**Porous Magnetic Cross-Linked Enzyme Aggregates (Pm-Cleas) of Porcine Pancreas Lipase as Biocatalysts For Hydrolysis of Tributyrin.**

José Renato Guimarães1, Raquel de Lima Camargo Giordano1, Roberto Fernandez-Lafuente2, Paulo Waldir Tardioli1\*

*1 Graduate Program in Chemical Engineering, Department of Chemical Engineering, Federal University of São Carlos, São Carlos, Brazil*

*2 Departmento de Biocatálisis, ICP-CSIC, Campus UAM-CSIC Madrid, Spain*

*\*Corresponding author: pwtardioli@ufscar.br*

**Highlights**

* Porous magnetic CLEAs (pm-CLEAs) of porcine pancreas lipase (PPL).
* High effectiveness factor and high thermal and operational stabilities.
* Easy recovery using external magnetic field and good reusability.

**1. Introduction**

Cross-linked enzyme aggregates (CLEAs) is an immobilization technique that does not require solid support, allows the use of semi-purified enzymes, and the biocatalyst has higher volumetric activity [1,2]. However, they present some problems, such as low mechanical resistance, difficulty of recovering and intraparticle diffusion limitations [2,3]. In this context, this work evaluated some strategies in the preparation of CLEAs of porcine pancreas lipase (PPL) to reduce these problems, such as: modification of the PPL surface with hydrophobic aldehyde, co-aggregation with protein feeders (soy protein (SP) and bovine serum albumin (BSA)), use of silica magnetic nanoparticles functionalized with amino groups (SMNPs) to aid the CLEA separation, and the use of starch as a pore forming agent.

**2. Methods**

The PPL surface was modified with dodecyl aldehyde (DDA) at a PPL:DDA mass ratio of 1:1 for 3 h at 25°C and pH 10 (100 mM sodium carbonate buffer) under 150 rpm stirring. After, sodium borohydride (1 mg/mL solution) was added to the solution and the reaction proceeded for 0.5 h. The modified enzyme was dialyzed at 4°C for 16 h. The aggregation/precipitation step was carried out by adding 3 mL of ethanol to 1 mL of modified PPL solution (5 mg/mL) prepared in 5 mM sodium phosphate buffer pH 7.0, containing 7.5 mg of BSA (or SP), 7.5 mg of SMNPs, and starch (0.8% w/v). The reaction proceeded for 0.5 h at 4°C under 150 rpm stirring. Glutaraldehyde was added to the suspension (5 µmoles of glutaraldehyde/mg total protein) and the cross-linking reaction proceeded for 15 h at 4°C under 150 rpm stirring. The precipitate was recovered by magnetic separation, washed, and resuspended in 3 mL of 5 mM sodium phosphate buffer pH 7.0. -amylase (100 L) was added in order to wash away the starch by hydrolysis at 25°C for 2 h. Porous magnetic CLEA of PPL (pm-CLEA) was physically and morphologically characterized, and applied in the hydrolysis of tributyrin at 40 °C, pH 8.0 for 4 h under 500 rpm stirring [4].

**3. Results and discussion**

The experimental strategies adopted in this work produced pm-CLEAs of PPL with immobilization yield (IY) around 100% and recovered activities (RA) between 67 and 81% for pm-BSA-CLEA and pm-SP-CLEA, respectively, suggesting that the proteins fedeers and nanoparticles reduced mass transfer problems in the CLEA supramolecular structure [4].

The highest activities for free PPL (32.2 ± 0.65 U/mg protein) and pm-SP-CLEA of PPL (24.13 ± 0.35 U/mg protein) were obtained at pH 8.0, while for pm-BSA-CLEA of PPL (19.14 ± 0.13 U/mg protein) was at pH 9.0. The pm-CLEAs of PPL were more thermally stable than the free enzyme, exhibiting maximum activity at 50°C, with pm-BSA-CLEA achieving 20% catalytic retention at 70 °C. The free PPL showed maximum activity under 500 rpm stirring, while the activities of the immobilized enzyme increased continuously within the range evaluated (250 to 1250 rpm). The pm-SP-CLEA and pm-BSA-CLEA exhibited high stability at 40 °C and pH 8.0, retaining approximately 50 and 80% of activity, respectively, after 10 h of incubation, while free PPL was inactivated after 2 h. These results suggest that pm-CLEAs have a fraction of PPL molecules more cross-linked to the nanoparticles, where stabilization effects should be higher due to the higher rigidity of this material compared to a protein, resulting in improved thermal stability to the immobilized enzyme.

The morphological characterization of pm-SP-CLEAs and pm-BSA-CLEAs using scanning electron microscopy (SEM) showed the presence of non-uniform and porous in the structure of the cross-linked PPL due to the hydrolysis of the starch molecules by the α-amylase, which could explain the high effectiveness factor (around η = 0,65), mainly for pm-CLEA of PPL prepared in presence of SP and SMNPs.

The hydrolysis profiles of tributyrin as a function of the time showed that the free enzyme rapidly loosed its catalytic activity, reaching yields of 36 and 52% for free PPL and pm-SP-CLEA, respectively. After five 4 h-batches, the hydrolysis yield of tributyrin decreased only 7% (from 52% to 45%), confirming the high mechanical and operational stability of pm-SP-CLEA.

**4. Conclusions**

Porous magnetic CLEAs co-aggregated with soy protein and magnetic nanoparticles showed good catalytic properties and performance/reusability in the hydrolysis of tributyrin. The strategies used in this work allowed reducing problems of low mechanical and operational resistance, improvements in intraparticle mass transport, and ease of recovery and reuse of the biocatalyst.

**Reference**

1. Cao, L.; Rantwijk, F.; Sheldon, R.A. Cross-Linked enzyme aggregates: A simple and effective method for the immobilization of *Penicillin Acylase*. Org. Lett. 2000, 2, 1361–1364.
2. Garcia-Galan, C.; Berenguer-Murcia, Á.; Fernandez-Lafuente, R.; Rodrigues, R.C. Potential of diferente enzyme immobilization strategies to improve enzyme performance. Adv. Synth. Catal. 2011, 353, 2885–2904.
3. Cui, J. D.; Jia, S. R. Optimization protocols and improved strategies of cross-linked enzyme aggregates technology: Current development and future challenges. Crit. Rev. Biotechnol. 2015, 35, 15–28.
4. Guimarães, J.R.; Giordano, R.L.C.; Fernandez-Lafuente, R.; Tardioli, P.W. Evaluation of strategies to produce highly porous cross-linked aggregates of porcine pancreas lipase with magnetic properties. Molecules, 2018, 23, 2993.