SEQUENTIAL ELUTION OF PROTEINS BOUND BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY AT SUBZERO TEMPERATURES

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1. Introduction

Use of uncharged hydrophobic absorbents attached to agarose may overcome the difficulty normally raised by the retention of solutes involving both electrostatic and hydrophobic mechanisms and then by the fact that the effects of changes in operating conditions on these two mechanisms are incompatible [1]. Since such an hydrophobic interaction chromatography theoretically operates by a single, specific retention mechanism, which should be quite sensitive to temperature, use of subzero temperatures might provide an extension of current possibilities for the purification of proteins. Recent study of a model system [2,3] has shown that retention was decreased as a function of decreasing temperature, a result expected on the basis of the extensive involvement of hydrophobic interactions, whereas other observations suggest that electrostatic mechanisms could interfere with hydrophobic ones [4–6]. Present data obtained between +20°C and −20°C with α-chymotrypsin, chymotrypsinogen A, ovalbumin and catalase, clearly demonstrate the involvement of both mechanisms of retention, but permit a good resolution in the fractionation of the mixture used as a model system for thermal retention and elution of protein.

2. Materials and methods

2.1. *Products*

The proteins, α-chymotrypsin, chymotrypsinogen A, ovalbumin and catalase were from Sigma. Chemicals were purchased from Merck except phenylbutylamine which was from Fluka. Ethylene glycol was purified by distillation under vacuum and stored in the dark at 4°C. Agarose CL 6B was from Pharmacia Fine Chemical products.

The buffers used were Tris or phosphate at 1 mM. When organic cosolvent was added to the buffers solutions in water or when the temperature was varied, the protonic activity (termed pa_H) was calculated to [7–9].

2.2. *Affinity gel*

The substituted agarose (Agarose PBA) was prepared as in [10] by activation with cyanogen bromide followed by the fixation of phenylbutylamine (PBA), a substrate analogue for α-chymotrypsin. Previous chromatographic experiments have shown that this gel does not pack anormally in mixed solvent ethylene glycol/water (1:1, v/v) and can be submitted to pressure of the order of 1 bar without loss of resolution.

2.3. *Chromatography*

Chromatography was performed with an apparatus already described but modified to use a column with an internal diameter of 1 cm [11]. The high bed gel was of 5 cm. The temperature was regulated with a Colora thermostat bath and measured inside the gel with a thermocouple associated with a millivoltmeter.

The column flowrate, regulated with a peristaltic pump was 0.4 ml.min^{-1}. The fractions were collected at room temperature. The concentrations of various proteins were determinated at 280 nm using an ISCO

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UA5 elugraphe connected at the bottom of the column.

2.4. *Gradient determinations*

Both gradients of organic cosolvent or salt were measured in each fraction (1 ml) with a conductivity meter (Radiometer CDM3) calibrated with standard solutions.

3. Results

3.1. *Behaviour of proteins in ethylene glycol/water mixture at various temperatures*

3.1.1. α-chymotrypsin

A buffered solution (Tris 1 mM, pH 8) of 20 mg α-chymotrypsin is fixed to agarose PBA preequilibrated in the same buffer, at +20°C. At this temperature an increase of ionic strength from 0–1 M KCl does not induce desorbtion. A linear gradient (at +20°C) of ethylene glycol desorbs the protein at the value of 38 ± 2% (by vol.) and this result is not dependent on ionic strength from 0–1 M KCl (fig.1). For a 38% volume ratio of ethylene glycol, in Tris buffer, the pa_H value is roughly the same as in pure water, i.e., 8.

As a solution of protein in ethylene glycol/Tris buffer (50:50, v/v) 1 mM, pa_H 8 at −20°C is applied to the top of the column precooled at this temperature in the same media ($pa_{H-20°C}$ 9.5), α-chymotrypsin is fully retained on the substituted gel. Same result is obtained in 30% ethylene glycol at −15°C. An increase of ionic strength (up to 1 M KCl) does not induce desorbtion. The elution of α-chymotrypsin can be achieved by increasing temp. to +20 ± 5°C.

The results obtained both at +20°C and −20°C are not dependent on the nature of the buffer. The same experiments performed in phosphate buffer 1 mM (pH 8; $pa_{H+20°C}$ 8.5 in 50% EGOH; $pa_{H-20°C}$ 8.8 in 50% EGOH) gave the same results. No retention is observed on the non-substituted agarose.

