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Detection and purification of two antibody–antigen complexes via selective adsorption on lowly activated anion exchangers

Manuel Fuentes, Benevides C. C. Pessela, Cesar Mateo, Roberto Munilla, Jose M. Guisán**, Roberto Fernandez-Lafuente*

Departamento de Biocatálisis, Instituto de Catálisis, CSIC, Campus UAM, Cantoblanco, 28049 Madrid, Spain

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Abstract

Taken advantage of the mechanism of adsorption of macro-molecules on ionic exchangers, (a multipoint interaction between the protein and the support), it is possible to selectively adsorb large proteins leaving small ones in the supernatant. Associated proteins should present a significant difference in its size as compared to the non-associated forms. Thus, the protein complexes may have much larger surfaces to interact with the support. Here, by selecting the support with the highest activation degree that was unable to adsorb the non-associated proteins, we have shown the simple and selective adsorption of immuno complexes (as a model), while antibodies and antigens remained in the supernatant. Therefore, it was possible to selectively adsorb on lowly activated supports (e.g., agarose 4BCL having only 1 μmol of amino groups per g of support) rabbit IgG/anti-rabbit immunoglobulins (immuno complex), while these supports were unable to adsorb the individual immunoglobulines. Similarly, horseradish peroxidase (HRP)/anti-HRP were selectively adsorbed on lowly activated supports, while the individual proteins were not adsorbed at all. Afterwards, the adsorbed associated proteins (purified at least from the non-associated counterparts and concentrated by the adsorption on the support) may be cross-linked with aldehyde-dextran and be desorbed from the matrix for their analysis. This strategy may permit very simple experiments to detect the presence of protein–protein complexes. Finally, we have shown the advantages of this technique compared to the use of one of the proteins previous immobilized on a support.

Keywords: Selective adsorption; Ion exchange; Multi-point interactions; Tailor-made supports; Purification of protein–protein complexes

1. Introduction

Ion exchange chromatography using commercial ionic supports (DEAE, CM, Mono Q, etc.) is a widespread technique used for protein purification [1–7]. Commercially available ion exchangers are normally highly activated with very strong ionic groups (DEAE agarose, Q sepharose, etc.). Thus, they can adsorb a high percentage of proteins from a given extract (near 80% of the proteins of a crude extract from *Escherichia coli* is adsorbed on DEAE) [8–14]. At first glance, a selective adsorption of a target protein complex onto these kind of matrices seems to be quite difficult.

However, there are two different hypothesis that make possible the use of these simple techniques in protein complex detection (Fig. 1):

(i) First, proteins become adsorbed on ion exchangers supports via a multi-point exchange process [12,14,15].
(ii) Second, the proteins forming a multi-molecular complex will have a much larger size to interact with large areas of the support.

Then, as the adsorption process requires the simultaneous interaction between several groups of the protein and several groups in the support, only large enough proteins (that is, covering a large area of the support) could be adsorbed on the

* Corresponding author. Tel.: +34 91 5854809; fax: +34 91 5854760.
E-mail addresses: jmguisan@icp.csic.es (J.M. Guisán), rfl@icp.csic.es (R. Fernandez-Lafuente).
** Co-corresponding author.

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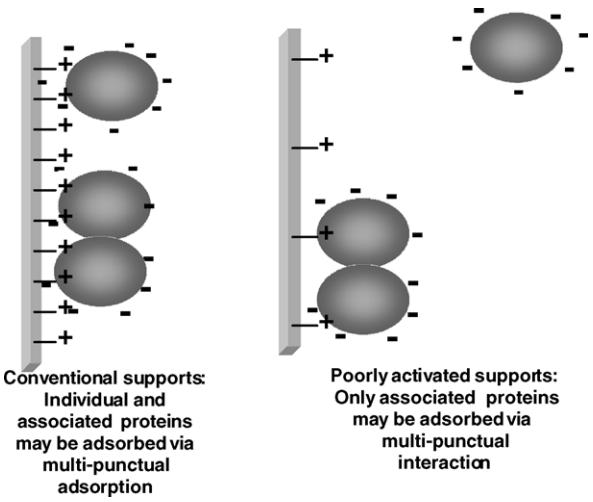

Fig. 1. Adsorption mechanism of protein complexes or non-associated proteins on differently activated anion-exchange supports.

matrix when using very lowly activated supports. This idea has been reported to be valid to purify large and multimeric proteins from monomeric and small ones [16].

