on the performance of CRL-CLEAs were studied. The differences in thermal stability, pH stability, organic solvent stability, and storage stability of CRL-CLEAs, CRL-BSA-CLEAs and CRL-FIL-CLEAs are discussed. FT-IR and SEM were used to characterize above lipase to explore the mechanism of improved lipase performance. This study is expected to enhance the activity and stability of CRL low-costly.

2. Experiment

2.1 materials

P-nitrophenyl palmitate (PNPP) was purchased from Aladdin (Shanghai, China). *Candida rugosa* lipase (CRL, type VII, L-1754) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (USA) and stored at 4 °C. 1-aminoethyl-3-methylimidazole bromide were purchased from LanZhou Greenchem ILs, Ammonium sulfate, Anhydrous dimethyl sulfoxide (DMSO), isopropanol (HPLC grade), ammonium

sulfate, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from SCRC (China). All other reagents used in the experiment were of analytical grade.

2.2 Preparation of immobilized CRL

2.2.1 Preparation of CRL-CLEAs

Ammonium sulfate (0.56 g) and 1 mL lipase solution (100 mg/mL) were mixed in a 2 mL centrifuge tube and allowed to stand for 30 mins until the lipase protein precipitated. 100 µl of glutaraldehyde (5%, v/v) was added to the centrifuge tube, and cross-linked in a shaker for 1 h at room temperature. The centrifuge tube was centrifuged at 10,000 rpm for 5 mins. The supernatant was removed, and washed with phosphate buffer solution (PBS, pH 7.5). The precipitate was retained for activity determination of lipase.

2.2.2 Preparation of CRL-BSA-CLEAs

1 mL of lipase solution (100 mg/mL) was thoroughly mixed with 0 mg, 5 mg, 20 mg and 50 mg BSA respectively in a 2 mL centrifuge tube. Ammonium sulfate (0.56g) was added to the centrifuge tube and mixed solution, allowed to stand for 30 mins until the lipase protein precipitated. The other steps are the same as Sect. 2.2.1.

2.2.3 Preparation of CRL-FIL-CLEAs

1 mL of enzyme solution (100 mg/mL) was thoroughly mixed with 100 mg, 200 mg, 300 mg, 400 mg, 500 mg 1-aminoethyl-3-methylimidazole bromide respectively in a 2 mL centrifuge tube. The other steps are the same as Sect. 2.2.2.

2.3 Activities assay of immobilized lipase

In a water bath shaker at 40°C, 0.03g PNPP was dissolved in 5 ml isopropanol solution. The obtained solution was stored at 4°C. PNPP isopropanol solution (16.5 mmol/L) and PBS (pH 7.5) were mixed into a milky white substrate solution at a ratio of 1: 9 (v/v). 0.02 g sample and 0.5 mL p-NPP substrate solution were added to a 2 mL centrifuge tube. The mixture was reacted at 35°C and 150 rpm for 10 mins, and then placed in a centrifuge at 12,000 rpm for 5 mins. 250 µL of the diluted supernatant was pipetted onto the microplate. The OD value was measured at 405 nm by a microplate reader (type: Tecan Infinite 200 Pro). The enzyme activity was defined as the protein amount of lipase required to catalyze the hydrolysis of PNPP to 1 µmol of PNP per minute at 35°C and pH 7.5.

$$\text{Enzymatic activity} = (A_1 - A_0 + C_0) / K (\mu\text{mol/ml}) \times V_1 (\text{mL}) \times n / [t (\text{min}) \times M (\text{g})]$$

Where A_1 and A_0 represented the OD value of the lipase sample and control solution, respectively. K and C_0 were the slope and intercept of the PNP standard curve, respectively. V_1 was the volume of the reaction solution (mL). n was the dilution factor. t was the reaction time (min). M was the mass of immobilized lipase in the reaction medium (g).

2.4 Effect of modifier amount and reaction temperature on lipase activity

At 35°C, the enzyme activity of free CRL, CRL-CLEAs, and CRL-BSA-CLEAs and CRL-FIL-CLEAs with different modifiers content (BSA and FIL) was determined. The activity assay method was the same as Sect. 2.3.

The enzymatic activities of free CRL, CRL-CLEAs, optimized CRL-BSA-CLEAs and optimized CRL-FIL-CLEAs were measured at 30°C, 35°C, 40°C, 45°C and 50°C, respectively. The activity assay method was the same as Sect. 2.3.

2.5 Michaelis–Menten kinetic parameters

The Michaelis constant (K_m) and maximum reaction rate (V_{max}) of free CRL, CRL-CLEAs and CRL-FIL-CLEAs were measured using PNPP with a concentration of 1 to 5 mmol/L. The activity assay method was the same as Sect. 2.3. The reaction time was 3 mins.

2.6 Stability assay of immobilized lipase

2.6.1 Thermal stability

Free CRL, CRL-CLEAs, CRL-BSA-CLEAs and CRL-FIL-CLEAs were treated in a 60 °C water bath to test the thermal stability of the immobilized lipase. The sample was taken out every 10 mins to detect the lipase activity. The activity assay method was the same as Sect. 2.3. The initial lipase activity was defined as 100% relative activity.

