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Stabilization of a Lipolytic Enzyme for Commercial Application

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Abstract: *Thermomyces lanouginosa* lipase has been used to develop improved methods for carrier-free immobilization, the Cross-Linked Enzyme Aggregates (CLEAs), for its application in detergent products. An activator step has been introduced to the CLEAs preparation process with the addition of Tween 80 as activator molecule, in order to obtain a higher number of the individual lipase molecules in the "open lid" conformation prior to the cross-linking step. A terminator step has been introduced to quench the cross-linking reaction at an optimal time by treatment with an amine buffer in order to obtain smaller and more homogenous cross-linked particles. This improved immobilization method has been compared to a commercially available enzyme and has been shown to be made up of smaller and more homogenous particles with an average diameter of 1.85 ± 0.28 μm which are 129.7% more active than the free enzyme. The CLEAs produced show improved features for commercial applications such as an improved wash performance comparable with the free enzyme, improved stability to proteolysis and a higher activity after long-term storage.

Keywords: enzyme stabilization; CLEAs; lipase; industrial application

1. Introduction

Over the last decade, there has been a trend in the industry to move towards more eco-friendly products as a way to boost a company's "green" credentials, and this is now driving economic growth [1].

In the laundry industry, many research and development projects have been focused on the creation of novel formulations using less polluting chemicals and more bio-based components including enzymes and natural surfactants. Enzymes are biodegradable and since they are usually used at 0.1%–1.0% of the substrate concentration, the contribution of the enzyme to the Biochemical Oxygen Demand (BOD) in the waste stream is negligible [2], which results in clear ecological benefits. In western Europe, consumers have shown a preference for liquid laundry detergent rather than powder detergent [3]. Compared to a powder formulation, liquid detergent is more concentrated, less environmentally aggressive and needs less packaging material, which results in a lower carbon footprint for the product.

In a typical biological laundry detergent formulation, there are at least three types of enzymes which address different types of stains, which include proteases, amylases, and lipases, but additional

enzymes can be added such as mannanases and cellulases. Detergent enzymes are always being challenged for their stability in laundry application because of the presence of proteases [4]. In the present investigation, lipase from *Thermomyces lanuginosa* was used to prepare cross-linked enzyme aggregates (CLEAs) with enhanced characteristics compared to the commercially available counterpart. *Thermomyces lanuginosa* (previously *Humicola lanuginosa*) lipase (TLL) is the enzyme responsible for the lipolytic activity of Lipex 16L, a commercial lipase preparation supplied by Novozymes. This enzyme has been used in a broad range of biotransformation [5–7]. Its structure has been solved at 1.8 Å resolution [8].The TLL shares a common α/β-hydrolase fold with other lipases and the typical Ser-His-Asp catalytic triad [9]. The structure contains a small loop of amino acids 86–93, referred to as a lid that covers the active site pocket. The lid can change from a "closed" to an "open" conformation when the lipase is adsorbed to an interface. This process is termed interfacial activation and it has been observed in many lipase enzymes [10,11]. In aqueous media, TLL is active over the pH range of 7.0–11.0; it maintains activity at 55–60 °C although the recommended temperature for applications is between 30 and 40 °C [12].

The immobilization of enzymes is a powerful tool to improve the enzyme rigidity and subsequent stability [13]. In some cases, immobilization has been shown to improve both activity and selectivity [14–16]. However, there is a delicate balance between increased rigidity and the flexibility needed for enzyme function [17,18]. The methods of enzyme immobilization can be divided into three categories: binding to a support (carrier), entrapment (encapsulation) and cross-linking [19].

The immobilization process often consists of a chemical attachment of the enzyme to an insoluble carrier. However, a distinct disadvantage of carrier-bound enzymes is the dilution of the overall catalytic activity resulting from the introduction of non-catalytic material, which can be a large proportion of the total mass [19,20]. Carrier-free immobilization techniques overcome this issue. One of the simplest ways to perform carrier-free immobilization of enzyme is using a chemical cross-linking reaction. The technique of protein cross-linking using the reaction of glutaraldehyde with reactive amine residues on the protein surface was initially developed in the 1960s [21,22]. However, this method had several drawbacks, such as low activity retention, poor reproducibility, low mechanical stability and difficulties in handling the gelatinous Cross-Linked Enzymes (CLEs). The cross-linking of a crystalline enzyme was first applied to stabilize enzyme crystals for X-ray diffraction studies [23]. Subsequently, these cross-linked enzyme crystals (CLECs) were successfully commercialized into industrial biocatalysts [24]. An inherent disadvantage of CLECs is the need to crystallize the enzyme, which is often a laborious procedure requiring enzyme of high purity. It was shown [25] that comparable results could be achieved by precipitating the enzyme and cross-linking the resulting physical aggregates.

