A new approach to the crystallization of proteins

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A new method for the crystallization of proteins was developed. Saturated solutions of a protein for crystallization are prepared by chromatography of adsorbed proteins from an insoluble polysaccharide matrix using gradients with decreasing ionic strength, decreasing concentration of organic solvents or an appropriate shift in pH. Insulin, α-chymotrypsin and lysozyme for which the X-ray crystal structures are known, were crystallized by this method. X-ray diffraction photographs were taken to demonstrate the quality of the crystals. In addition to this analytical application, the method may be useful for the crystallization and the simultaneous purification of larger amounts of proteins.

Protein crystallization *α-Chymotrypsin* *Lysozyme* *Insulin* *Polysaccharide matrix* *Chromatography*

1. INTRODUCTION

The traditional methods and reagents for the crystallization of biological macromolecules have been described by Mc Pherson [1]. The most commonly used techniques are the introduction of precipitating agents into aqueous buffered solutions of the macromolecules via vapour phase or liquid phase diffusion. Selection of the most favourable conditions for the crystallization of a given protein or other macromolecule is tedious and time-consuming and depends not only on the knowledge of the properties of macromolecules but also on the proper matching of numerous other parameters necessary for crystallization. The selection of appropriate conditions is usually further hampered by difficulties in reproducing experiments when material-saving micromethods have to be used. Here we present a new approach which allows the selection of crystallization parameters during the continuous process based on reverse-gradient chromatography on polysaccharide gels. This approach is exemplified for lysozyme [2], α-chymotrypsin [3,4] and insulin [5], which have previously been crystallized and their structure determined by X-ray analysis.

2. MATERIALS AND METHODS

Lysozyme from hen egg white was purchased from Sigma (St. Louis, MO), porcine insulin was from 'Polfa' (Tarchomin, Poland), α-chymotrypsin from bovine pancreas was from Koch-Light Laboratories (Suffolk, England), Sepharose 4B and Sephadex G-25 were the products of Pharmacia (Uppsala, Sweden). Polyethylene glycol of M_r 6000 (PEG-6000) and 2-methyl-2,4-pentanediol (MPD) were from Serva, Feinbiochemica (Heidelberg, FRG). Isopropanol and ammonium sulphate were from Merck (Darmstadt, FRG). All other reagents were of analytical grade and purchased from POCH (Gliwice, Poland). Saturated salt solutions were prepared at 22°C. Their dilution is given as a percent related to total saturation (10%).

3. RESULTS AND DISCUSSION

Proteins and nucleic acids can be precipitated from aqueous solutions by salting-out with in-

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creasing ionic strength. In the presence of water-insoluble polysaccharides salting-out results in a precipitation of the macromolecules on the polymer surface [6,7]. The reverse solubilization can be achieved by a gradual decrease of the ionic strength in the elution buffer. This principle previously applied for purification of proteins [7] and nucleic acids [6] is utilized in this work to prepare saturated solutions of proteins for crystallization. In extension of the previous work, in which mainly changes in ionic strength were used to elute the proteins, we also used shifts in both pH and the concentration of organic solvents to achieve elution of the saturated protein solutions for crystallization experiments.

For the crystallization of lysozyme a Sepharose 4B chromatography column was used. The enzyme could be precipitated on a column with different precipitating agents. Either NaCl, isopropanol, PEG-6000 or MPD was used at different pH conditions. The amount of lysozyme adsorbed on the column was varied as well. An example of lysozyme crystallization after its elution from a Sepharose 4B column on an NaCl reverse linear gradient is shown in fig.1. The peak of lysozyme appears at 8.6% saturation of NaCl. The concentration of the protein in this fraction corresponds approximately with the turbidity point which was independently determined for each precipitating agent. At this concentration of NaCl (tubes 8–10) crystallization of lysozyme occurs. The results of more systematic studies of the lysozyme crystallization are summarized in table 1. Using the chromatographic procedure to prepare saturated protein solutions, the crystals formed at pH 4.8 and pH 7.5, provided NaCl or PEG-6000 were used as precipitating agents. The crystallization was not dependent on the concentration of lysozyme and took place in 2–3 days at room temperature. In the case of isopropanol as precipitating agent crystals were observed only at pH 7.5. Crystals did not grow in either acidic or neutral pH if ammonium sulphate or MPD were used for precipitation.