2.6.2 PH stability

Five equal samples of free CRL, CRL-CLEAs, CRL-BSA-CLEAs and CRL-FIL-CLEAs were placed in buffers with pH 6.0, 6.5, 7, 7.5 and 8.0, respectively. The samples were soaked in buffer solutions of different pH for 2h, and then centrifuged at 12000rpm for 5 mins. The activity assay method was the same as Sect. 2.3. The initial lipase activity was defined as 100% relative activity.

2.6.3 Storage stability

Free CRL, CRL-CLEAs, CRL-BSA-CLEAs and CRL-FIL-CLEAs were stored at 25°C. The samples taken every day were measured for lipase activity. The activity assay method was the same as Sect. 2.3. The initial lipase activity was defined as 100% relative activity.

2.6.4 Reusability

After each cycle, the immobilized lipase was centrifuged and washed 3 times with PBS (pH 7.5). The new substrate was added with the separated lipase to measure the retained lipase activity. The activity assay method was the same as Sect. 2.3. The initial lipase activity was defined as 100%.

2.6.5 Organic tolerance stability

Free CRL, CRL-CLEAs, CRL-BSA-CLEAs and CRL-FIL-CLEAs were soaked in isopropanol, acetonitrile, dimethyl sulfoxide and ethyl acetate for 1 h, respectively. The mixed solution was centrifuged at 10,000 rpm for 5 mins. The precipitate was retained and washed with buffer to determine the lipase activity. The activity assay method was the same as Sect. 2.3. The initial lipase activity without organic solvent treatment was defined as 100%.

3. Results And Discussion

3.1 Effect of the modifier amount and reaction temperature on lipase activity

With the increase of BSA and ionic liquid content, the activity of CLEAs increased first and then decreased (Fig. 2(a)). Ammonium sulfate was used as a precipitating agent and the relative activity of CRL-BSA-CLEAs reached the highest value when the pH was 7.5 and the BSA addition was 20 mg/mL at 35°C. BSA is a lysine-rich protein that acts as a spacer and protein supplement in CLEAs. The narrow CLEAs pores can affect substrate diffusion, and the lysine-rich surface of BSA prevents excessive cross-linking of lipase molecules. Low addition of BSA led to the formation of more CLEAs and increased the activity of immobilized lipase. Excessive addition of BSA protein reacted with glutaraldehyde, resulting in a decrease in the amount of cross-linking of lipase. At the same time, excessive cross-linking can cause blockage of the active site. Therefore, excessive addition of BSA resulted in a decrease in enzyme activity. Pattarapon et.al controlled the amount of BSA added to observe the activity of *Aspergillus oryzae* lipase aggregates. They came to a similar conclusion^[15]. When the amount of BSA added was 6 mg, its activity was increased to 80% compared to the immobilized lipase without BSA. When the added amount was increased to 14 mg, the recovery activity of the immobilized lipase dropped to 32%. It can be seen that the appropriate addition of BSA is beneficial to the improvement of the immobilized lipase activity. In this study, the optimal addition amount of BSA was 20 mg/mL.

Under the same conditions, the relative activity of CRL-FIL-CLEAs reached the maximum when the ionic liquid was added at 200 mg/mL (Fig. 2(a)). As the ionic liquid content increased, the activity of CRL-FIL-CLEAs gradually decreased. There are interactions between lipase molecules and functional groups of ionic liquids such as electrostatic, hydrogen bonding, hydrophobicity, and van der Waals forces. The conformation of the lipase may change to a certain extent under these interactions. The exposure of the active site facilitates the substrate to enter and react with the lipase. On the other hand, the immobilized lipase interacted with the imidazole group of IL through π - π stacking, so as to better maintain its structural integrity. Therefore, the activity of the lipase was improved after being modified by the ionic liquid. After exceeding the optimal dosage, the CRL-FIL-CLEAs activity decreased. It may be that the excess ionic liquid interacts with the catalytic triad of the lipase. The interaction causes the closed catalytic conformation of lipase and reduces the accessibility of the substrate and the active center^[16]. Sou et al. used ionic liquid-modified nanoparticles as a Lipoprotein Lipase (PPL) immobilization carrier. Compared with the unmodified immobilized lipase and free lipase, the activity of the modified

immobilized lipase increased by 1.3 times and 2.8 times, respectively ^[17]. This indicated that the ionic liquid was also helpful to improve the properties of the enzyme in the immobilization with carrier. In this experiment, the optimal addition amount of ionic liquid was 200 mg/mL.