Cross-linked enzyme aggregates (CLEAs) have been described [25,26], and are receiving increasing interest from the industrial sector [25,27]. The production of CLEAs involves the precipitation of enzymes from aqueous solution followed by the cross-linking of the protein aggregates (Figure 1).

This cross-linking occurs between amino groups of the lysine residues on the surface of the enzyme and the *N*-terminal amino group. The cross-linking reagent which is usually glutaraldehyde [28], results in the formation of covalent bonds, rendering the CLEAs permanently insoluble. The mechanical resistance of the CLEA particle is lower than that of the CLEC [29], but it has the advantage of using impure enzyme preparations and can be carried out more rapidly since it involves enzyme precipitation and not crystallization [15]. However, CLEAs present extended diffusional limitations in mass transfer [30], and the cross-linking efficiency is dependent on the number of accessible lysine residues in the protein, making it not an ideal method for some proteins. This issue may be overcome by precipitating the protein with a polymer containing numerous free amino groups, such as poly-lysine or polyethylene imine [15,16], by modifying the target protein in order to introduce more lysine residues into the sequence by site-directed mutagenesis or by adding bovine serum albumin as a "proteic feeder" during the preparation of the CLEAs to facilitate their formation [31].

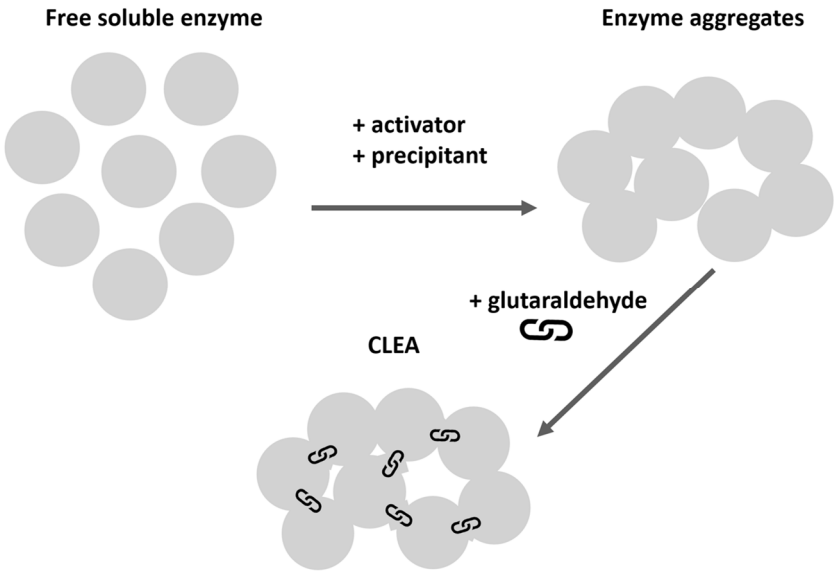

Figure 1. Schematic representation of the cross-linked enzyme aggregate (CLEA) production process.

Regardless of the choice, both immobilization strategies have a common feature of not requiring a support since the biocatalyst is predominantly the enzyme [32]. Hyperactive lipase CLEAs have been made in the presence of surfactants and detergents [27,33]. These compounds are added to the enzyme prior to cross-linking and initiate the process of interfacial activation, opening the lipase "lid", by binding to the hydrophobic active site of the enzyme [10]. Cross-linking of the enzyme molecules in this "open" conformation can produce lipase CLEAs with over 100% activity [34]. It is assumed that these "activating" molecules are washed out of the lipase active site when the CLEAs are washed after the cross-linking reaction, since enzymatic activity towards known substrates can be readily detected. The CLEA technology offers many advantages for industrial applications as it is simple and amenable to rapid optimization, which translates to low costs and short time-to-market processes [26].

