Production and Characterization of Reinforced Cross-linked HEWL Crystals (RCLECs)

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Introduction

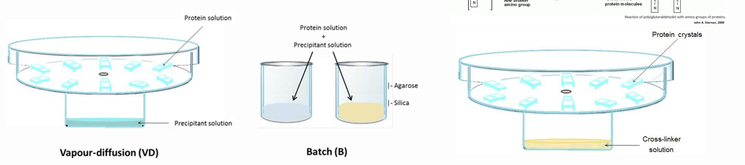
Biocatalysts are employed in several industrial and pharmaceutical processes for the production of novel compounds and/or natural products owing to the versatility that enzymes offer. One of the most common strategies to increase the lifetime and efficiency of enzymes is their immobilization in different materials. Cross-linked enzyme crystals (CLEC) provide a powerful solution to improve the lifetime and recoverability, which results in a higher efficiency. Due to difficulties in obtaining the crystalline material the focus of the research has shifted towards the use of cross-linked enzymes aggregates (CLEAs)[1]. Yet, there is great interest in the development of new methodologies/strategies to produce CLECs for biotechnological applications.

Aim of the work

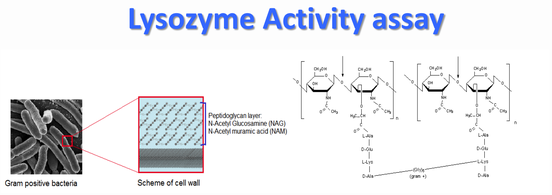
We have explored the use of agarose[2] and silica gels[3] to produce reinforced enzyme crystals and to control the diffusion of cross-linkers within the crystals while avoiding any osmotic stress. The proof of concept and the definition of standard procedures have been carried out using the model protein lysozyme (HEWL). The production of Reinforced Cross-linked Enzyme Crystals (RCLECs) and preliminary characterization of lysozyme activity measurements are shown in this poster.

Methodology

Crystallization and Cross-Linking set-ups



Lysozyme Activity assay



Methodology

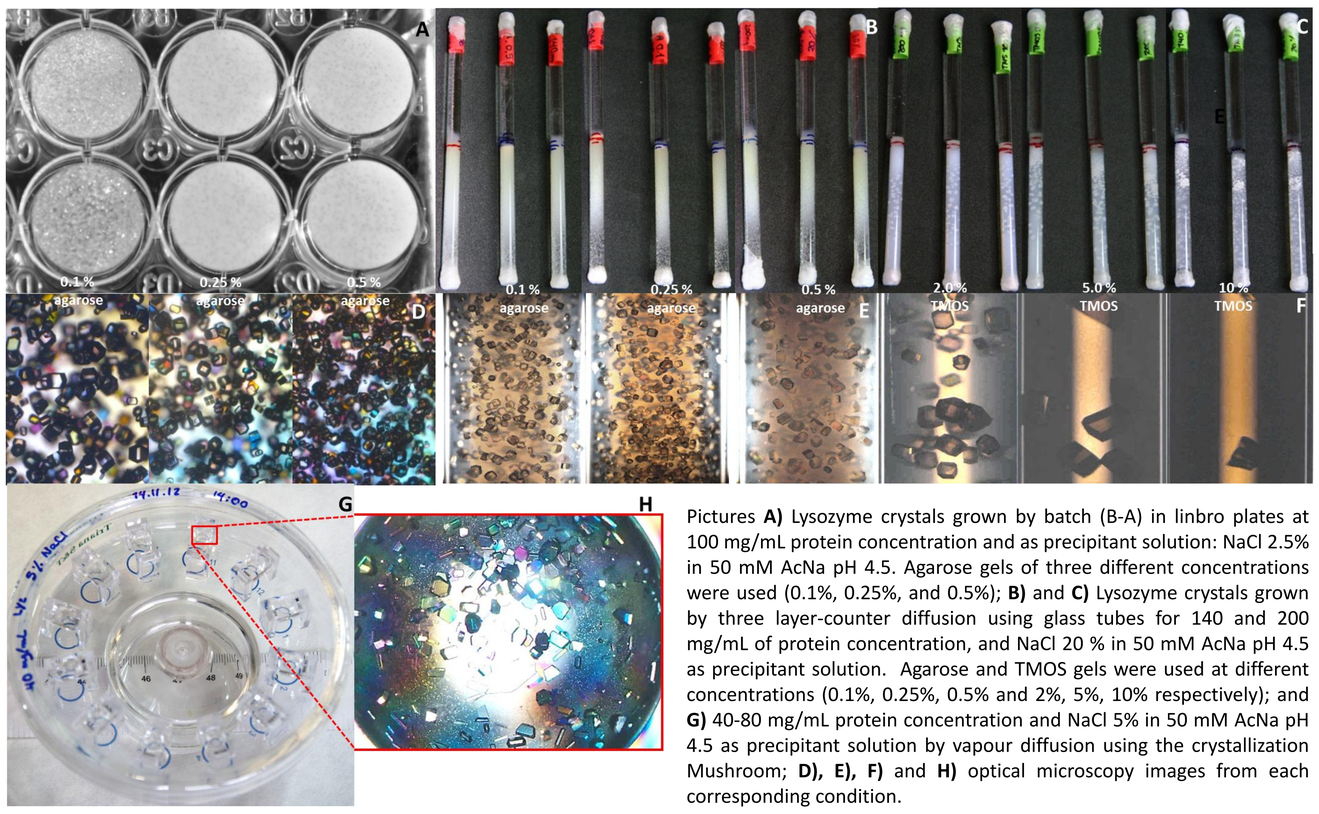
Enzymatic reaction of crystalline lysozyme was tested using two protocols: *The **turbidimetric assay** (a modification of Shugar method[4]) and the **Fluorescence method**[6].* Lysozyme hydrolyzes the β-glycosidic linkage between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls. The turbidimetric assay was performed using a 0.30 mg/mL *Micrococcus lysodeikticus* suspension and analyzing the change in absorbance at 450 nm using a Varian Cary-100 spectrophotometer with a 1 cm path length quartz cells. Lysozyme activity was followed by the clarification of the insoluble polymers of bacterial cell-walls. Alternatively the we used a fluorescence assay that follows the formation of 4-MeU when the enzyme hydrolyze the substrate (GlcNAc)3-MeU). The fluorescence spectra were recorded with a Cary Eclipse Fluorescence Spectrophotometer using 1 cm path length quartz cells. In both cases the temperature of reaction was maintained at 20° C.