Figure 2(b) shows that the activities of both free lipase and immobilized lipase enhanced and then decreased with the increased temperature. Under the condition of pH 7.5, the activity of free lipase reached the highest (11.65 U/mg protein) at 35°C. CRL-CLEAs (2.86 U/mg protein), CRL-BSA-CLEAs (3.98 U/mg protein), CRL-IL -CLEAs (5.51 U/mg protein) showed maximum activity at 40°C, 40°C and 45°C, respectively. It can be seen that the thermal stability of lipase after immobilization was improved compared with that of free enzyme. And CLEAs modified with ionic liquids had higher temperature tolerance. It may be due to the higher conformational integrity of the immobilized lipase modified by ionic liquid. The covalent bond formed between glutaraldehyde and protein reduced conformational flexibility and protected the enzyme from deformation caused by heat exchange. Therefore, the optimum temperature of the enzyme molecule was changed ^[18]. Persson et. al. proposed that there is an electrostatic interaction between the ionic liquid and the enzyme molecule, which keeps the enzyme molecule rigid ^[19]. This situation was conducive to the improvement of the stability of the enzyme structure. Sou et. Al. used imidazole-based ionic liquid modified chitosan, combined with magnetic nanoparticles as a carrier for immobilizing PPL ^[20]. The optimal temperature of immobilized enzyme was 5°C higher than that of free enzyme, which was consistent with our experiment results of immobilized enzyme without carrier. This shows that ionic liquids were obviously helpful for improving the temperature tolerance of enzymes.

3.2 Kinetic parameters of free lipase and immobilized lipase

The effect of adding ionic liquid on the kinetic parameters of cross-linked enzyme aggregates was investigated. By Lineweaver-Burk diagram (Fig. 3), the kinetic parameters of CRL-CLEAs and CRL-FIL-CLEAs were determined. V_{max} and K_m represent the maximum reaction rate of the enzyme and the affinity to the substrate, respectively. The K_m value of CRL-FIL-CLEAs (4.80 mM) was smaller than that of CRL-CLEAs (8.06 mM), while the V_{max} of CRL-CLEAs and CRL-FIL-CLEAs were 555.56 U/mg protein and 500 U/mg protein, respectively.

Table.1 shows the CRL-CLEAs activity without any additives was 2.86 U/mg protein, which was lower than the free lipase activity (11.65 U/mg protein). Cross-linking resulted in fewer active sites attached to the substrate. After adding BSA, the activity increased to 3.98 U/mg protein, which may be due to the addition of more amino groups on the surface of the enzyme. A previous study showed a finding similar to this result, that the prepared CLEAs recovered 80% of its activity (compared with free enzyme) after adding BSA ^[12]. After adding ionic liquid to CRL-CLEAs (activity 5.51 U/mg protein), the activity was 1.9 times that of the immobilized enzyme without adding modifier. It can be seen that using ionic liquids as modifiers can effectively improve the activity of cross-linked enzyme aggregates. The maximum reaction rate of the enzyme decreased slightly, but the affinity for the substrate was improved after adding the

ionic liquid. It was explained that adding ionic liquids for modification had a positive effect on improving the activity of immobilized enzymes. The addition of ionic liquid may stabilize the open state of the lipase lid structure. This state was conducive to the substrate to enter the catalytic center of the enzyme to achieve an increase of activity ^[21]. Sou et al. used ionic liquid-modified magnetic nanoparticles as a carrier for immobilization of lipase. The immobilized lipase showed high activity, which was 1.6 times higher than the immobilized lipase without adding ionic liquid ^[22]. Water contact angle analysis showed that the introduction of ionic liquid increased the hydrophobicity of the carrier, which caused the lipase to open the lid and its active site became more accessible. Ionic liquid modification performed well in both carrier and non-carrier immobilized enzymes, indicating that the introduction of ionic liquid had a positive effect on the activity of immobilized enzymes.

3.3 Stability assay of immobilized lipase

Figure 4(a) shows the activity of free lipase and immobilized lipase in phosphate buffer at 60°C for different time. At pH 7.5, free enzymes, CRL-CLEAs, CRL-BSA-CLEAs, and CRL-FIL-CLEAs were uniformly maintained at 60°C. The samples were taken out every 10 mins to measure the enzyme activity and calculate the residual rate. Temperature had a great influence on free enzyme activity. Temperature increased and the free enzyme activity dropped sharply. After 50 mins, the residual activity was only 17% of the activity under the optimal conditions. The activity of CRL-FIL-CLEAs was much less affected by temperature. After 50 mins, the immobilized enzyme still maintained 61% of the relative activity. CRL-CLEAs and CRL-BSA-CLEAs kept 22% and 42% relative activity under their optimal conditions, respectively. It indicated that ionic liquids provided better heat resistance for cross-linked enzyme aggregates. This may be due to the fact that the ionic liquid may dissociate the hydrogen bonds that maintain the integrity of the α -helix and β -sheet structure, resulting in the unfolding of all or part of the protein ^[23]. Therefore, the immobilized enzyme modified by ionic liquid had better temperature tolerance ^[24]. Lau et al. bathed the lipase in ionic liquid [BMIM] [PF₆] at 80°C for 20 hours and found that the activity of the lipase increased by 20% ^[25]. Jiang et al. pointed out that ionic liquids can maintain the tight conformation of the enzyme and reduce the destruction rate of its secondary structure at high temperatures ^[26]. The discovery was consistent with our experimental results, so ionic liquid can improve the thermal stability of the immobilized enzyme.