Since enhanced activity and stability are good requisites for industrial enzymes, these technologies have received increasing interest in recent years from the industry [10,15,33]. This is particularly true for the production of "bio-based" liquid laundry detergent, where the detergent enzymes have to remain stable during the detergent shelf life of the product and to remain active when used in the wash process.

This paper describes the use of a carrier-free immobilized enzyme technique to determine if CLEAs generated from the commercially available *T. lanuginosa* lipase will have improved stability in detergent surfactants and will be more resistant to proteolysis.

2. Results

2.1. T. lanouginosa Lipase (Lipex) CLEA Hyperactivation

A selection of compounds were identified which may have potential beneficial effects on the activity retained by the Lipex CLEA (Table 1). These included numerous non-ionic surfactants, ether compounds, waxes and oils, natural fats, natural detergents, inhibitors and amino acids. These compounds were added at varying concentrations to the CLEA for 5 min prior to precipitation. The surfactant, oil, and wax may trigger the opening of the active site by lid movement and will bind to the hydrophobic active site.

Table 1. A list of the compounds tested in the Lipex CLEA (cross-linked enzyme aggregate) production highlighting the concentration range tested, the final concentration of the compound where the highest activity was observed and the value of the highest activity retained.

Compound	Final Concentration Test (Concentration that Shows the Highest Activity Retained)	Highest Activity Retained (%)
Lipex 16L	-	100.0
Lipex CLEAs without compound	-	86.3
Tween 85	2.4% v/v	47.5
Tween 80	6.5–50.4 mM (19 mM)	129.7
Tween 40	19.5 mM	55.6
Tween 20	6.7–27.1 mM	57.8
Span 20	72.1 mM	35.0
Span 60	58.1 mM	55.4
Span 80	58.3 mM	61.2
Tributyrin	110–661 mM (220 mM)	36.2
Sodium dodecyl sulfate	5.8–115.6 mM (5.8 mM)	21.6
Orlistat	67–336 µM (67 µM)	45.7
Arginine	1.9–9.6 mM (1.9 mM)	3.5
Coconut oil	1.65%–3.3% v/v (1.65 v/v)	31.6
Rhamnolipid	1.6%–9.9% v/v (3.3 v/v)	51.8

The phenomenon of interfacial activation of lipases by additives such as surfactants, crown ethers or amines is well documented [27,35] and generally ascribed to the lipase adopting a more active conformation under these conditions. On preparing Lipex CLEAs, most of the tested compounds produced a negative effect with the exception of Tween 80, which had 129.7% retained activity as compared to the free (native) enzyme, and 150.3% retained activity as compared to the Lipex CLEAs prepared without surfactant. Such hyperactivity of CLEAs in the presence of surfactants, when compared to the free enzyme, has previously been reported by López-Serrano et al. for *T. lanuginosa* lipase [36].

2.2. CLEAs Suspension in Formulation

During the experimentation, it could be observed that enzyme CLEAs do settle during storage, which needs to be taken into consideration for future product formulation (Figure 2).

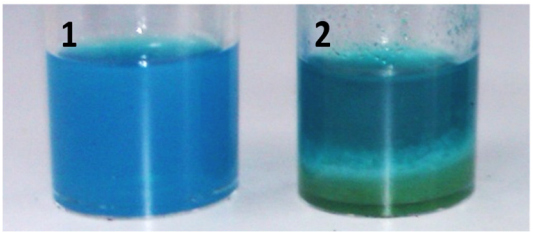

Figure 2. Settling of CLEAs in liquid formula. Formulation containing soluble enzyme (**left**) was compared with formulation containing lipase CLEA only (**right**). The settlement of the CLEA enzyme is clearly visible.

The fact that the CLEAs are settling in solution would result in product heterogeneity in liquid solutions. The suspending properties of the structured liquid were studied for the ability to keep the Lipex CLEA in solution during long-term storage. The addition of a low-value material such as citrus pulp, which is a waste product from other industrial processes, and the addition of clay both show potentially interesting results (Figure 3).