The crystallization of insulin was achieved by chromatography on Sephadex G-25. This material is more stable in acidic solutions and at elevated temperatures compared to the Sepharose matrix.

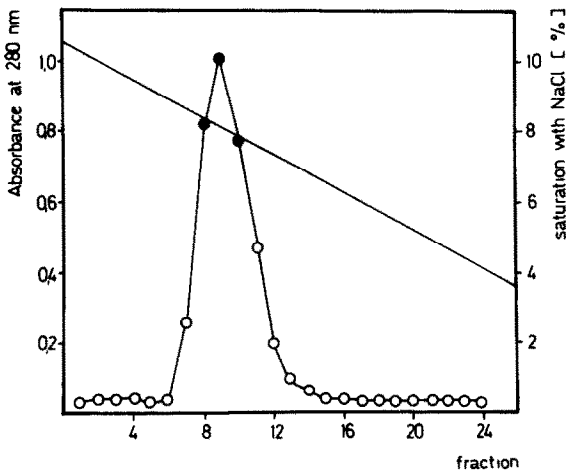

Fig.1. Crystallization of lysozyme. A Sepharose 4B column (0.5 × 5.7 cm) was equilibrated with 11% saturated NaCl in 20 mM Tris/HCl buffer, pH 7.5, containing 10 mM $MgCl_2$. Lysozyme (1 mg in 10 μl buffer) was applied to the column and 10 μl of the same buffer with 22% saturated NaCl was added. The precipitated protein was eluted with a linear gradient from 11% saturated to 0% NaCl (2 × 3.5 ml). Fractions of 250 μl were collected in glass tubes at a flow rate of 5 ml/h. (●) Crystallization of the protein occurred within 2–3 days at room temperature.

Table 1

Crystallization of lysozyme from various eluates of Sepharose 4B columns

Gradient starting from	Amount of lysozyme (mg)		
	1.0	2.5	5.0
NaCl saturated to 11%	+ (+)	+ (+)	+ (+)
38% isopropanol	+ (−)	+ (−)	+ (−)
$(NH_4)_2SO_4$ saturated to 8%	− (−)	− (−)	− (−)
52% MPD	− (−)	− (−)	− (−)
20% PEG-6000	+ (+)	− (−)	+ (+)

A column of Sepharose 4B (0.5 × 5.7 cm) was equilibrated with a buffer containing 20 mM Tris/HCl, or 50 mM sodium citrate, pH 4.8 (data in parentheses), 10 mM $MgCl_2$ and precipitating agents in indicated concentration. A gradient (2 × 3.5 ml) up to 0% of the precipitating agent was applied. 250 μl fractions were collected. The crystallization of the fraction with highest protein concentration is indicated. +, good crystals; −, no crystals or microcrystalline precipitate

The protein was precipitated at pH 6.4 and then eluted from the column at 50°C by a pH gradient from pH 6.4 to pH 5.4 (fig.2). The insulin-saturated solutions were then treated with $ZnSO_4$, placed in a thermos flask at 50°C and slowly cooled. Crystallization occurred in 2 or 3 fractions after 18 h. The variation of sodium citrate buffer concentration in the range between 100 and 150 mM did not have any effect on the crystallization of the insulin. The experiment in fig.2 demonstrates, in addition, that the principle of reversed-gradient chromatography, using solutions of decreasing ionic strength [7], can be extended to variation of pH at constant salt concentration.

Protein precipitation on a Sephadex G-25 column by addition of an organic solvent and its elution by decreasing a gradient of isopropanol is