The immobilized complexes may be cross-linked (if desired), for example, using aldehyde-dextran [17,18] and further analyzed following the desired protocols.

Thus, in this study, we have explored the advantages of using lowly activated anionic exchanger supports for the selective adsorption of protein complex. As a first approximation to check the feasibility of the system, we have analyzed the adsorption of a well characterized system: immuno complex [rabbit IgG and anti-rabbit immunoglobulins, horseradish peroxidase (HRP) and anti-horseradish peroxidase (anti-HRP)], and their individual complexes on different activated matrices.

2. Materials and methods

2.1. Materials

Agarose (4% cross-linked agarose beads) was a kind gift from Hispanagar (Burgos, Spain). Sodium periodate was from Merck (Darmstadt, Germany). Ethylenediamine, sodium borohydride, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), rabbit IgG purified immunoglobuline (rabbit IgG) and monoclonal anti-rabbit immunoglobulins peroxidase conjugate (developed in mouse) (anti-rabbit), polyclonal anti-horseradish peroxidase (anti-HRP) (developed in rabbit) (that does not inhibit the HRP activity), horseradish peroxidase (HRP), dextran (M_r 20000 Da) from *Leuconostoc mesenteroides* were supplied by Sigma (St. Louise, MO, USA). Rabbit IgG were purified by passing the commercial sample by a column of monoaminoethyl-*N*-aminoethyl agarose (MANAE)-agarose immobilized anti-rabbit immunoglobulin; and, anti-rabbit immunoglobulins peroxidase conjugate were purified by passing the commercial sample by a column of MANAE-agarose immobilized anti-HRP immunoglobuline [19]. HRP was purified by a column of MANAE-agarose immobilized anti-HRP, and anti-HRP was purified by passing the commercial sample by a column of glyoxyl agarose-immobilized HRP [19]. Aldehyde-dextran was prepared as described by Fuentes et al. [17]. Trimethylaminoborane was purchased from Fluka (Buchs, Switzerland). Other reagents were analytical grade.

2.2. Methods

All experiments were performed at least, by triplicate, and experimental error was never over 5%.

2.2.1. Preparation of MANAE-agarose

The protocol was similar to the previously described Fernandez-Lafuente et al. [11], but using glyoxyl agarose [15] with different activation degrees. Ten milliliter of agarose (4BCL) containing the desired amount of glyoxyl groups were suspended in 90 mL 1 M ethylenediamine pH 10.05 and gently stirred for 2 h. Then, 1 g of solid $NaBH_4$ was added and the support was reduced for 2 h. The reduced gels, MANAE, were filtered and sequentially washed with 100 mL of 0.1 M sodium acetate, 1 M NaCl at pH 5.0, with 100 mL of 0.1 M sodium hydrogencarbonate buffer, 1 M NaCl at pH 10.0 and finally with 500 mL of deionized water. The full conversion of glyoxyl to MANAE groups permitted that the concentration of amino groups corresponded to the initial concentration of glyoxyl group of the agarose [11]. The supports were further modified with formaldehyde to eliminate any primary amino group, to permit the final chemical treatment of the immobilized proteins with aldehyde-dextran without of covalent reaction with the support [20,21].