The pH of the reaction medium plays a crucial role in the stability and enzyme activity of the enzyme. Figure 4(b) shows the effects of different pH environments on immobilized lipase. Free lipase, CRL-CLEAs, CRL-BSA-CLEAs, CRL-FIL-CLEAs were soaked in phosphate buffer at different pH for 2 h. Free lipase (100%) showed the greatest relative activity at pH 7.0, CRL-CLEAs (95%), CRL-BSA-CLEAs (92%) and CRL-FIL-CLEAs (98%) at pH 7.5. This showed that after the immobilization treatment, the pH stability of CRL was improved. After the free enzyme with positively charged cross-linked with glutaraldehyde, all available amino groups on the surface of the enzyme were coupled to glutaraldehyde. The result was that CRL carried a negative charge, and the optimal pH eventually shifted toward the alkaline direction ^[27]. This change in the optimal pH of CRL-FIL-CLEAs may be attributed to the introduction of ionic liquids, which will further affect the surface charge of immobilized lipases ^[28]. Zhang et al. also reported that the

pH stability of immobilized enzymes modified by ionic liquid aggregates was improved [29]. Ionic liquids can reduce the negative impact of pH on the conformation of lipase to a certain extent, thereby improving the stability of immobilized enzymes on pH.

The storage stability of the immobilized enzyme is the key to the industrial application of the enzyme. As shown in Fig. 4(c), the storage stability of free CRL, CRL-CLEAs, CRL-BSA-CLEAs, and CRL-FIL-CLEAs was investigated. The enzyme preparation was stored at 25 °C, and the enzyme activity was measured every 24 h, and the residual rate of the enzyme activity was calculated. It can be seen that free CRL, CRL-CLEAs, CRL-BSA-CLEAs, and CRL-FIL-CLEAs all decreased over time. However, CRL-BSA-CLEAs and CRL-FIL-CLEAs had better storage stability than CRL-CLEAs. On the fourth day, CRL-FIL-CLEAs retained 37% of the activity, CRL-BSA-CLEAs and CRL-CLEAs only retained 33% and 32% of the activity, respectively. It showed that using ionic liquid to modify the immobilization of lipase can successfully improve storage stability. The decrease in enzyme activity was mainly due to the loss of water molecules, which was necessary to maintain the enzyme conformational stability and enzyme catalytic activity. The presence of ionic liquid increased the rigidity of the enzyme and slowed the loss of bound water. The storage stability of the immobilized enzyme had been maintained [30]. HajKacem et al. modified the immobilized enzyme after mixing different ionic liquids, and the stability of the immobilized enzyme was greatly improved [31]. Consistent with our experimental results, it was verified that ionic liquids can enhance the storage stability of enzymes.

The reusability of immobilized enzymes is a key factor in the industrial application of enzymes. Figure 4(d) shows the residual activity of the immobilized enzyme after five cycles. Compared with CRL-CLEAs and CRL-BSA-CLEAs, cross-linked enzyme aggregates modified by ionic liquid showed higher stability and reusability. As the number of repetitions increased, the enzyme activity gradually decreased. After repeated use for five times, the residual activity of CRL-FIL-CLEAs was 51%, while the residual activities of CRL-BSA-CLEAs and CRL-CLEAs were only 33% and 22%. This result can be attributed to the increased interaction between the enzyme and the substrate due to the modification of the ionic liquid. The active conformation of the enzyme was protected due to the reduction of enzyme loss. Xiang et al. modified immobilized enzymes with imidazole ionic liquids with different anions. After four times of repeated use, the activity of the modified immobilized enzymes retained 17%-26.4% [24]. Ionic liquids strengthened the hydrophobic force, hydrogen bond force and adsorption between the enzyme and the carrier. The structure of the enzyme was difficult to be destroyed during repeated use, thereby improving the reusability of the immobilized enzyme.

Figure 5 shows the activity of immobilized enzymes in different organic solvents. The stability of CRL-FIL-CLEAs in isopropanol (logP, 4.3) had been improved, but not good in acetonitrile (logP, 6.2), dimethyl sulfoxide (logP, 7.2) and ethyl acetate (logP, 4.3). Jia et al. reported that IL can significantly improve the organic solvent tolerance of CRL, PPL and CALB [32]. However, CRL-CLEAs did not have the protection of the carrier, and the enzyme molecules were in direct contact with organic solvents. Therefore, the immobilized enzyme was greatly affected by organic solvents. The addition of BSA and IL did not solve

this problem well. Laane et al. found that the biocatalytic activity in organic solvents was lower in polar solvents with $\log P < 2$, moderate in solvents with $\log P$ between 2 and 4, and higher in non-polar solvents with $\log P > 4$. But this result was not consistent with the law of this experiment^[33]. Yang et al. found that in addition to the polarity of the organic solvent, there are other factors that affect the stability of the enzyme in the organic solvent. Such as the spatial structure of solvent molecules, molecular size and the ability to enter and exit the active site of the enzyme^[34]. Pramod et al. pointed out that the enzyme activity was inversely proportional to the polarity of the organic medium used^[35]. This may explain the result that CRL-FIL-CLEAs maintained good activity in isopropanol solution in this experiment.