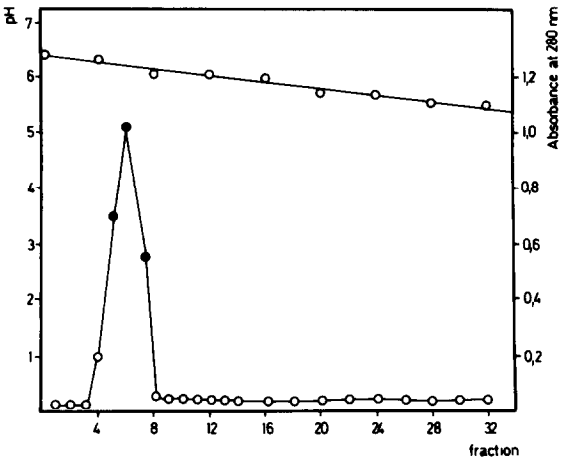

Fig.2. Crystallization of insulin. A jacketed column of Sephadex G-25 was equilibrated with 50 mM sodium citrate buffer, pH 6.4, at 50°C. Insulin solution (1.25 mg) in 50 μl of 30 mM HCl was applied to the column and additional 50 μl of 50 mM sodium citrate, pH 6.4, were added to achieve a quantitative precipitation of the protein. Elution was performed with a linear gradient of 2 × 3.5 ml from 50 mM sodium citrate, pH 6.4, to 50 mM sodium citrate, pH 5.4. Fractions of 200 μl were collected at a flow rate of 6 ml/h. To protein-containing fractions 5 μl of 75 mM $ZnSO_4$ was added. These fractions were put into a thermos flask at 50°C and slowly cooled. After 18 h the crystals started to occur (●). No differences were observed by variation of the sodium citrate concentration in the elution buffer in the range between 100 and 150 mM.

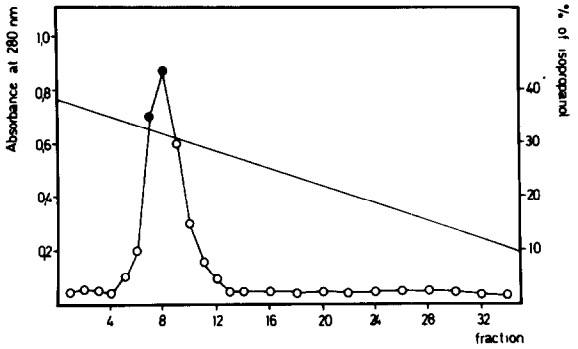

Fig.3. Crystallization of α-chymotrypsin. The same column of Sephadex G-25 as described in fig.2 was used. α-Chymotrypsin (1.05 mg in 50 μl of 50 mM Tris/HCl, pH 6.8) applied to the column which was previously equilibrated with the same buffer containing 38% isopropanol at room temperature. The column was washed with a linear gradient of isopropanol from 38 to 0% (2 × 3.5 ml) in the same buffer. Absorbance at 280 nm was monitored. Fractions of 200 μl were collected and stored at 15°C. The protein crystallized after three days (●). Similar results were obtained under analogous conditions using a linear gradient of MPD and ammonium sulfate in the above buffer. In these cases the concentration boundaries of the precipitating agents were 45 to 0% and 44 to 0%, respectively.

demonstrated for α-chymotrypsin in fig.3. The protein was eluted as a single peak and in fractions of the highest protein concentration, crystals formed within 3 days at room temperature. The crystallization was not dependent on the amount of protein applied to the column. The concentration of the organic solvent in the corresponding fraction is the most important parameter which determines the crystallization process. Similar results were obtained for MPD and ammonium sulfate, when used for the precipitation of the protein. Here, however, gradients from 45 to 0% MPD and 44% saturation of ammonium sulfate were utilized to achieve the elution and crystallization of the protein.

In all cases the crystals formed were analysed by taking X-ray diffraction photographs, which were compared with published data [2–5].

4. CONCLUSIONS

We have demonstrated that crystallization of proteins can be combined with a common purifica-

tion procedure using reverse-salt-gradient chromatography. Saturated solutions of proteins can be produced by this method under very mild and reproducible conditions. An oversaturation leading to formation of an amorphous precipitate can be avoided by this chromatographic procedure. In addition, to decreasing ionic strength, pH shifts and decreasing concentrations of organic solvents (or precipitating agents) can also be used to generate saturated protein solutions. This method allows rapid crystallization of small quantities of protein and in addition the possibility for fast optimization of the crystallization conditions. Since the capacity of the columns is very high, also large quantities of a protein can be purified and crystallized in one step by the described procedure. Although in our approach easily crystallizable proteins as lysozyme and α-chymotrypsin were used, we could demonstrate in the case of insulin that this method considerably accelerates the crystallization process.

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