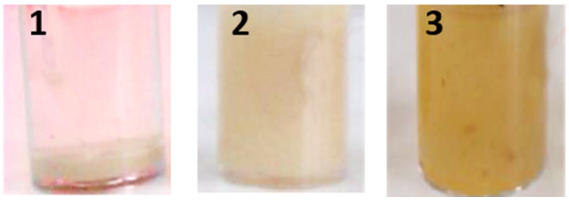

Figure 3. A figure showing the settling of the Lipex CLEA with and without the addition of citrus pulp. **1**. Water–100 mg Lipase CLEA, **2**. 0.25% citrus pulp/water–100 mg Lipase CLEA, **3**. 0.1% citrus pulp/0.4% clay–100 mg Lipex CLEA.

The results shown in Figure 3 show that a structuring agent such as citrus pulp or a citrus pulp/clay combination can maintain the Lipex CLEA in a dispersed state and no settling is observed during a four-week storage period. Since homogeneity of the product is crucial to obtain optimal cleaning performance, these structuring agents will be included in new detergent formulation compositions.

2.3. CLEA Particle Size Determination

The particle size distribution of differently produced CLEAs might have an impact on the activity performance of the resulting products. Kartal and Kilinc [27] showed that increasing the particle size of the CLEA resulted in lower activity because of the loss of enzyme flexibility caused by excessive cross-linking and/or diffusion limitations [27]. The CLEA particle size gradually increased as a result of the cross-linking times and the process needed to be fine-tuned in order to obtain a positive effect. Too short cross-linking times can lead to a low activity because of insufficient cross-linking, whereas too long cross-linking times may lower activity due to increasing CLEA particle size. If the particle size of the CLEA becomes larger, the enzyme molecules inside the CLEA particle would have no chance to reach the substrate [27].

Thus, a particle size comparison of Lipex CLEAs produced with and without the introduction of a termination step to control particle size could reveal the impact on the improved stability of the enzyme and improved wash performance (Table 2). The different CLEA samples were equally prepared for these experiments with a controlled repeatable aggregation process prior to the measurement and were analyzed with a sedimentometer. The resulting data have proved evidence for the crucial role of the terminator step for the production of smaller CLEAs with higher activity, due to the fact that more enzyme molecules are able to interact with the substrate.

Table 2. Comparison of the particle size distribution across the different Lipex CLEAs.

Sample	Velocity (cm/s)	Diameter (μm)	Mean Diameter (μm)	Retained Activity Compared to Free Enzyme (%)
Lipex CLEA without terminator step	4.86×10^{-5} 8.96×10^{-5} 9.05×10^{-5} 8.33×10^{-5}	2.99 4.05 4.07 3.91	4.01	67.9 ± 4.5
Lipex CLEA with terminator step	1.43×10^{-5} 1.32×10^{-5} 2.84×10^{-5} 2.08×10^{-5}	1.62 1.56 2.28 1.96	1.85	123.8 ± 8.0

2.4. Lipex CLEA Stability

The Lipex CLEA stability in different storage conditions was studied and, after each time point, the residual activity of each sample was determined by measuring the hydrolytic enzyme activity of the enzyme using the substrates as described in Material and Methods.

The Lipex CLEAs are more stable than soluble Lipex 16L when stored in Tris-HCl pH 8.0 buffer at 4 °C with a retained activity after 12 weeks of 87.6% compared to the 75.9% obtained for the Lipex 16L (Figure 4).

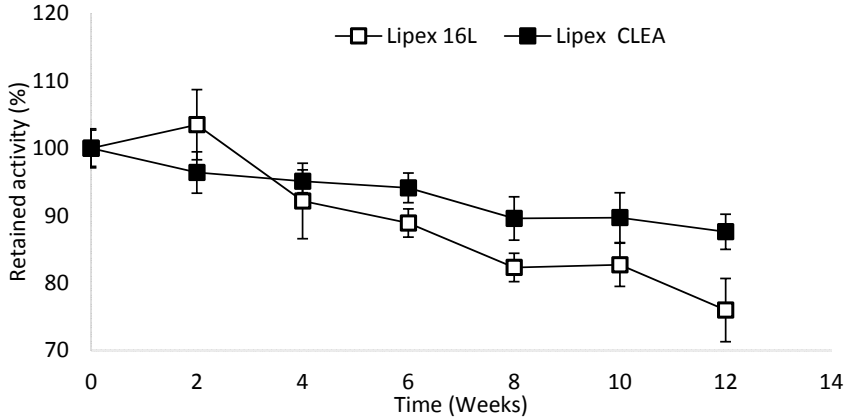

Figure 4. The average activity of the soluble Lipex 16L and Lipex CLEA over 12 weeks incubation at 4 °C.