3.4 Characterization of free lipase and immobilized lipase

FTIR spectroscopy analyzed the chemical composition of free CRL and CRL-FIL-CLEAs (Fig. 6). After CRL was immobilized, it can be seen that the FTIR spectrum had changed significantly. The main protein absorption band caused by peptidyl vibration appeared in the $1200\text{--}1900\text{ cm}^{-1}$ spectral region. The amide I band ($1600\text{--}1700\text{ cm}^{-1}$) was mainly due to C = O tensile vibration. The amide II band ($1510\text{--}1580\text{ cm}^{-1}$) was attributed to N-H bending and C-N tensile vibration. The amide III band ($1200\text{--}1400\text{ cm}^{-1}$) was attributed to N-H bending, C-C and C-N tensile vibrations^[36]. The protein amide I band contained a wealth of information about protein secondary structure. The most sensitive spectral region of the protein structural components in the enzyme secondary structure was located in the amide I band, which played a vital role in the catalytic activity. The peak near 1660 cm^{-1} in the spectrum of free enzyme and CRL-FIL-CLEAs represented the amide I band of the enzyme, confirming the presence of CRL in these samples. The absorption band at 572 cm^{-1} indicated that CLEAs had been successfully prepared. At the same time, a prominent peak at 1432 cm^{-1} was observed in CRL-FIL-CLEAs, but this peak did not appear in the spectrum of free enzyme. It showed that the functional group was formed during the cross-linking process. The reason was that the propyl chain of glutaraldehyde had an absorption peak at the C = N vibration at 1432 cm^{-1} . The Schiff base bond was successfully formed by the reaction of glutaraldehyde with the terminal amine group of lipase during the formation of CLEA. Miao et al. used CaCO_3 particles as a template to synthesize porous cross-linked enzyme aggregates by in-situ co-precipitation^[37]. Absorption peaks were also observed at 572 cm^{-1} and 1456 cm^{-1} , and the conclusion was similar to this experiment. Therefore, the successful preparation of CLEAs was confirmed by infrared spectroscopy.

As shown in Fig. 7, the morphology of the free CRL and the prepared CRL-BSA-CLEAs and CRL-FIL-CLEAs were studied and analyzed. The structure of lipase aggregates can be divided into two types^[38]. One type was aggregates forming a typical "ball", such as CALB, and the second type was aggregates forming a less clear structure, such as Fig. 7(a). In this study, the pyramid-like structure in the structure shown by CRL-FIL-CLEAs was presumed to be the solid state of the ionic liquid after freeze-drying (Fig. 7(c)). It can be seen that the lipase showed an aggregated state after being precipitated by ammonium sulfate (Fig. 7(b)). The enlarged image showed dense pores between the particles. These pores were conducive to substrate entry, which could increase the substrate affinity and activity of CLEAs. Compared with CRL-BSA-CLEAs, the pyramid-like structure in CRL-FIL-CLEAs seemed to be more conducive to substrate

access to the active site of the enzyme. CRL-BSA-CLEAs had smaller pores, and agglomeration and tight aggregation were observed. Such a structure may have a steric hindrance effect on the substrate. The substrate reactant was restricted from entering the internal CLEAs due to diffusion limitation, thus showing a smaller substrate conversion rate. Diffusion restriction restricted the substrate from entering the internal CLEAs, thus showing a lower substrate conversion rate. Especially in the case of insoluble substrates, so the affinity to the substrate was lower than CRL-FIL-CLEAs. On the contrary, compared with CRL-BSA-CLEAs, CRL-FIL-CLEAs particles were smaller, and had a finer internal pore structure and a larger internal surface area. The support of the pyramid-like structure provided more channels, making it easier for the substrate molecules to enter the lipase catalytic site (Fig. 7(d)). This structure not only reduced the mass transfer limitation, but also improved the catalytic efficiency of CRL-FIL-CLEAs.

4. Conclusion

In this study, ionic liquids with amino groups were used as a new functional molecular modifier, and finally CRL-FIL-CLEAs were obtained. At the same time, we compared catalyst properties with unmodified CLEAs (CRL-CLEAs), traditional CLEAs (CRL-BSA-CLEAs) and free CRL. The results showed that the activity of CRL-FIL-CLEAs (5.51 U/mg protein) was 1.9 times that of CRL-CLEAs (2.86 U/mg protein) and 1.4 times that of CRL-BSA-CLEAs (3.98 U/mg protein). CRL-FIL-CLEAs maintained 61% activity at 60 °C for 50 mins, while the free enzyme only maintained 17% activity. After repeated use for five times, CRL-FIL-CLEAs (51%) retained higher activity than CRL-CLEAs (22%). The organic tolerance test also showed that the tolerance of CRL-FIL-CLEAs to isopropanol was better than other immobilized lipase. Combining FT-IR and SEM characterization, we found that CRL-FIL-CLEAs particles have a larger surface area, making it easier for substrate molecules to enter the lipase catalytic site. Therefore, the catalytic efficiency of CRL-FIL-CLEAs was improved. Furthermore, we performed a kinetic analysis of CLEAs before and after modification. The results showed that the K_m value (4.80 mM) of CRL-FIL-CLEAs was less than CRL-CLEAs (8.06 mM). It can be inferred that the affinity of CRL-FIL-CLEAs to the substrate increased. In summary, CRL-FIL-CLEAs could be used as an efficient biocatalyst, advanced CLEAs technology after adding ionic liquids could broaden the application range of lipase immobilization.