2.2.2. Determination of peroxidase activity

Peroxidase activity was determined using H_2O_2 as oxidizing substrate and ABTS as reducing substrate [22]. Activity was followed spectrophotometrically by recording the increase in the absorbance at 430 nm promoted by the ABTS oxidation product. Experimental conditions were 1 mM ABTS and 1 mM H_2O_2 in 50 mM sodium phosphate buffer at pH 6 and 25 °C.

2.2.3. Immuno complex preparation

2.2.3.1. Rabbit IgG/anti-rabbit IgG complex. As standard, 2.5 mg of purified rabbit IgG was added to 10 mg of a purified anti-rabbit in 5 mM sodium phosphate buffer pH 7 and left to interact for a minimum of 2 h at room temperature [19].

2.2.3.2. HRP/anti-HRP complex. As standard, 1 mg of purified anti-HRP was added to 10 mL of purified HRP solution (1 mg/mL) in 5 mM sodium phosphate pH 7 and left to interact for a minimum of 2 h at room temperature [19].

2.2.4. Adsorption of protein on aminated supports

2.2.4.1. Adsorption of the individual molecules on different aminated supports. One milliliter of anion exchanger support was suspended in 10 mL of different protein solutions (0.05 mg of protein per mL of 5 mM sodium phosphate buffer at pH 7 and 25 °C). During adsorptions, samples were withdrawn from the supernatant and the suspension and the protein concentration was determined by Bradford's method [23] and/or HRP activity [22]. After 24 h, the derivatives were washed with an excess of distilled water and stored at 4 °C.

2.2.4.2. Adsorption of mixed proteins. One milliliter of anionic exchanger support was suspended in 10 mL of immuno complex prepared as described above in 5 mM sodium phosphate buffer pH 7.0 and 25 °C. During adsorptions, samples were withdrawn from the supernatant and the suspension and the peroxidase activity and protein content was determined as described previously. After 24 h, the matrix with the adsorbed protein was washed with an excess of distilled water and stored at 4 °C.

2.2.5. Cross-linking of the adsorbed proteins

One gram of the matrix with the adsorbed proteins or the soluble proteins were added to 5 mL of cross-linking solution (200 mM sodium phosphate buffer pH 7 contained 150 mg of aldehyde-dextran M_r 20,000 Da) in presence of 150 mM trimethylaminoborane during 24 h at 25 °C [18]. Then, the Schiff's bases, formed between the primary amino groups of the protein and the aldehyde groups of the polymer, were reduced by addition of 3 mg/mL of sodium borohydride at pH 10. After 30 min, the pH was decreased at pH 7 by addition of diluted HCl. The low protein load of the support and the high concentration of aldehyde-dextran prevent any undesired cross-linking [17].

2.2.6. Gel filtration assays

Gel filtration analysis was performed using a glass column packed with agarose 4BCL (column bed volume: 100 mL). The column was previously equilibrated with 500 mL of the elution buffer (50 mM sodium phosphate buffer, pH 7.0). All experiments were carried out at 25 °C with a flow rate of 0.5 mL/min employing an isocratic pump (Pharmacia) and detecting the absorbance at 280 nm (UV detector, Pharmacia). The eluted samples were collected in 1 mL aliquots and peroxidase activity or protein concentration was determined by the methods previously described.

2.2.7. Adsorption of peroxidase on immobilized anti-HRP

One gram of immobilized anti-HRP (1 mg/g) [17] was added to 10 mL of HRP (0.25 mg) in 200 mM sodium phosphate buffer pH 7. In some cases, HRP was previously mixed with soluble anti-HRP. The adsorbed HRP was determined by quantifying the difference in HRP activity in the supernatant and suspension at different time, using the method described above. The experiments were carried out at least in triplicate, typically yielding an experimental error of lower than 5%.