To simulate the storage of the Lipex CLEA in a commercial laundry formulation, further stability tests have been performed by incubating the samples at 37 °C for four weeks in a liquid laundry formulation with and without protease (Figure 5).

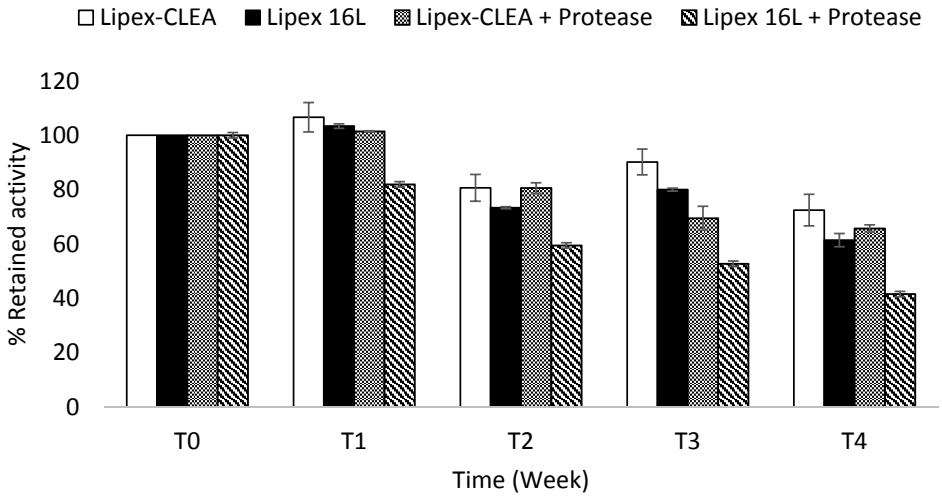

Figure 5. The Lipex-CLEA residual activities following 4 weeks storage at 37 °C in laundry formulation with and without protease.

The results in Figure 5 show that the residual activities of the Lipex-CLEA which had been stored for four weeks (without protease) was higher than that observed for the soluble Lipex. The difference in residual activities between the CLEA and the soluble lipase was approximately 10%. In addition to the increased enzyme activity, an increase in enzyme stability was also observed with the cross-linked lipases in the presence of protease. The Lipex CLEA was found to be more stable in comparison to soluble lipase. After four weeks, the Lipex-CLEA possessed a residual activity of ≥65% while a substantial drop in residual activities was observed with Lipex 16L (~40%). It can be concluded that the cross-linked Lipex (Lipex CLEA) possesses a superior activity and stability in the presence and absence of protease in laundry formulation compared to soluble Lipex 16L.

2.5. Wash Performance

The micro-titre End-point Stain Removal Assays have been performed using the freshly prepared Lipex CLEA and the soluble enzyme against two types of lipid stain and the results show that the cleaning effect is comparable (Figure 6) with a slight advantage for the Lipex-CLEA on beef fat stains.

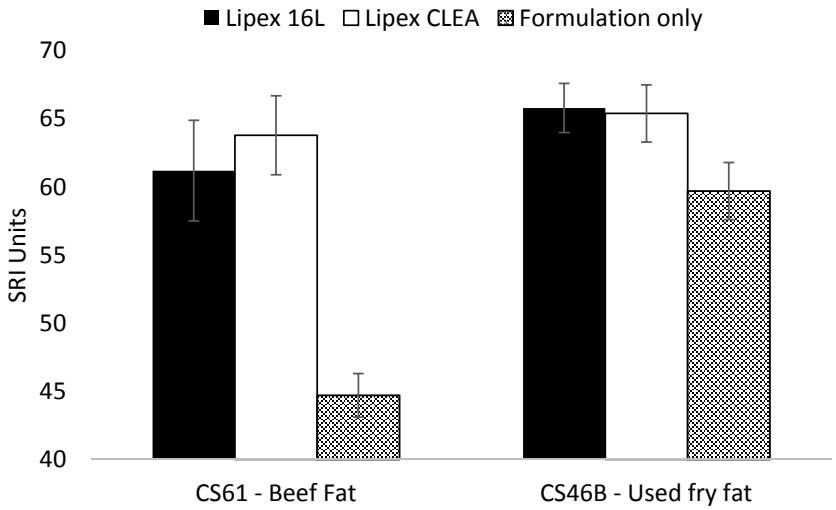

Figure 6. The Lipase end-point stain removal assays using CS61 (Beef Fat stained cloth) and CS46B (Used Fry Fat stained cloth) treated with Lipex 16L, Lipex CLEA, or laundry formulation with no enzyme.

