Affinity chromatographic studies on antigen-antibody dissociation

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An analytical affinity chromatography assay has been developed for the investigation of the dissociation of antigen-antibody complexes. Albumin-coupled Sepharose 4B and anti-albumin has been used as a model system. At extremely low or high pH, in the presence of highly concentrated chaotropic ions at pH 7 or by elution with 100% ethylene glycol after pretreating with high pH buffer, most of the bondings could be ruptured. The latter two-step desorption procedure provides recovery of intact antibody with high yield. The technique was also utilized for the preparation of antibody against human growth hormone.

Antigen-antibody complex *Affinity chromatography* *Mild desorption*

1. INTRODUCTION

One of the advantages of the affinity chromatography technique is its ability to isolate a solute from a biological material selectively. However, the slow adsorption-desorption kinetics of many proteins usually causes contamination of the eluted protein, implying a drawback in the use of the technique for the preparative work as well as for the quantitative analysis purposes. A way to overcome the problem in the former case was described in [1]: one-step purification of lectin from raw extract by affinity chromatography was made possible by extensive washing of the column material on a glass filter prior to desorption and then exposing the desorbed material to a second adsorption-desorption procedure on a smaller column packed with fresh material. Another key problem in affinity chromatography is to determine optimal conditions for the release of the adsorbed solute from the column. Low dissociation constants of many antigen–antibody complexes require drastic conditions for desorption of the adsorbed component from the immunoaffinity column. Thus, powerful desorption agents, such as low pH buffers, guanidine–HCl or chaotropic ions, have been employed frequently (publications until 1971 reviewed in [2]). As the elution with such agents usually leads to loss of the immunological activity of the antigen or antibody, desorption under mild conditions is thus desirable.

This paper describes a study on antigen-antibody dissociation by means of an analytical affinity chromatography assay developed for the purpose. Using HSA–anti-HSA as a model system, the effect of various desorption agents, as well as the conditions for almost full dissociation with intact immunological activity, were investigated. The findings were applicated to the immuno-complex HGH–anti-HGH.

2. EXPERIMENTAL

2.1. *Chemicals*

HSA was obtained from Kabi (Stockholm). Immunoglobulin fraction of rabbit antiserum against HSA was from Dakopatts (Copenhagen). CNBr-activated Sepharose 4B was from Pharmacia (Upp-

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Abbreviations: HSA, human serum albumin; EG, ethylene glycol; HGH, human growth hormone

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sala), agarose, type Indubiose A_{27} from Reactifs IBF (Villeneuve) and EG from Merck (Darmstadt).

2.2. *Apparatus*

For the affinity chromatographic procedure the gel chromatographic isolation equipment in [3,4] was used. As immunoadsorbent columns (1 cm × 4 mm i.d.) graduated 2-ml pipettes were utilized as before but were cut off leaving 5 cm and marked at a bed height of 1 cm. The agarose gel puncher and template used in the immunodiffusion tests were from the workshop of the Biomedical Center (Uppsala).

2.3. *Procedures*

2.3.1. Preparation of immunoadsorbent columns

HSA was coupled to CNBr-activated Sepharose 4B according to manufacturer's instructions [5]. The product was suspended in a buffer (0.1 M Tris–CH_3COOH, pH 7.8, containing 0.5 M NaCl) and packed in the columns. After equilibrating with 4–6 ml buffer using the peristaltic pump (flowrate 6 ml/h) the packings were capped and stored at +5°C.

2.3.2. Affinity chromatographic process

Sets of 3 columns (V_t = 125 μl) were loaded with 50 μl anti-HSA. After washing with two drops of the buffer and draining, the packings were capped and incubated for 30 min at room temperature. Then the empty space was refilled with the buffer and the columns were connected to the pump and washed with 3–4 ml buffer. The columns were then placed on the fraction collector over sets of 16 small test tubes marked for 250 μl, and desorption of the adsorbed antibody was effected in two steps by elution with 2 ml of the appropriate agent (fig.1). Prior to each elution step, the eluent in the pump and the column capillary as well as in the space above the bed surface was replaced by the desorption eluent to be used. The absorbance of the individual fraction was measured at 280 nm against the respective desorption agent.

2.3.3. Immunodiffusion test

To check the immunological stability of the antibody against risks of denaturation by the desorption agents, the antibody was incubated with the respective eluent as follows. Using a disposable Pasteur pipette, one drop anti-HSA and one drop eluent were mixed on a parafilm placed in a petri dish, moistened by means of a wet filter paper in the bottom. After incubation for 16 h at room temperature, two drops of 1 M Tris–CH_3COOH buffer, pH 8.6, was added to each mixture (final antibody dilution: 1/4). Except for guanidine–HCl, the incubation with other salts was done with the double salt concentration for obtaining the final eluent concentration as given in the legend to fig.1. Double diffusion immunoprecipitations [6] were done in 1% agarose in 0.1 M Tris–CH_3COOH buffer, pH 8.6. Agarose plates (1–2 mm thick) were prepared from hot agarose solution (3 ml) cast on a glass plate (7.5 × 2.5 cm). Wells of 2 mm diameter were punched at a free distance of 5 mm between the center well and the outer ones, and the gel plates were placed in moistened petri dishes. For the immunodiffusion test, 5 μl treated antibody was pipetted into a center well and 5 μl of a serial dilution of a 5 mg/ml HSA solution was pipetted into an appropriate outer well. The plates were observed ocularly for the appearance of precipitation lines after 16 h storage at room temperature under humid atmosphere.