3. Results and discussion

3.1. Adsorption of individual proteins on differently activated supports

Fig. 2 shows that most of the anti-rabbit becomes adsorbed on anionic exchanger highly activated (40 μmol/g) at pH 7 in 5 mM sodium phosphate buffer. In contrast, by decreasing the activation degree of the support (1 μmol/g) the amount of adsorbed anti-rabbit was significantly decreased, with no detectable adsorption using these lowly activated supports after 32 h of incubation. Similar results were achieved with Rabbit IgG and the other IgGs. HRP could not be adsorbed in supports having 1 μmol/g, although could be fully adsorbed on supports having 10 μmol/g or more.

3.2. Adsorption of antibody/antigen preparations on differently activated supports

3.2.1. Adsorption of rabbit IgG/anti-rabbit preparations on differently activated supports

Rabbit IgG and anti-rabbit were mixed at pH 7 and under conditions (an excess of rabbit IgG*)* where most of the anti-rabbit is involved in the formation of the immuno complex (rabbit IgG versus anti-rabbit*)* (this was confirmed by gel filtration at pH 4 of the aldehyde-dextran cross-linked proteins, results not shown) [17]. This immunocomplex may be even three-fold larger than the independent proteins implied.

Then, different anionic exchangers were added to the solution. While the isolated anti-rabbit was not adsorbed on supports having 1 μmol of amino MANAE groups/g (see above), in the presence of the rabbit IgG the anti-rabbit resulted almost fully adsorbed on these supports. The ionic

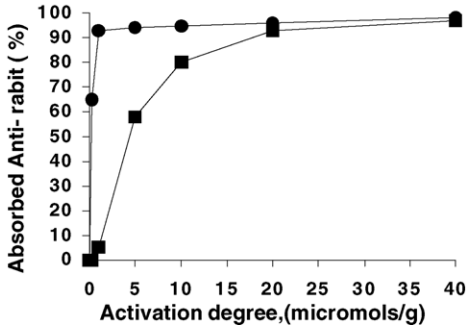

Fig. 2. Adsorption of anti-rabbit or rabbit IgG/anti-rabbit on MANAE-agarose 4BCL with different activation degree. Adsorption was performed in 5 mM sodium phosphate at pH 7.0 and 25 °C. Other specifications are described in Section 2.2. Squares: anti-rabbit adsorbed on different aminated supports. Circles: immuno complex adsorbed on different aminated supports.

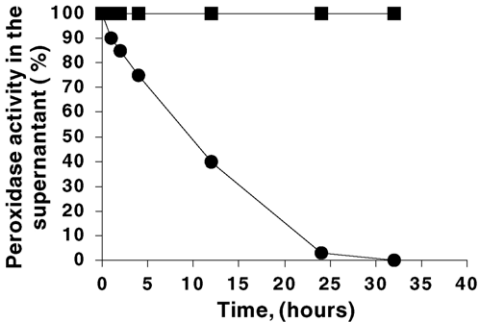

Fig. 3. Adsorption course of anti-rabbit or rabbit IgG/anti-rabbit on MANAE-agarose 4BCL 1 μmol/g. Adsorption was performed in 5 mM sodium phosphate buffer at pH 7.0 and 25 °C. The percentage of adsorbed immuno complex, on poorly MANAE-agarose, was determinated by determination of peroxidase activity (as described in Section 2.2) of the supernatants of the suspensions during adsorption. Other specifications are described in Section 2.2. Squares: supernatant of the anti-rabbit during adsorption on MANAE-agarose (1 μmol/g). Circles: activity of the supernatants of the suspensions during adsorption of immuno complex on MANAE-agarose (1 μmol/g).

adsorption was very slow in MANAE-agarose activated with 1 μmol/g; the full adsorption of immuno complex took 24 h (Fig. 3).

This result suggested that the support poorly activated might be adsorbing the large immunocomplex formed in presence of both molecules, while the individual antibodies could not be adsorbed. This was confirmed by analyzing the adsorbed proteins.

