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Breakage of lysozyme crystals due to compressive stresses during cake filtration

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HIGHLIGHTS

- Breakage of lysozyme crystals below 1×10^5 Pa is evidenced.
- Morphology dependent differing breakage mechanisms of lysozyme crystals.
- Filtration properties are influenced but not dominated by crystal breakage.

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ABSTRACT

Compared to other organic and inorganic crystals, protein crystals are much more sensitive to any kind of mechanical stress. The present work focuses on the influence of compressive stresses during cake filtration on lysozyme crystals. Crystals of two different morphologies, isometric and needle-shaped, are considered. Breakage of both crystal types is observed. However, not only the extent, but also the type of breakage mechanism differs for the two types of crystals. Isometric crystals only exhibit breakage of edges away from the whole crystals, whereas needles are broken totally. Compressibility and general cake resistance of needle-shaped crystals are higher than those of isometric crystals. It can be concluded that the breakage of crystals contributes to the general compressibility and resistance of filter cakes, but the influence of absolute size and morphology is much higher.

1. Introduction

Crystallization is a common method for the purification of proteins and has many advantages to chromatographic processes used for purification purposes today. Crystallization is comparatively low-priced, easily scalable and, under ideal crystallization conditions, a highly selective separation process. Furthermore, the dry crystalline product is sufficiently stable for storage and is a formulation suited for the direct application of drugs. Protein crystallization is followed by solid–liquid separation e.g., filtration or centrifugation. Crystallization, chromatography, and solid–liquid separation steps are commonly the parts of a typical downstream processing of a protein. All of the process steps aim to shorten the process time and reduce the costs. At first glance, filtration is a gentle, simple, and inexpensive separation process compared to centrifugation. For conventional inorganic and organic crystalline systems, crystal breakage does not occur at common pressure differences in cake filtration processes (Alles and Anlauf, 2003; Wiedemann, 1996; Bentz, 2009). Nevertheless, protein crystals are much more sensitive to any kind of mechanical stresses. Breakage at low filtration pressures cannot be excluded. The mechanical sensitivity is due to the weak molecular bonds, which are mostly hydrogen bonds, and high water content resulting from the size of the protein molecules (Tachibana et al., 1999; Tait et al., 2008; Zamiri and De, 2010). Breakage of protein crystals may influence cake filtration by increasing cake resistance and compressibility. This may lead to increasing filtration times and a longer downstream processing time, respectively. These bad filtration properties were noticed before for lysozyme crystals as model proteins

Abbreviations: CSD, crystal size distribution; C–P, cell compression–permeability cell; pcr, particle concentration ratio

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(Cornehl et al., 2013). Furthermore, a decreasing particle size aggravates subsequent product formulation steps like drying.

Breakage of protein crystals of two different morphologies, isometric and needle-shaped, is investigated. The morphology of the crystals influences mainly the stress distribution in the filter cake and, hence, the intensity of the load acting on the crystal, which in turn is responsible for the breakage event (Mütze, 2011). Lysozyme is chosen as a model protein, because it is studied very well and crystallization is easy to control. The pressure on the filter cakes is generated by two different devices: A compression–permeability cell (CP cell) for investigations at pressures higher than 1×10^5 Pa and a pressure nutsche for investigations at pressures below 1×10^5 Pa. The choice of two different devices for different pressure regions has experimental reasons, as will be explained later. The main experimental results are the change of crystal size distribution, specific cake resistance, and porosity of the cake due to compressive stresses. Based on the data of specific cake resistances and porosities versus different pressures, compressibility is evaluated. Potential correlations between breakage and compressibility are discussed. Furthermore, the influence of compression time on breakage is highlighted. This allows the equilibrium state of the filter cake regarding filtration properties and crystals size to be determined. Filtration behaviors and pressure stabilities of isometric and needle crystals are compared as regards their suitability for separation by cake filtration.

2. Material and methods

2.1. Crystallization and detection of crystal size distribution (CSD)

Lysozyme is provided by OVOBEST Eiprodukte GmbH and Co. KG, Germany. Two different crystallization conditions were chosen to obtain isometric and needle-shaped lysozyme crystals. To produce isometric crystals, 100 g/l of lysozyme is dissolved in 25 mM sodium acetate buffer at pH 4. Afterwards, the solution was mixed with the same amount of a salt/buffer solution containing 80 g/l sodium chloride. In total, 5 l slurry was crystallized in a double-jacket receiver tank at 20 °C and the solution is stirred for 24 h at 300 rpm with a turbine stirrer of IKA® type R1313. The salt/buffer solution was added very slowly to prevent a local peak of salt concentration which would have caused an amorphous precipitation of lysozyme.