Proof of concept

The model protein lysozyme was used as test enzyme since crystallization in gels[5] enzymatic activity determination[4, 6] and cross-linking protocols[7] are well known. We have grown HEWL crystals in solution and compared with crystal grown in agarose and silica gel for stability, crosslinking efficiency and retention of enzymatic activity.

CRYSTALLIZATION

Lysozyme was crystallized by: batch (B-A in agarose gel) using limbro plates; counter diffusion (Three Layer: 3L-CD in agarose and TMOS gels) and by vapour diffusion (sitting drop: SD-VD in solution) using the crystallization Mushroom[8].



Pictures **A)** Lysozyme crystals grown by batch (B-A) in linbro plates at 100 mg/mL protein concentration and as precipitant solution: NaCl 2.5% in 50 mM AcNa pH 4.5. Agarose gels of three different concentrations were used (0.1%, 0.25%, and 0.5%); **B)** and **C)** Lysozyme crystals grown by three layer-counter diffusion using glass tubes for 140 and 200 mg/mL of protein concentration, and NaCl 20 % in 50 mM AcNa pH 4.5 as precipitant solution. Agarose and TMOS gels were used at different concentrations (0.1%, 0.25%, 0.5% and 2%, 5%, 10% respectively); and **G)** 40-80 mg/mL protein concentration and NaCl 5% in 50 mM AcNa pH 4.5 as precipitant solution by vapour diffusion using the crystallization Mushroom; **D), E), F)** and **H)** optical microscopy images from each corresponding condition.

CROSS-LINKING

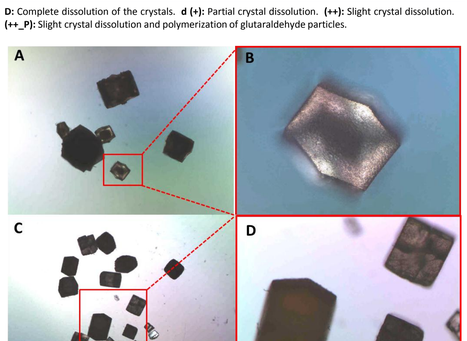
The cross-linking reaction was optimized by varying the concentration of glutaraldehyde and the incubation time. The efficiency of the cross-linked product was evaluated by visual inspection of the dissolution of lysozyme crystals versus time (Table 1). In the case of counterdiffusion experiments we evaluated only the cross-linked concentration.