Further wash performance experiments have been carried out using a Terg-O-Tometer which is an instrument able to reproduce washing conditions on a laboratory scale. Adapting a real laundry product condition, the cleaning effects of lipases/enzyme CLEAs were screened following storage at 37 °C for four weeks in the presence of protease as described in Material and Methods. Figure 7 shows that with Lipex-CLEA (in the presence of protease), a cleaning effect on the CS61 stain was obtained after four weeks of incubation. In comparison to the soluble Lipex, the Lipex-CLEA showed ~2 SRI units better cleaning than soluble-Lipex on CS61 stains (Figure 7).

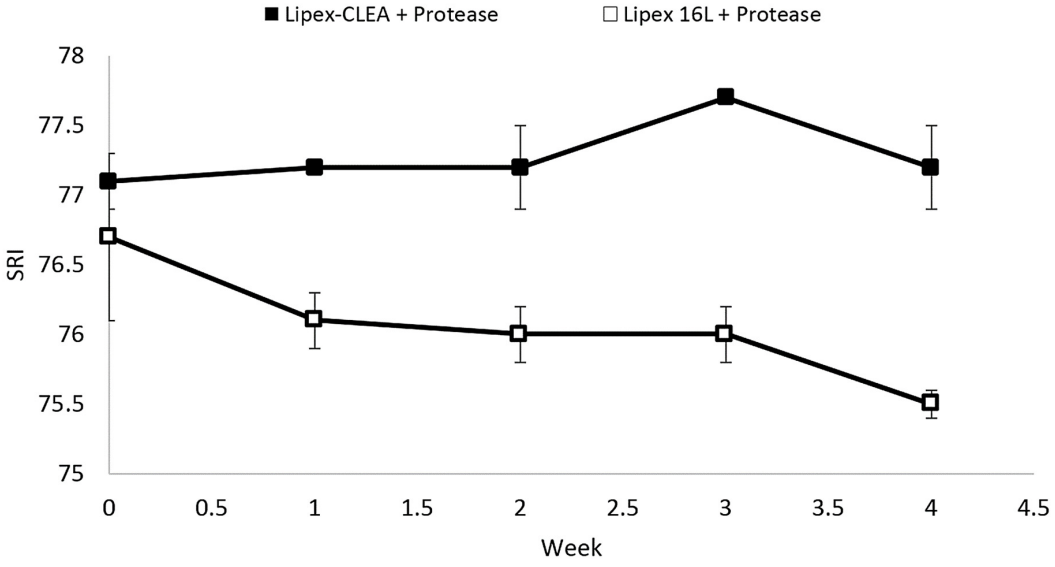

Figure 7. The effect of Lipex-CLEA and Lipex 16L on beef fat stain (CS61) over 4 weeks storage at 37 °C.

3. Discussion

The CLEA enzyme immobilization technology has many advantages in the context of industrial applications. The method is simple and amenable to rapid optimization, which translates to low costs and short time-to-market [20]. The project aimed to develop and optimize the production of a cross-linked enzyme aggregate lipase product. This has led to the development of a Lipex CLEA which has been shown to be more homogenous, stable for longer in wash formulation, less protease-sensitive and to have a better wash performance after long-time storage when compared with the commercially available equivalent enzyme. The overall product yield by the process is below 50%, which still requires optimization for a commercial process to be economically viable. There is the opportunity that the remaining amount of unmodified enzyme material, which is still active, could be fed back into the cross-linking process to enhance the total overall product yield.

The Lipex CLEAs have been obtained by improving the cross-linking method in the presence of Tween 80 as an activator/enhancer. This compound most likely binds to the active site region of the enzyme rendering it in its open conformation, while undergoing the precipitation and cross-linking reaction. The introduction of a reaction to stop the cross-linking reaction, using amine buffer, into the production process has led to highly homogeneous CLEAs with a small particle size distribution as shown by the sedimentometer experiments.