3. RESULTS AND DISCUSSION

Elution profiles of anti-HSA are given in fig.1. For a quantitative estimating of the desorption effect of the eluents, the peak areas on the chromatograms were also measured (table 1). The large effect obtained at low pH is in agreement with common observations in affinity chromatography. The acid buffer presumably causes conformational changes which abolish most of the affinity between antigen and antibody. Furthermore, the desorption effect proved markedly enhanced at extremely high pH. On the other hand, the highest recovery of antibody from the immobilized antigen was achieved by elution with chaotropic ions at high concentration (fig.1, table 1) indicating hydrophobic interactions as essential forces maintaining the complex. It has generally been assumed that chaotropic ions change the structure of water in the proximity of hydrophobic regions [7]. As is known, strongly adsorbed substances can be desorbed by adding a polarity-reducing agent such as EG to the high pH buffer, e.g., in the proportion

Table 1

Effect of desorption agent on the release of anti-HSA

Peak in fig.1	Elution step	Elution agent	Elution peak area on the chromatograms (mm^2)
a	1	0.1 M Gly–HCl, pH 2.3	317.5
	2	100% EG	66.2
	1	0.1 M Gly–HCl, pH 2.3	313.7
	1	0.1 M Gly–HCl, pH 2.3	281.2
b	1	0.1 M Gly–NaOH, pH 9.8	93.6
	2	100% EG	302.5
c	1	0.1 M Gly–NaOH, pH 11.5	237.7
	2	100% EG	275.0
d	1	0.1 M Tris–CH_3COOH, pH 7.7, containing 2 M NaCl	106.0
	2	100% EG	295.0
e	1	2 M trichloroacetic acid–NaOH, pH 7.0	408.2
	2	100% EG	90.0
f	1	6 M guanidine–HCl, pH 3.0	361.2
	2	100% EG	106.2
	1	0.1 M Gly–NaOH, pH 9.8, containing 50% EG	102.0
g	1	0.1 M Gly–NaOH, pH 9.8, containing 50% EG and 0.2 M NaCl	148.5
	2	6 M guanidine–HCl, pH 3.0	338.7
	1	0.1 M Gly–NaOH, pH 9.8, containing 50% EG and 1 M NaCl	200.0
	1	0.1 M Gly–NaOH, pH 9.8, containing 50% EG and 2 M NaCl	95.8
h	1	0.02 M Gly–NaOH, pH 9.8, containing 96% EG	287.5
	2	6 M guanidine–HCl, pH 3.0	163.5
i	1	100% EG	165.0
	2	6 M guanidine–HCl, pH 3.0	321.2

1:1 [8,9]. Hydrophobic affinity chromatography has been employed since its introduction [8] for dissociation of many antigen–antibody complexes [10,11]. A preserving effect of EG on proteins is documented [11,12]. Concentrations of 20–60% EG were reported as used to rupture hydrophobic bondings [8,9,11,13,14]. However, dissociation obtained in this way was usually relatively poor [11,14]. Certainly, addition of NaCl to diluted EG enhances the dissociation ability [8]. As seen in table 1, the strongest effect is attained in the presence of 1 M NaCl. To determine whether higher concentrations of EG might promote the desorption capacity, the elution was performed with 100% EG (fig.1i) and the elution peak was compared with that obtained by means of a conventional elution (fig.1g). As the chromatogram illustrates, EG by itself has no noticeable effect. On the other hand, 96% EG at pH 9.8 proved highly effective in dissociating the complex (fig.1h). The strongest effect of EG, however, was attained by elution with 100% EG after the column had