Declarations

Acknowledgments

The work was funded by the National Natural Science Foundation of China (No. 21406093), Natural Science Foundation of Jiangsu Province (BK20140529), Open Project Program of State Key Laboratory of Food Science and Technology of Jiangnan University (SKLF-KF-201919), Key University Science Research Project of Jiangsu Province (14KJB530001), China Postdoctoral Science Foundation (2014M550271), and Priority Academic Program Development of Jiangsu Higher Education Institutions.

Compliance with Ethical Standards

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted. We confirm that the manuscript has not been published or under consideration for publication elsewhere. Further, this submission has been approved by the institution where the study was conducted. All authors are aware of the submission and agree with its publication.

Authorship contribution statement

Xia Jiaojiao: Investigation, Writing-Original Draft. Yan Yan: Conceptualization, Methodology, Formal analysis. Zou Bin: Conceptualization, Supervision, Resources, Writing-Review & Editing. Adesanya Idowu Onyinye: Investigation, Resources, Writing-Review & Editing

References

1. Jones, J. U. M. H. S. P. T. (1994) The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. *Structure*, 2:293-308.
2. Liu, J., Ma, R.-T. and Shi, Y.-P. (2020) "Recent advances on support materials for lipase immobilization and applicability as biocatalysts in inhibitors screening methods"-A review. *Anal. Chim. Acta*, 1101, 9-22.
3. Le Joubioux, F., Ben Henda, Y., Bridiau, N., Achour, O., Graber, M. and Maugard, T. (2013) The effect of substrate structure on the chemoselectivity of *Candida antarctica* lipase B-catalyzed acylation of amino-alcohols. *J. Mol. Catal. B-Enzym.* 85-86, 193-199.
4. Jamwal, S., Ranote, S., Dautoo, U. and Chauhan, G. S. (2020) Improving activity and stabilization of urease by crosslinking to nanoaggregate forms for herbicide degradation. *Int. J. Biol. Macromol.* 158, 521-529.
5. Kubiak, M., Storm, K.-F., Kampen, I. and Schilde, C. (2019) Relationship between Cross-Linking Reaction Time and Anisotropic Mechanical Behavior of Enzyme Crystals. *Cryst. Growth Des.* 19, 4453-4464.
6. Voberkova, S., Solcany, V., Vrsanska, M. and Adam, V. (2018) Immobilization of ligninolytic enzymes from white-rot fungi in cross-linked aggregates. *Chemosphere*, 202, 694-707.
7. Bilal, M., Zhao, Y., Noreen, S., Shah, S. Z. H., Bharagava, R. N. and Iqbal, H. M. N. (2019) Modifying bio-catalytic properties of enzymes for efficient biocatalysis: a review from immobilization strategies viewpoint. *Biocatal. Biotransfor.* 37, 159-182.
8. Zerva, A., Antonopoulou, I., Enman, J., Iancu, L., Rova, U. and Christakopoulos, P. (2018) Cross-Linked Enzyme Aggregates of Feruloyl Esterase Preparations from *Thermothelomyces thermophila* and *Talaromyces wortmannii*. *Catalysts*, 8, 208-223.
9. Badoei-dalfard, A., Karami, Z. and Malekabadi, S. (2019) Construction of CLEAs-lipase on magnetic graphene oxide nanocomposite: An efficient nanobiocatalyst for biodiesel production. *Bioresource Technol.* 278, 473-476.