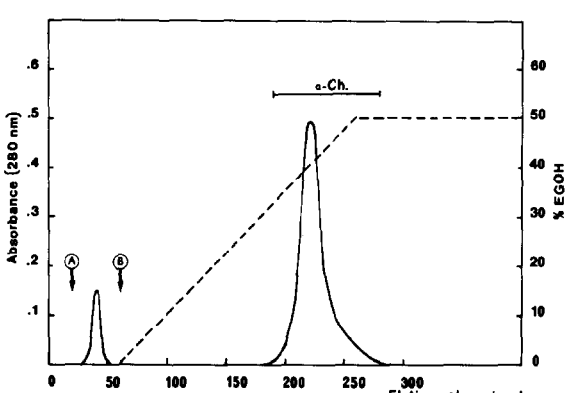

Fig.1. Elution pattern of a +20°C chromatography of α-chymotrypsin with an ethylene glycol gradient. Agarose PBA column. (A) Introduction of the protein. (B) Application of the gradient. Left scale, A_{280}; right scale, ethylene glycol gradient, v/v.

3.1.2. Chymotrypsinogen A

Chymotrypsinogen A applied to the column at +20°C (20 mg in Tris buffer 1 mM, pH 8 is slightly lowered on the agarose PBA (by a factor 1.2 c.f. column void vol.). A v/v of 30 ± 2% ethylene glycol suppresses this delay and in this condition the protein is obtained in the void volume.

In the presence of 30% EGOH, at −15°C, a full retention of the protein is observed. The elution is obtained by increasing temp. to +4 ± 5°C.

The results obtained are not dependent on both the ionic strength (up to 1 M KCl) and the nature of the buffer (Tris or phosphate). As in the case of α-chymotrypsin, no retention occurs on the non-substituted gel.

3.1.3. Ovalbumin and catalase

These two proteins are always obtained in the void volume of the column, both at +20°C in the absence or in the presence of 50% EGOH, or at −20°C in presence of 50% EGOH showing that no interaction occurs between the proteins and the agarose PBA.

3.2. *Fractionation of the protein mixture as a function of temperature*

Figure 2 summarizes the set of operations allowing: first to separate ovalbumin and catalase from α-chymotrypsin and chymotrypsinogen A which are retained at −15°C in presence of 30%, v/v, ethylene glycol/water; secondly to obtain the elution of chymotrypsinogen A by increasing temp. to +4°C; thirdly, in presence of 50%, v/v, ethylene glycol/water at +20°C to obtain the elution of α-chymo-

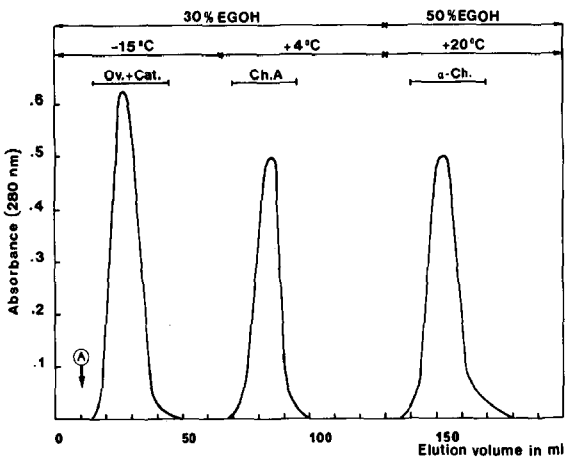

Fig.2. Sequential elution of proteins on agarose PBA column. (A) Introduction of the protein mixture. (Ov., ovalbumin; Cat., catalase; Ch-A, chymotrypsinogen A; α-Ch., α-chymotrypsin.)

trypsin. Note that this 'sequential' elution of different proteins, mostly based on temperature variations, can be carried out at leisure provided selected temperatures are kept constant during elution of each kind of protein.

4. Discussion

Retention of α-chymotrypsin at subzero temperatures clearly indicates that electrostatic mechanisms of binding are involved along with hydrophobic mechanisms. A similar conclusion can be reached to explain the retention of chymotrypsinogen A in such temperature conditions. Recourse must be made to different types of gels to clear this problem and yet to use subzero temperatures to decrease retention of these proteins.

Retention at low temperatures and stepwise elution by increasing temperatures, obtained in the present case, represent a model of a useful application of temperature effects on hydrophobic interactions chromatography and might be generalized to other protein mixtures, opening a new dimension in protein fractionation with mixed solvents as a function of temperature.

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