Needle-like growth of lysozyme crystals was achieved by a slight variation of the crystallization conditions. A total slurry volume of 1.5 l was crystallized. The slurry contained 40 g/l lysozyme and 88 g/l sodium chloride in 25 mM sodium acetate buffer at pH 4. The ratio of the lysozyme to salt solution volume was 3:2 compared to 1:1 for isometric crystals. The slurry was crystallized in a 2 l beaker glass at 480 rpm with a floating magnetic stir bar made by NALGENE (DS6630-0400) for 24 h in a room having a temperature of 20 °C. The geometric conditions during crystallization are shown in Table 1.

Table 1
Geometric conditions during crystallization of lysozyme.

	Isometric crystals	Needle-shapedcrystals
Container volume (l)	7	2
Filling volume (l)	5	1.5
Container diameter (cm)	15	12.5
Stirrer diameter (cm)	7	6.25
Height of suspension level (cm)	30	12.5
Stirrer position from container bottom (cm)	19.5	1.7

To evaluate the extent of crystal breakage, the crystal size distribution (CSD) has to be analyzed before and after compression and these values have to be compared. For this purpose, microscopies were taken by a transmitted-light microscope at a magnification of 40× (isometric crystals) and 400× (needle-shaped crystals). Afterwards, the microscopies were converted into binary pictures and the contours were sharpened using Adobe® Photoshop. With the open-source java software ImageJ, particle sizes and areas were measured. The measured size parameter was the maximum Feret diameter. With the help of the data collected, the relevant size distribution parameters were calculated.

Before taking microscopies of the slurries, they were diluted to separate the crystals from each other. The necessary dilution for crystal needles is achieved at slurry to mother liquor ratio of 1:200. A ratio of 1:80 is required for the isometric crystals. To evaluate the size distributions after compression, pieces of the filter cake were resuspended gently in the mother liquor by a slow rotation of the sample. As was shown by the experiments, the influence of the resuspension procedure on the CSD was negligible. A filter cake piece of approximately 0.5 g (wet mass) was resuspended and the resulting slurry was diluted at a ratio of 1:10 for the needle-shaped and 1:30 for isometric crystals.

Sometimes, the dilution of the needle-shaped crystals was not sufficient to separate the crystals from each other. In these cases, the particles were measured manually using ImageJ. All samples were analyzed in triplicate and at least 1000 particles were measured per sample.

For a general evaluation of crystal breakage, the characteristic values or relative changes of particle size distribution are compared with one another. The respective parameter was obtained by dividing the values of 10%, 50%, and 90% of the particle collective size after and before compression.

The mechanisms of crystal breakage were discussed on the basis of the particle concentration ratio (pcr) according to Espig and Reinsch (1998). By means of this function, two particle size distributions are related to each other and breakage behavior can be evaluated over the whole range of particle sizes (Mütze, 2011). The pcr is calculated by dividing the density distributions of the stressed particle system ($q_{r,B}(x)$) by the density distribution of the unstressed crystals ($q_{r,0}(x)$)

$$\mathrm{pcr}(x)=\frac{q_{r,B}(x)}{q_{r,0}(x)}. \tag{1}$$

2.2. Mechanical single crystal analysis

Single lysozyme crystals were analyzed mechanically using a Hysitron TriboIndenter Ti 900 (Minneapolis MN) equipped with a flat punch probe of 100 μm in diameter. The system is designed for a load of 0.1 μN and a displacement of 0.2 nm. The lysozyme crystals were added to the mother liquor in a liquid chamber consisting of a glass slide and a plastic ring attached by silicon grease. By repeated pumping with a disposable pipette, the crystals were distributed homogeneously and allowed to settle for 15 min afterwards. This is resulting in a random orientation of the non-bonded crystals on the glass slide. Within this procedure the crystals are in the mother liquor at all time, to avoid influence of drying to their mechanical properties like observed by Tait et al. (2008).

To ensure compression between two parallel surfaces, the tilt of the flat punch probe was analyzed using the in-situ imaging mode of the TriboIndenter. The sample was placed on an adjustable table and the glass slide was aligned parallel to the probe. Prior to each test, the particle was found to be aggregated or to have the form of a single crystal using the optics integrated in the TriboIndenter (see also Arfsten et al., 2008).