**Enhancement of mechanical properties of cross-linked enzyme crystals.**

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**Highlights**

* Investigation of mechanical properties of cross-linked enzyme crystals
* Enhancement of crystal stability due to cross-linking immobilization
* Changing of mechanical properties of protein crystals through protein engineering
* Determination of correlation between crystal structure and cross-linking progress

**1. Introduction**

Due to their highly selective reactions, safety and sustainability, industrial use of particular biocatalysts, like protein enzyme crystals has been expanded to many manufacturing sectors including the pharmaceutical production. The catalytic activity of those particles correlates with particle size and must maintain as high as possible during enzyme-catalyzed reactions. Hence, the knowledge of mechanical properties is necessary to avoid particles breakage by downstream processing and thereby, changing or loss of the catalytic activity. Mechanical properties of protein enzyme crystals depend on the structure of individual protein molecules, packing density, as well as the conformation within a 3-dimensional structure of protein particles. In recent years, there has been an increasing interest in the improvement of protein performance using immobilization method or due to protein engineering, which aims at the modification of protein sequence, and hence, its structure, in order to create enzymes with improved functional properties.

**2. Methods**

The purpose of this study was to enhance mechanical stability of protein crystals due to cross-linking immobilization method on the one hand and additional protein engineering for intensified cross-linking on the other hand. For this reason, halohydrin dehalogenase wild type (HheG) was crystallized as model protein, cross-linked using glutaraldehyde and mechanical tested in liquid environment with the aid of an atomic force microscopy (AFM), as described previously1. Those measurements allowed investigation of hardness and Young’s modulus of prismatic and basal crystal faces of hexagonal crystal’s prism depending on the cross-linking duration time. Moreover, a breakage behaviour of cross-linked enzyme crystals, compared to the native crystals is examined using micro-compression tests. In the second step, additional lysine residuals, which can form covalent bonds with the glutaraldehyde-linker, were introduced on the halohydrin dehalogenase wild type (HheG) surface. Then, mutated HheG protein were crystallized, cross-linked and mechanical tested in nanoscale using AFM-based nanoindentation technique again. Due to exchanging of amino acid residuals, increased cross-linking and hence, higher mechanical stability of enzyme crystals was expected.

**3. Results and discussion**

One of the most significant findings to emerge from this study is that the cross-linking reaction takes place in the first 24-hours. In this time both, hardness as well as Young’s modulus increase almost threefold, compared to the reaction time of 4 hours and stay constant at ca. 11.5 MPa (prismatic face) and 500 MPa, respectively. The second major finding was the evaluation of 30%-higher mechanical stability for the basal face. The evidence of this study suggest an anisotropic behavior within the three-dimensional crystal lattice, which was subsequent analyzed using a mathematical model. The applied model enables quantification of theoretical bonds for cross-linking within a supercell and qualitative visualization of potential cross-linking bridges within the crystal structure. The results show that the measured anisotropy might be caused by different direction-dependent amount of bonds on the one hand, and distinct channel orientation within the crystals on the other hand. Interestingly, because of the protein engineering both, hardness and Young’s modulus slightly decreased, compared to the mechanical properties of wild type crystals (see Figure 1). However, the statistical consideration of the results showed a significant reduction of the distribution width, indicating an improved crystal packing density with less flaws.



**Figure 1.** Hardness distribution of HheG wild type and mutant protein crystals. Note that the mean value of mutant HheG crystals decreases by ca. 40%, compared to the wild type crystals.

Additionally, the results of the micro-compression tests show considerable higher mechanical stability against normal forces for cross-linked HheG wild type crystals, compared to the native crystals.

**4. Conclusions**

The present study was designed to enhance mechanical stability of enzyme crystals due to cross-linking immobilization and protein engineering. Using AFM-nanoindentation technique, we were able to investigate small changes in the mechanical properties, like hardness and Young’s modulus on distinct crystal faces dependent on the cross-linking duration time. Due to explanation of measured phenomena using a mathematical model, which bases on the crystal structure, this project provides an important opportunity to advance the understanding of the structure - properties correlation of protein particles and creates fundamentals for future studies on desired formulation of reinforced catalytic active crystalline biocatalysts.

**References**

[1] Kubiak, M.; Solarczek, J.; Kampen, I.; Schallmey, A.; Kwade, A.; Schilde, C. Micromechanics of Anisotropic Cross-Linked Enzyme Crystals. *Cryst. Growth Des.* **2018**, *18*, 5885–5895.