Table 1: *C*ross-linking screening study for lysozyme crystals at different concentrations of glutaraldehyde and incubation times.

	[Glutaraldehyde]							
Incubation Time	**0.8 %**	**1.6 %**	**2.5 %**	**3.0 %**	**3.5 %**	**4.0 %**	**5.0 %**	**6.0 %**
10 min	D	D	D	D	D	D	d (+)	d (+)
60 min	D	D	D	d (+)	(++)	(++)	(++)	(++)
120 min	D	D	D	d (+)	D (+)	(++)	(++)	(++)
180 min	D	d (+)	d (+)	(++)	(++)	(++)	(++)	(++)
24 Hrs.	d (+)	(++)	(++)	(++)	(++)	(++)	(++)	(++)
48 Hrs	d (+)	(++)	(++)	(++_P)	(++_P)	(++_P)	(++_P)	(++_P)
168 Hrs	(++_P)	(++_P)	(++_P)	(++_P)	(++_P)	(++_P)	(++_P)	(++_P)

D: Complete dissolution of the crystals. **d (+):** Partial crystal dissolution. **(++):** Slight crystal dissolution. **(++_P):** Slight crystal dissolution and polymerization of glutaraldehyde particles.

CROSS-LINKING CRYSTALS ISOLATED FROM THE GEL . Optical microscopy images of: A) lysozyme (200 mg/mL) crystals grown in agarose gel by 3L-CD using glass tubes and cross-linked with 3% glutaraldehyde. B) and D) Crystals grown from agarose gel were cleaned and recovered by gel melting in Milli-Q water at 80 °C while crystals grown in silica gels were recovered mechanically rubbed between two Ethylene Vinyl Acetate slides (EVA foam) and cleaned with Milli-Q water. C) Lysozyme (200 mg/mL) crystals grown in 2 % TMOS by 3L-CD and cross-linked with 2.5% glutaraldehyde.



References

[1] Brady, D. et al. *Biotech. Letters,* 31 (2009) 1639; **[2]** Gavira, J. A.; García-Ruiz, J. M. *Acta Crystallogr*., Sect. D 2002, 58, 1653–1656; **[3]** Gavira, J. A.; et al. *Cryst. Growth Des.,* 2013, 13 (6), pp 2522–2529; **[4]** Shugar, D. *Biochim. biophys. Acta (Amst.),* 8 (1952) 302; **[5]** Lorber, B. et al., Prog. Biophys. Mol. Biol. 2009, 101 (1), 13–25; **[6]** Yang, Y. & Hamaguchi, K. *Journal of Biochemistry* 88, 829-836 (1980); **[7]** Migneault, I., et al. *Review. BioTechniques*, Vol. 37 (2004) 5; **[8]** García-Ruiz J. M. & González-Ramírez L. A., *Protein crystallizations 2nd* Ed. Terese Bergfors; **[9]** Adelaine K. W. Leung, et al. *J. Appl. Cryst*. (1999). 32, 1006-1009.

Acknowledgements

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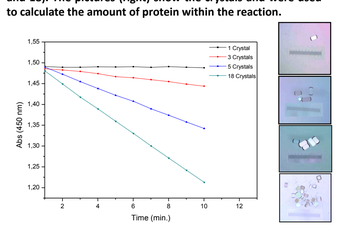
CHARACTERIZATION

ENZYMATIC ACTIVITY: We have determined the retention of enzymatic activity of reinforced lysozyme crystals using two methods as described in M&M. Initial assay using cell-wall fragments did not produce the expected activity when compared with the solution enzyme. We conclude that cell-wall fragments cannot diffuse within the crystal channels. Therefore a more suitable substrate of small size was successfully used.

Protein concentration in the crystals: In order to evaluate the enzymatic activity we need to know the amount of protein in the crystal. Two methods were used: **i)** direct weighted of the lyophilized crystals or **ii)** indirectly estimation from the calculated volume from optical microscopy images. In order to validate the indirect method, lysozyme crystals grown in solution were photographed, dissolved and the amount of protein measured Spectrophotometrically at 280 nm. Different number of cross-linked crystals (lyophilized and no lyophilized) were weighed in an analytical micro-balance with a precision of 10^5g. The amount of protein in the crystals was calculated taking 1.2 g/cm^3 as HEWL crystal density[9].