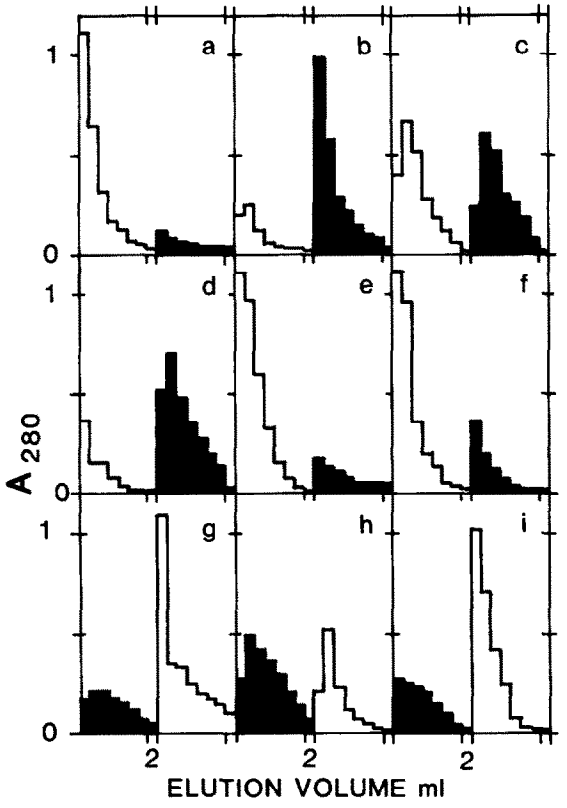

Fig.1. Effect of desorption agent on the recovery of anti-HSA from the immunoadsorbent column. Each packing was loaded, incubated and washed under identical conditions. Two-step desorption was carried out by means of the following agents. The first peaks of the chromatograms (a–f) obtained by elution with (a) 0.1 M Gly–HCl, pH 2.3; (b) 0.1 M Gly–NaOH, pH 9.8; (c) 0.1 M Gly–NaOH, pH 11.5; (d) 0.1 M Tris–CH_3COOH, pH 7.7 containing 2 M NaCl; (e) 2 M trichloroacetic acid–NaOH, pH 7.0; (f) 6 M guanidine–HCl, pH 3.0 and the second peaks obtained by elution with 100% EG throughout; the first peaks of the chromatograms (g–i) obtained by elution with (g) 0.1 M Gly–NaOH, pH 9.8, containing 50% EG and 0.2 M NaCl; (h) 0.02 M Gly–NaOH, pH 9.8, containing 96% EG and (i) 100% EG and the second peaks obtained by elution with 6 M guanidine–HCl, pH 3.0.

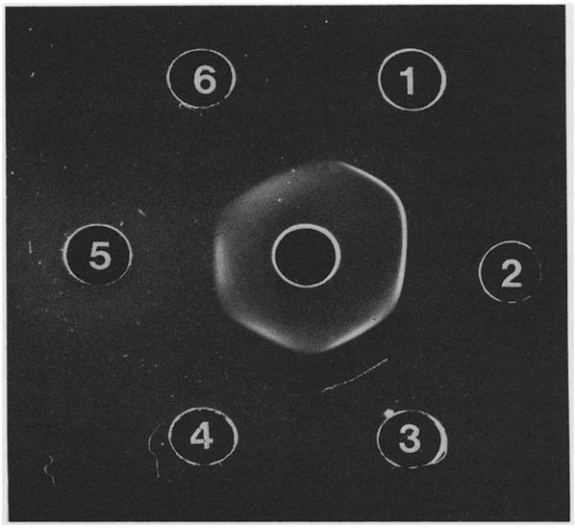

Fig.2. Typical HSA–anti-HSA precipitation lines in agarose gel obtained by immunodiffusion test. Center well containing anti-HSA incubated with EG, and outer wells containing (1) 0.15, (2) 0.31, (3) 0.63, (4) 1.25, (5) 2.50 and (6) 5.00 mg/ml HSA.

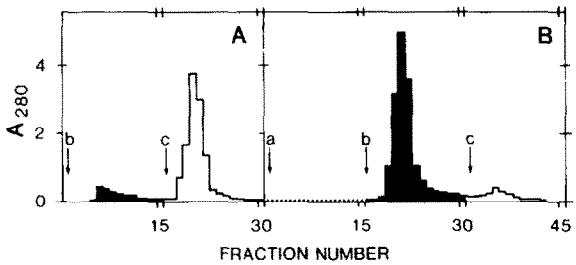

Fig.3. Preparation of anti-HGH. Affinity chromatographic conditions: HGH-Sepharose 4B column (0.9 cm × 9 mm i.d.); starting buffer, 0.1 M Tris–CH_3COOH (pH 7.7) containing 0.5 M NaCl; flow rate, 1 ml/h for sample loading and 6 ml/h for washing and desorption; sample, 3 ml goat antiserum dialyzed against the buffer; fraction volume, 0.3 ml. Desorption chromatograms: arrows indicate starting of elution with (b) 0.1 M Gly–NaOH, pH 9.8, containing 50% EG and (c) 0.1 M Gly–HCl, pH 2.3, in A and (a) 0.1 M Gly–NaOH, pH 9.8, (b) 100% EG and (c) 0.1 M Gly–HCl, pH 2.3, in B.

previously been treated with a high pH buffer (fig.1b). Probably, a powerful resolving of the complex by a polarity-reducing agent takes place after preceding releasing of some key sites at the binding area by altering the degree of ionization of the involved groups. A study of the immunological stability of the antibody against the desorption agents showed that immunodiffusion-precipitation lines (fig.2) could be obtained for all eluents used except for the buffer at pH 2.3 and the guanidine–HCl, pH 3.0 or 7.0. An application of the findings for the preparation of anti-HGH is given in fig.3.

Fig.3A shows a conventional [15] desorption procedure and B illustrates the presented technique. The pretreatment of the immunoaffinity column with a high pH buffer did not cause any desorption, while a subsequent elution with 100% EG provided almost total recovery of the antibody.

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