10. Jia, J., Zhang, W., Yang, Z., Yang, X., Wang, N. and Yu, X. (2017) Novel Magnetic Cross-Linked Cellulase Aggregates with a Potential Application in Lignocellulosic Biomass Bioconversion. *Molecules*, 22,269-283.
11. Hero, J. S., Romero, C. M., Pisa, J. H., Perotti, N. I., Olivaro, C. and Martinez, M. A. (2018) Designing cross-linked xylanase aggregates for bioconversion of agroindustrial waste biomass towards potential production of nutraceuticals. *Int. J. Biol. Macromol.* 111, 229-236.
12. Razib, M. S. M., Abd Rahman, R. N. Z. R., Shariff, F. M. and Ali, M. S. M. (2020) Biochemical and Structural Characterization of Cross-Linked Enzyme Aggregates (CLEAs) of Organic Solvent Tolerant Protease. *Catalysts*, 10, 55-71.
13. Qiu, X., Wang, S. S., Miao, S. S., Suo, H. B., Xu, H. J. and Hu, Y. (2021) Co-immobilization of laccase and ABTS onto amino-functionalized ionic liquid-modified magnetic chitosan nanoparticles for pollutants removal. *J. Hazard. Mater.*, 401, 123353-122288.
14. Xu, C., Suo, H. B., Xue, Y., Qin, J., Chen, H. Y. and Hu, Y. (2021) Experimental and theoretical evidence of enhanced catalytic performance of lipase B from *Candida antarctica* acquired by the chemical modification with amino acid ionic liquids. *Mol. Catal.*, 501, 111355-111367.
15. Hu, Y., Tang, S., Jiang, L., Zou, B., Yang, J. and Huang, H. (2012) Immobilization of *Burkholderia cepacia* lipase on functionalized ionic liquids modified mesoporous silica SBA-15. *Process Biochem.* 47, 2291-2299.
16. Kim, H. S., Eom, D., Koo, Y.-M. and Yingling, Y. G. (2016) The effect of imidazolium cations on the structure and activity of the *Candida antarctica* Lipase B enzyme in ionic liquids. *Phys. Chem. Chem. Phys.* 18, 22062-22069.
17. Suo, H., Xu, L., Xue, Y., Qiu, X., Huang, H. and Hu, Y. (2020) Ionic liquids-modified cellulose coated magnetic nanoparticles for enzyme immobilization: Improvement of catalytic performance. *Carbohydr. Polym.* 234, 115914-115922.
18. Parveen, S., Asgher, M. and Bilal, M. (2021) Lignin peroxidase-based cross-linked enzyme aggregates (LiP-CLEAs) as robust biocatalytic materials for mitigation of textile dyes-contaminated aqueous solution. *Environ. Technol. Inno.* 21, 101226-101256.
19. Persson, M. and Bornscheuer, U. T. (2003) Increased stability of an esterase from *Bacillus stearothermophilus* in ionic liquids as compared to organic solvents. *J. Mol. Catal. B-Enzym.* 22, 21-27.
20. Suo, H., Xu, L., Xu, C., Chen, H., Yu, D., Gao, Z., Huang, H. and Hu, Y. (2018) Enhancement of catalytic performance of porcine pancreatic lipase immobilized on functional ionic liquid modified Fe₃O₄-Chitosan nanocomposites. *Int. J. Biol. Macromol.* 119, 624-632.
21. Zhang, C., Zhang, L., Zhang, Y., Huang, H. and Hu, Y. (2016) Study on the Stability and Enzymatic Property Improvement of Porcine Pancreas Lipase Modified by Ionic Liquids Using Molecular Simulation. *Acta Chim. Sinica.* 74, 74-80.
22. Suo, H., Xu, L., Xu, C., Qiu, X., Huang, H. and Hu, Y. (2019) Enhanced catalytic performance of lipase covalently bonded on ionic liquids modified magnetic alginate composites. *J. Colloid Interf. Sci.* 553,

23. Wahlstroem, R. M. and Suurnaekki, A. (2015) ChemInform Abstract: Enzymatic Hydrolysis of Lignocellulosic Polysaccharides in the Presence of Ionic Liquids. *J. Cheminformatics*, 46, 336-356.
24. Xiang, X., Ding, S., Suo, H., Xu, C., Gao, Z. and Hu, Y. (2018) Fabrication of chitosan-mesoporous silica SBA-15 nanocomposites via functional ionic liquid as the bridging agent for PPL immobilization. *Carbohydrate Polymers*, 182, 245-253.
25. Lau, R. M., van Rantwijk, F., Seddon, K. R. and Sheldon, R. A. (2000) Lipase-catalyzed reactions in ionic liquids. *Org. Lett.* 2, 4189-4191.
26. Jiang, Y., Guo, C., Xia, H., Mahmood, I., Liu, C. and Liu, H. (2009) Magnetic nanoparticles supported ionic liquids for lipase immobilization: Enzyme activity in catalyzing esterification. *J. Mol. Catal. B-Enzym.* 58, 103-109.
27. Amaral-Fonseca, M., Kopp, W., Camargo Giordano, R. d. L., Fernandez-Lafuente, R. and Tardioli, P. W. (2018) Preparation of Magnetic Cross-Linked Amyloglucosidase Aggregates: Solving Some Activity Problems. *Catalysts*. 8, 496-517.
28. Lai, J.-Q., Li, Z., Lue, Y.-H. and Yang, Z. (2011) Specific ion effects of ionic liquids on enzyme activity and stability. *Green Chem.* 13, 1860-1868.
29. Zhang, C., Dong, X., Guo, Z. and Sun, Y. (2018) Remarkably enhanced activity and substrate affinity of lipase covalently bonded on zwitterionic polymer-grafted silica nanoparticles. *J. Colloid Interf. Sci.* 519, 145-153.
30. Fan, Y., Wang, X., Zhang, L., Li, J., Yang, L., Gao, P. and Zhou, Z. (2018) Lipase-catalyzed synthesis of biodiesel in a hydroxyl-functionalized ionic liquid. *Chem. Eng. Res.Des.* 132, 199-207.
31. HajKacem, S., Galai, S., Hernandez Fernandez, F. J., Perez de los Rios, A., Smaali, I. and Quesada Medina, J. (2020) Bioreactor Membranes for Laccase Immobilization Optimized by Ionic Liquids and Cross-Linking Agents. *App. Biochem. Biotech.* 190, 1-17.
32. Jia, R., Hu, Y., Liu, L., Jiang, L., Zou, B. and Huang, H. (2013) Enhancing Catalytic Performance of Porcine Pancreatic Lipase by Covalent Modification Using Functional Ionic Liquids. *Acs Cata.* 3, 1976-1983.
33. Laane, C., Boeren, S., Vos, K. and Veeger, C. (2009) Rules for Optimization of Biocatalysis in Organic Solvents. *Biotechnol. Bioeng.* 102, 2-8.
34. Yang, L., Dordick, J. S. and Garde, S. (2004) Hydration of enzyme in nonaqueous media is consistent with solvent dependence of its activity. *Biophys. J.* 87, 812-821.
35. Wangikar, P. P., Michels, P. C., Clark, D. S. and Dordick, J. S. (1997) Structure and Function of Subtilisin BPN' Solubilized in Organic Solvents. *J. Am. Chem. Soc.* 119, 70-76.
36. Salgin, S., Cakal, M. and Salgin, U. (2020) Kinetic resolution of racemic naproxen methyl ester by magnetic and non-magnetic cross-linked lipase aggregates. *Prep. Biochem. Biotech.* 50, 148-155.
37. Miao, C., Li, H., Zhuang, X., Wang, Z., Yang, L., Lv, P. and Luo, W. (2019) Synthesis and properties of porous CLEAs lipase by the calcium carbonate template method and its application in biodiesel