A technology has been developed to stabilize the Lipex enzyme to make it more appropriate for commercial applications in the detergent industry. This methodology could be applied to help stabilize other enzymes that are used in commercial detergents

4. Materials and Methods

4.1. Materials

Lipex 16L^{TM} was obtained from Novozymes (Bagsværd, Denmark), Lipex CLEA was produced in-house according to the process developed, Lipase-sensitive stains: CS61–Beef fat stain, and C46B-Used fry fat (CFT BV) were from Warwick Equest Ltd (Durham, United Kingdom). All other chemicals were obtained from Sigma-Aldrich (Irvine, United Kingdom). All chemicals used were of analytical grade.

4.2. Synthesis of Lipex CLEA

At room temperature in a 50 mL Falcon tube, Lipex 16L (liquid concentrated enzyme solution) was prepared to a final concentration of 0.69 μM with 30 mM MES-NaOH, pH 7.5, 150 mM NaCl, and 1 mM $CaCl_2$ to a final volume of 5 mL. Tween 80 was added to a final concentration of 19 mM in order to activate the Lipex by locking the lid in the open conformation and the solution was left stirring at room temperature on a magnetic plate at 150 rpm for 5 min. Ammonium sulphate was slowly added to a final concentration of 80% and the solution stirred at 150 rpm for 30 s prior to the addition of glutaraldehyde at a final concentration of 5 mM. The CLEA reaction was stirred for 17 h at 4 °C at 150 rpm in a clear 50 mL Falcon tube. The reaction was stopped with 27 mL of amine buffer (1 M Tris-HCl pH 8.0). The Lipex CLEA was mixed for 5 min with the amine buffer using a Pasteur pipette and centrifuged at 24,000 *g* for 40 min at 4 °C. To wash the Lipex CLEA, 27 mL of dH_2O was added to the sample. The Lipex CLEA was mixed for 5 min with the water using a Pasteur pipette and centrifuged at 24,000 *g* for 40 min at 4 °C. The supernatant was decanted and 30 mL of dH_2O was added to the CLEA pellet. The CLEA samples were mixed with the dH_2O using a Pasteur pipette until the CLEA particles were dispersed evenly, and then centrifuged at 24,000 *g* for 40 min at 4 °C. This was repeated three times in order to wash the CLEA thoroughly. The CLEA pellet was dispersed in 5 mL of 30 mM MES-NaOH, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$ or in 5 mL of Unilever formulation and mixed at 150 rpm for 17 h at 4 °C in order to obtain a homogeneous preparation of CLEA particles in suspension. The final enzyme preparation was stored at 4 ± 1 °C. At the end of the process, 57% of the enzyme was cross-linked with a final concentration of Lipex in CLEA of 0.40 μM. It is important to note that the not cross-linked enzyme is still active and can be used for further applications, which would make the process more cost-efficient.

4.3. Size Determination of the CLEA

The different samples have been measured with a Turbiscan MA 2000 sedimentometer (Sci-Tec Inc., Sandy Hook, CT, USA) to scan a vertical cylinder of sample over a distance not exceeding 80 mm with 40 µm resolution. The rate of scanning is variable from 1 min up to several hours and can follow the kinetics of sedimentation. The temperature was set to 21 °C. The scanning frequency was varied according to the rate of sedimentation, initially set at the minimum of one scan per minute. From the rate of change of the sedimentation height of the material in the tube, the particle velocity was calculated, from which the particle size was calculated using the Stokes–Einstein equation. As these are related to particle size and phase volume, it is possible to evaluate the maximum and minimum sizes of particles contained in the material.

4.4. Activity Assay

The standard activity test [33] was performed in a 96-well micro-titre plate with a *p*-nitrophenyl (PNP) ester as the substrate. A 20 µL volume of diluted enzyme sample was added to each well of a standard micro-titre plate followed by 100 µL of 100 mM Tris-HCl pH 8.5, 60 µL water and 20 µL of 1 mM PNP-laurate in water adjusted to pH 4.5. The activity was measured by monitoring the release of free PNP at 405 nm during the 15 min incubation at room temperature.

