Protein crystallisation: an attractive alternative to protein purification

The production of pure crystals is a method that is normally used for the determination of the spatial structure of a protein using X-ray defraction. Crystalline proteins have a very regular structure, meaning that contaminations can to a large extent be excluded. Therefore, protein crystals only contain a small number of foreign substances, which makes them a lot more stable in solution than proteins are. Due to the aforementioned properties, crystallisation opens up a broad range of applications in industrial protein purification processes. For example, the method can be used for the purification, formulation or storage stabilisation of proteins.

The biopharmaceutical producers hope that this method will help them to replace efficient but expensive purification steps (such as affinity chromatography) with relatively simple and less expensive steps that are just as efficient.

But this is not the only advantage: crystallisation saves a lot of time, said Prof. Roland Wagner, head of development at Rentschler Biotechnologie. The Laupheim-based CRO is a partner in a BMBF-funded project dealing with the technical crystallisation of proteins, which also involves Biberach-based Boehringer Ingelheim and the University of Karlsruhe.

Challenge: Production of crystals from complex protein mixtures

In principle, the problem of protein crystallisation has long been solved, said Roland Wagner. Protein crystallisation has been used since the enzyme urease was successfully crystallised in 1926, and follows the same principles as the crystallisation of low molecular substances (Jones, Ulrich: Industrielle Kristallisation von Proteinen – Eine Frage der Aktivität, in: Chemie Ingenieur Technik 2005; 77, No. 10).
The fine difference is that these crystals are produced from highly pure protein solutions. It is far more difficult to crystallise proteins contained in “dirty” protein solutions, for example proteins found in the fermentation broth after the upstream process. That is where the cooperative project starts.

Any contamination leads to an impure, unfinished crystal; purification is incomplete, said Wagner. The project partners face the challenge of producing good, pure crystals under these difficult conditions, crystals that can cope with the highly complex components of the protein-containing media that interfere with the crystal.

The goal of the project partners is therefore to create conditions that are suitable for creating pure crystals in complex protein mixtures. The Baden-Württemberg research partners are not the only ones pursuing this goal. Technical protein crystallisation is also used in pharmaceutical research, said Wagner. However, the BMBF-funded venture is nevertheless worth the effort, because the technical realisation of protein crystallisation pursued by other institutions is either not disclosed or is protected by patents, and is therefore not available to other manufacturers.

Protein solubility depends on many parameters

Protein solubility is a complex function of a number of different factors. In contrast to low molecular substances, proteins in aqueous solutions cannot be crystallised by evaporating the solvent. Therefore, it is necessary to use certain salts that lead to the precipitation of the proteins.

Sulphate anions and ammonium sulphate have proven to be the best and cheapest substances for the precipitation of proteins. However, other precipitants such as low molecular polyethylene glycol can also be used. Another major factor is the pH of the solution, which has a considerable effect on protein stability and solubility.

Although the crystallisation of proteins can be achieved with precipitants, a number of important questions have to be solved before the industrial production of crystalline proteins becomes possible. These include the reproducibility and the robustness of the crystallisation method and its suitability for pharmaceutical production according to good manufacturing conditions (GMP).

Another important factor is time. Industrial crystallisation needs to be carried out in hours rather than days and must lead to high yields at the same time as guaranteeing the economy of the entire process. The precipitating agents must not be toxic and it must be possible to remove them completely from the system after crystallisation. Another important prerequisite is the ability to store the crystalline suspension at room temperature in order to enable the simple handling of the substances.

Smaller volumes, greater stability

Crystallisation begins with an already purified protein, and then slowly decreases the material's level of purity. The crystallisation of a pure protein is aimed at establishing the stable formulation of proteins in crystalline form.

The commercial benefit of this method will be to decrease the volume of the bulk substance and presumably also to reach a degree of stability that enables the storage of the product without the need for cooling. In the next step, the proteins will be crystallised in the prepurification step. This can also replace chromatography. Subsequently, an investigation will also be carried out to determine whether the method is also suitable as capturing method, which would lead to enormous cost reductions compared to the currently used affinity chromatography methods.

Initially, the researchers will look for crystallisation conditions on the microlitre scale. Steam diffusion methods such as those used in X-ray structure analyses will be used to enable experiments to be carried out with small amounts of protein. The formation of crystals will be monitored under the microscope. Using such approaches it is sometimes possible to harvest crystals manually to analyse them. In the next step, a few parameters will be systematically changed in order to
find the best conditions.

Upscaling in the stirrer tank

Once the small-scale conditions have been established, the protein crystallisation will be upscaled in stirrer tanks. This is a well-known process and upscaling is possible at any time. Of great advantage are small stirrer tanks that enable the precise control of the stirring speed and temperature, which can also easily be transferred to a larger scale. Special focus needs to be put on the compatibility of the crystals' size distribution and morphology with subsequent separation methods.

If the project is successful, then Wagner envisages that the partners will be able to implement the concept in the production process in about two years' time.

Second Rentschler project: the purification of polymer-modified proteins using standard chromatography

In a second project, which is also funded by the BMBF, Rentschler and its industrial and academic partners are looking into the purification of polymer-modified proteins. This project involves Biotest AG, a biotechnology company located in the city of Dreieich and an immunology and haematology specialist, Tosoh Bioscience, a Stuttgart-based producer of chromatographic materials; the Göttingen-based company Sartorius, a purification specialist; the Department of Molecular Processing of Bioproducts at the University of Karlsruhe; the Department of Thermodynamics at the University of Kaiserslautern and the Institute of Cell Biology and Immunology at the University of Stuttgart.

The project partners are using a kind of standardised chromatography method to reduce the costs arising from the purification of the proteins. By coupling big polymers to proteins, it is possible to generate a holistic process that is largely independent from the actual target protein, and can thus be used for almost all modified proteins.

The same matrix for a broad range of different proteins

The objective of the cooperative project is to use the same matrix materials for the purification of all proteins. This would drastically reduce the development costs. It is assumed that the costs can be reduced by coupling the proteins to a big polymer, with the result that the matrix is no longer interested in the small protein, but rather in the big polymer: the matrix binding properties of the protein would then largely be defined by the polymer, which is an important prerequisite for successful separation.

The first step involves the modification of the proteins by combining them with polyethylene glycol (PEG). Established methods will then be used to purify the proteins. The chromatography matrix will have a high affinity to the polymer and will bind it. The research partners from the universities of Karlsruhe, Kaiserslautern and Stuttgart will focus on the investigation of the basic biochemical and biophysical binding of the polymer to the protein.

If the partners succeed, the method will help save considerable sums of money due to the fact that up to ten subsequent steps are normally required to purify proteins like antibodies that cannot be purified using affinity chromatography. The new method would help reduce the number of steps by virtually half and make cost savings of around 30% in the development process.
Hope for rapid integration in the production process

Wagner envisages that it will be possible to rapidly integrate this standardised purification method in the production process due to the immense experience of the researchers with pegylated proteins. The chemistry and binding physics are known and protein pegylation is not new, as the water-soluble polymer is already used as a drug carrier in the pharmaceutical industry, in industrial applications and cell biological research.