3.2.2. Adsorption of HRP/anti-HRP preparations on differently activated supports

Individual HRP nor anti-HRP did not become adsorbed on supports activated with 1 μmol/g, but when mixed, more than 90% of the HRP activity become adsorbed after 24 h (here, we used an excess of antibody).

3.3. Gel filtration studies of the cross-linked protein adsorbed on the differently activated supports

Different samples of adsorbed proteins were chemically treated with aldehyde-dextran, to fix their association state, and then, analyzed by gel filtration under dissociation conditions (pH 4) to prevent association during analysis [17,19].

Using the lowly activated supports where anti-rabbit had been adsorbed in the presence of rabbit IgG, the gel filtration shows a single peak that corresponds to the cross-linked immuno complex (CL-immuno complex). Very interestingly, the presence of individual cross-linked anti-Rabbit (CL-anti-rabbit) or cross-linked rabbit IgG (CL-rabbit IgG) was no detected, confirming that this smaller molecules are not ionic adsorbed on the lowly activated MANAE-agarose (1 μmol/g) (Fig. 4A). Even more interestingly, when only 10 μg of one of the antibodies was used versus 1 mg of the other, only the immuno-complex were detected, with no traces of the other antibody (results not shown).

However, the excess of rabbit IgG or anti-rabbit (detected by peroxidase activity) contaminated the CL-immuno complex when the adsorption was performed on highly activated supports (that was able of adsorbing both individual molecules or immuno complex) (results not shown).

To ensure that these results were not an artifact promoted by the solid-phase cross-linking, rabbit IgG or anti-rabbit individually adsorbed on highly activated MANAE-agarose (40 μmol/g) were treated with aldehyde-dextran. The gel filtration chromatogram shows only a single peak in a similar elution volume as aldehyde-dextran treated rabbit IgG or anti-rabbit in soluble conditions (Fig. 4B).

In a similar way, only the immunocomplex could be detected when mixing HRP and anti-HRP and adding supports activated with 1 μmol/g (Fig. 5A). However, the gel filtration assays of the proteins adsorbed proteins on 40 μmol/g when mixing an excess of HRP and anti-HRP shows two peaks corresponding to the CL-inmunocomplex adsorbed and the excess of CL-HRP adsorbed (Fig. 5 B) (as shown by the analysis of the individual proteins).

Fig. 6 shows that immobilized anti-HRP (1 mg) was able to adsorb all the peroxidase (0.25 mg) when used pure. However, when this enzyme was previously mixed with growing concentrations of soluble antibody, the immobilized anti-HRP become unable to capture HRP, very likely because the enzyme was already interacting with the soluble antibody and the interaction with the immobilized antibody implied the breaking of that interactions. However, our system was fully efficiently on all conditions.