4.5. CLEAs Dispersion in Aqueous Environment

Having observed that CLEAs are settling in solution, the suspending properties of a structured liquid were tested for keeping the Lipex-CLEAs in solution during long-term storage. Three test tubes containing 100 mg of Lipex CLEAs were set up with only water, 0.25% citrus pulp/water and 0.1% citrus pulp/0.4% clay in laundry formulation, respectively. The tubes were monitored for four weeks at room temperature for observation of potential settling of the clear particles.

4.6. Storage Stability

The long-term stability of the CLEAs in buffer (100 mM Tris-HCl pH 8.5) has been evaluated over 12 weeks. During the experiment, the CLEAs have been stored at 4 °C in the cold room and were mixed for 17 h at 150 rpm prior to each point of activity measurement. In addition, to simulate the storage of the Lipex CLEA in a commercial laundry formulation, further stability tests were performed by incubating the samples at 37 °C for four weeks in a laundry formulation with and without protease (125 µL enzyme sample in 2.375 mL laundry formulation without enzymes). The activity was measured with the standard hydrolytic assay, taking the activity measured at time 0 as 100%.

4.7. Wash Performance Methods

4.7.1. Micro-Titre End-Point Stain Removal Assays

Fabric lipase-sensitive stains, CS61 (Beef fat on cotton) and C46B (Used fry fat and violet dye on cotton), were hole-punched into discs and transferred to 96-well micro-titre plates (MTP). The stained clothes were washed to remove any residual free stain. The wash liquor contained the following reagents: Enzyme (Lipex or CLEA Lipex) 10 mg/L final; 20 µL of laundry formulation (Persil^{TM}) containing no enzymes 0.8 g/L final; Water 160 µL.

The reaction was incubated at 40 °C for 1 h with shaking at 250 rpm. The clothes were washed by adding 200 µL of distilled water to each well, followed by shaking at 900 rpm for 1 min. The wash liquor was then removed. This procedure was repeated three consecutive times, then the clothes were dried overnight at room temperature. After drying, the stain plates were digitally scanned and their deltaE measured. This value is used to express cleaning effect and is defined as the colour difference between a white cloth and that of the stained cloth after being washed. Mathematically, the definition of deltaE is:

$$\text{deltaE} = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$$

where ΔL is a measure of the difference in darkness between the washed and white cloth; Δa and Δb are measured for the difference in redness and yellowness, respectively, between both cloths. From this equation, it is clear that the lower the value of deltaE, the whiter the cloth. With regard to this colour measurement technique, reference is made to Commission International de l'Eclairage (CIE); Recommendation on Uniform Colour Spaces, colour difference equations, psychometric colour terms, supplement no. 2 to CIE Publication, no. 15, Colorimetry, Bureau Central de la CIE, Paris 1978. The cleaning effect is expressed in the form of a stain removal index (SRI):

$$\text{SRI} = 100 - \text{deltaE}. \text{ The higher the SRI, the cleaner the cloth, SRI} = 100 \text{ (white)}.$$

4.7.2. Terg-O-Tometer Wash Performance

To better simulate home washing condition, a Terg-O-Tometer (Testfabric Inc.West Pittston, Pennsylvania, PA, USA) has been used. Terg-O-Tometer is a laboratory-scaled multiple washing device that is used for laboratory evaluation of perfumes and fragrances, soaps, detergents, dyes and surfactants. This instrument simulates the action of an agitator-type washer and performs the wash in all its six beakers (wash vessels) under controlled conditions of temperature and speed of agitation. Terg-O-Tometer experiments have been performed using stain cloths and the clean ballast cloths. Ballast was a mixture of woven cotton (10 × 10) and polyester squares (10 × 10). In the Terg-O -Tometer wash study, frying fat with violet dye (CS46B) and beef fat with dye (CS61) cotton stains were included and the dimension was 7.0 × 3.5 cm^2. After washing, the cloths were removed from the wash liquor and the cotton stain cloths were separated from the ballast cloths. The ballast cloths were discarded and the cotton stain cloths were retained. Further cotton stain cloths were rinsed in 1 litre of water and allowed to dry flat at room temperature in the dark overnight. The dried cotton stain cloths were read using an X-Rite instrument (X-rite Pantone, Poynton, United Kingdom) with a UV-excluded light source and the deltaE whiteness measured relative to an unwashed white fabric.

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Conflicts of Interest: The authors declare no conflict of interest.

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