Turbidimetric method (Shugar).

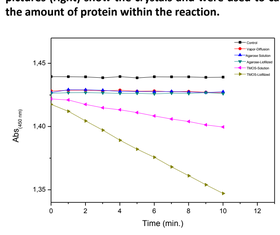
Enzymatic reaction of no cross-linked lysozyme crystals (1, 3, 5 and 18). The pictures (right) show the crystals and were used to calculate the amount of protein within the reaction.



UNTREATED CRYSTALS

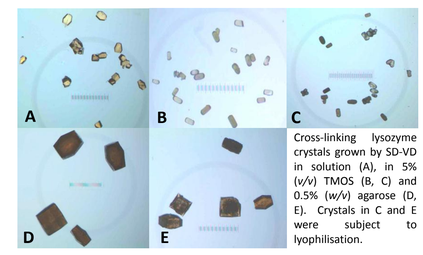
	[Lysozyme] µg/mL	
Sample	**Measured** $_{(280\ nm)}$	**Calculated**
1 crystal	3.4	4.32
3 crystals	10.6	32.4
5 crystals	21.1	38.4
18 crystals	35.0	43.2

Enzymatic reaction of cross-linked lysozyme crystals. The pictures (right) show the crystals and were used to calculate the amount of protein within the reaction.



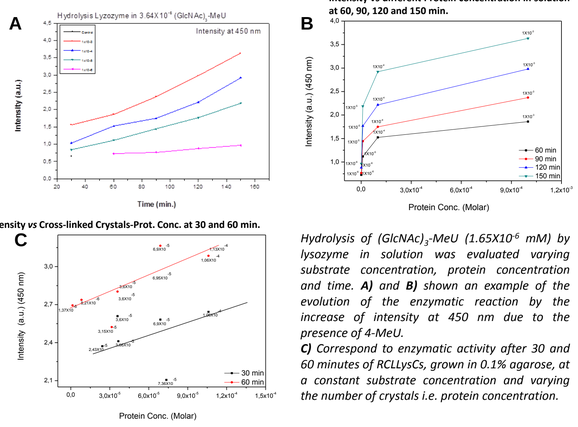
TREATED CRYSTALS

	[Lysozyme] µg/mL	
Sample	**Measured** $_{(280\ nm)}$	**Calculated**
Vapor diffusion	-	126.24
Agarose no lyophylized	-	1344
Agarose lyophylized	-	840
TMOS no lyophylized	-	98.4
TMOS lyophylized	-	96.97



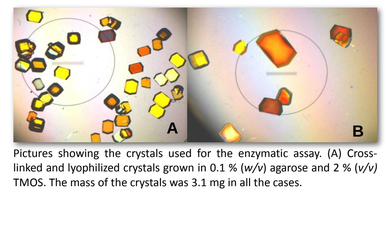
Cross-linking lysozyme crystals grown by SD-VD in solution (A), in 5% (*v/v*) TMOS (B, C) and 0.5% (*w/v*) agarose (D, E). Crystals in C and E were subject to lyophilisation.

Fluorescence methods. *Hydrolytic reaction of $(GlcNAc)_3$-MeU)* catalyzed of lysozyme



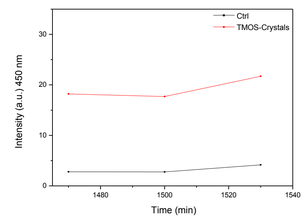
*Hydrolysis of $(GlcNAc)_3$-MeU ($1.65X10^{-6}$ mM) by lysozyme in solution was evaluated varying substrate concentration, protein concentration and time. **A)** and **B)** shown an example of the evolution of the enzymatic reaction by the increase of intensity at 450 nm due to the presence of 4-MeU.*

***C)** Correspond to enzymatic activity after 30 and 60 minutes of RCLLysCs, grown in 0.1% agarose, at a constant substrate concentration and varying the number of crystals i.e. protein concentration.*