production. Rsc Adv. 9, 29665-29675.

38. Wahab, M. K. H. A., El-Enshasy, H. A., Abu Bakar, F. D., Murad, A. M. A., Jahim, J. M. and Illias, R. M. (2019) Improvement of cross-linking and stability on cross-linked enzyme aggregate (CLEA)-xylanase by protein surface engineering. Process Biochem. 86, 40-49.

Tables

Table.1 Kinetic parameters of CRL, CRL-CLEAs and CRL-FIL-CLEAs

Sample ^a	Kinetic parameters ^b		Enzyme assay ^c
	V_{max} (U/mg protein)	K_m (mM)	Optimum activity (U/mg protein)
CRL	126.58±5.33	0.92±0.08	11.65±0.23
CRL-CLEAs	555.56±21.26	8.06±0.42	2.86±0.15
CRL-FIL-CLEAs	500.16±18.32	4.80±0.31	5.51±0.18

^a CRL-BSA-CLEAs 3.98±0.19 U/mg protein

^b Kinetic reaction conditions: temperature 35°C and pH 7.5

^c Optimum enzyme activity assay conditions: optimum temperature (The activity of CRL at 35°C, the activity of CRL-CLEAs and CRL-BSA-CLEAs at 40°C, and the activity of CRL-FIL-CLEAs at 45°C were defined as 100%.) and pH 7.5

Figures

Figure 1

Figure 2

(a) Effect of modifier amount on lipase activity (temperature 35°C, pH 7.5. the activity under BSA addition were 20mg/ml (3.28 U/mg protein) and ionic liquid addition were 200mg/ml (3.68 U/mg protein) were defined as 100%) (b) Effect of reaction temperature on lipase activity (temperature 35°C, pH 7.5. The optimal activity of free lipase, CRL-CLEAs, CRL-BSA-CLEAs and CRL-FIL-CLEAs were defined as 100% in table 1).

Figure 3

Kinetic parameters of CRL-CLEAs and CRL-FIL-CLEAs (Reaction temperature: 35°C, pH 7.5).

Figure 4

(a) Thermal stability of free lipase and immobilized lipase (The optimal activity of free lipase, CRL-CLEAs, CRL-BSA-CLEAs and CRL-FIL-CLEAs were defined as 100% in table 1.), (b) PH stability of free lipase and immobilized lipase (Reaction temperature: 35°C, pH 7.5), (c) Storage stability of free lipase and immobilized lipase (Reaction temperature: 35°C, pH 7.5. The initial activity was defined as 100%), (d) Reusability of immobilized lipase (Reaction temperature: 35°C, pH 7.5. The initial activity was defined as 100%).

Figure 5

Organic tolerance stability of Free CRL, CRL-CLEAs, CRL-BSA-CLEAs and CRL-FIL-CLEAs (Reaction temperature: 35°C, pH 7.5. The initial activity was defined as 100%).

Figure 6

FT-IR spectra of free lipase and CRL-FIL-CLEAs.

Figure 7

(a) SEM of CRL-BSA-CLEAs (4000 $\times$, 30 μ m), (b) SEM of CRL-BSA-CLEAs (10000 $\times$, 10 μ m), (c) SEM of CRL-FIL-CLEAs (4000 $\times$, 30 μ m), (d) SEM of CRL-FIL-CLEAs (10000 $\times$, 10 μ m).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.docx](#)