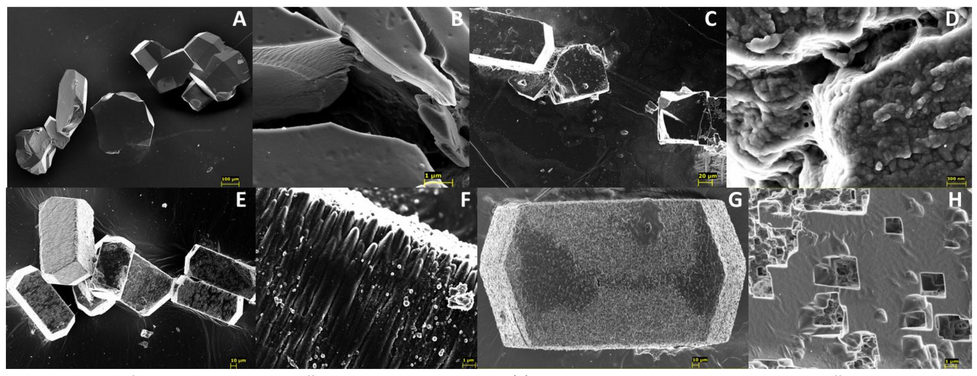


Pictures showing the crystals used for the enzymatic assay. (A) Cross-linked and lyophilized crystals grown in 0.1 % (*w/v*) agarose and 2 % (*v/v*) TMOS. The mass of the crystals was 3.1 mg in all the cases.

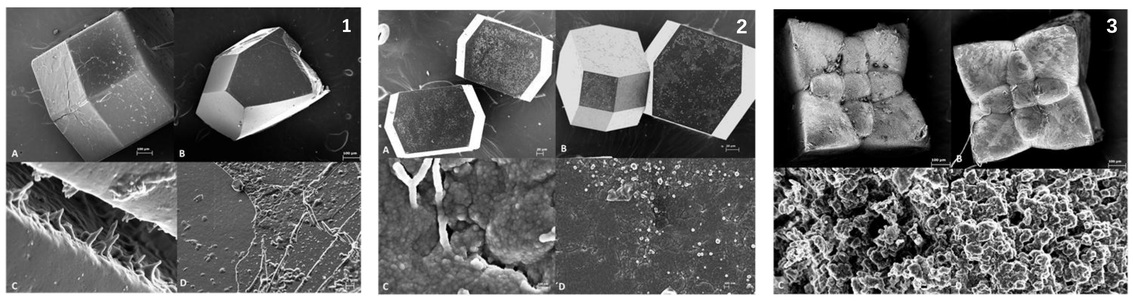
Correspond to enzymatic activity vs time of RCLLysCs, grown in 2.0% TMOS at a constant substrate concentration.



SURFACE CHARACTERIZATION: OF HEWL CRYSTALS GROWN IN SOLUTION AND AT DIFFERENT AGAROSE AND TMOS CONCENTRATIONS, BEFORE AND AFTER THE DEHYDRATION TREATMENT.



FESEM micrographs of HEWL crystals obtained by different techniques and treatments. **(A)** Lysozyme crystals grown in solution by the Vapor Diffusion technique using the crystallization Mushroom and **(B)** a high magnification image. **(C)** RCLPCs crystals growth in agarose gel by counter-diffusion technique using glass tubes and **(D)** a high magnification image of the crystal. **(E)** Cross-linked and lyophilized RCLPCs grown in agarose gel by the counter-diffusion technique using glass tubes and **(F)** a high magnification image. **(G)** Cross-linked and lyophilized lysozyme crystals grown in TMOS gel and **(H)** high magnification image of those crystals.



FESEM micrographs, **1**: A) and B) cross-linked lysozyme crystals lyophilized and not lyophilized respectively, obtained by SD-VD in solution, C) and D) the corresponding magnification images.
2: A) and B) RCLPCs lyophilized and not lyophilized respectively, crystals were obtained by CD technique in agarose gel, C) and D) the corresponding magnification images.
3: A) and B) RCLPCs lyophilized and not lyophilized respectively, obtained by CD technique in TMOS gel, C) and D) the corresponding magnification images.

Conclusions

- We have successfully produced lysozyme crystals in agarose and silica gels that were gently cross-linked in a diffusion mass transport environment.
- The reinforced crystals thus obtained can be easily manipulated and extracted from the gel matrix, although some side effect on the activity was detected in the case of agarose after the recovering procedure by increasing the temperature. Silica grown crystals, on the other hand, could be lyophilize and storage while keeping their enzymatic activity.
- Preliminary studies of activity using both turbidimetry and fluorescence methods showed that the size of the crystals is a key factor to obtain high yields.
- Although we are still analysing the side effects on the activity due to the different treatments, we may conclude that silica-RCLECs is potentially an excellent industrial product.