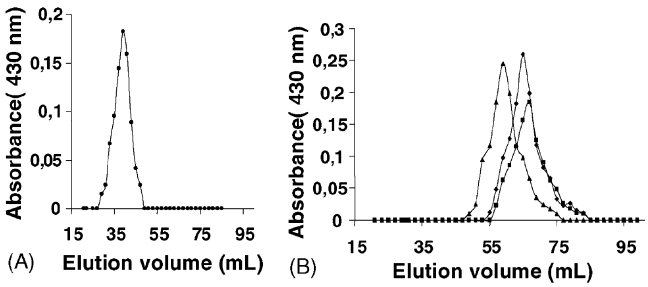

Fig. 4. (A) Gel filtration analysis of cross-linked adsorbed immuno complex (CL-immuno complex). One milliliter of rabbit IgG and anti-rabbit preparation were adsorbed on poorly MANAE-agarose (1 μmol/g) and cross-linked with aldehyde-dextran. This was loaded in a glass column packed with 100 mL of agarose 4BCL. Flow rate was 0.5 mL/min. The CL-immuno complex (circles) was eluted from the column using 100 mM sodium acetate pH 4. Other details are described in Section 2.2. (B) Gel filtration analysis of cross-linked anti-rabbit (CL-anti-rabbit). One milliliter of solution containing untreated anti-rabbit (squares), 1 mL of a CL-anti-rabbit (soluble anti-rabbit was modified with aldehyde-dextran (M_r 20,000) as described in Section 2.2) solution (rhombus), 1 mL of CL-anti-rabbit (adsorbed anti-rabbit IgG was cross-linked with aldehyde-dextran (20,000) as described in Section 2.2) solution (triangles). The samples were loaded in the glass column packed with 100 mL of 4BCL agarose. Flow rate was 0.5 mL/min. Other details are described in Section 2.2. The samples were eluted in 100 mM sodium phosphate pH 7.

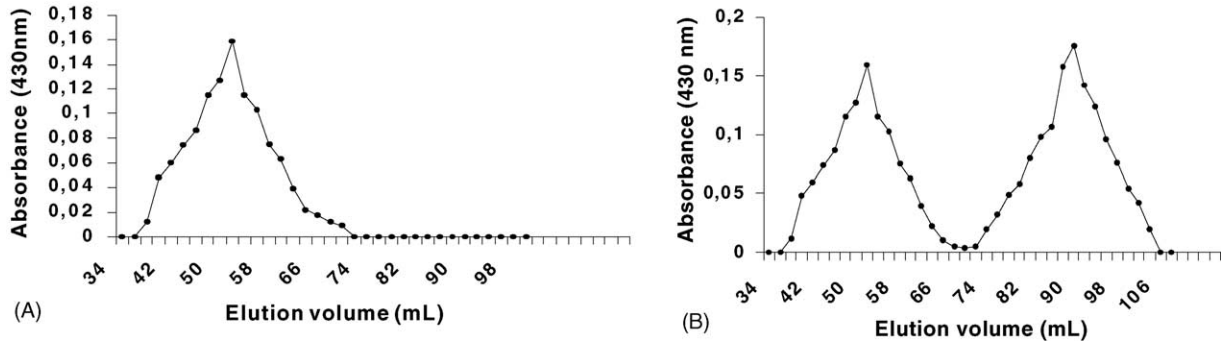

Fig. 5. (A) Gel filtration analysis of cross-linked adsorbed protein mixtures 1 mL of HRP (10 mg) and anti-HRP (1 mg) adsorbed on poorly MANAE-agarose (1 μmol/g) and cross-linked with aldehyde-dextran (M_r 20,000) was loaded in the glass column packed with 100 mL of agarose 4BCL. Flow rate was 0.5 mL/min. The proteins (circles) were eluted from the column using 100 mM sodium acetate pH 4. Other details are described in Section 2.2. (B) Gel filtration analysis of cross-linked HRP derivatives. One milliliter HRP (10 mg) and anti-HRP (1 mg) adsorbed on MANAE-agarose (40 μmol/g) and cross-linked with aldehyde-dextran (20,000), was loaded in the glass column packed with 100 mL of agarose 4BCL. Flow rate was 0.5 mL/min. The inmunocomplex was eluted from the column using 100 mM sodium acetate buffer pH 4. Other details are described in Section 2.2.

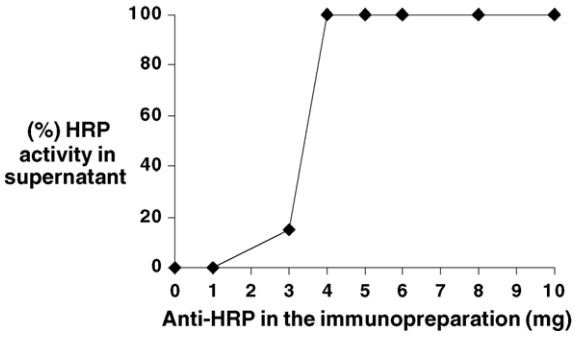

Fig. 6. Adsorption of HRP (0.25 mg) on immobilized anti-HRP (1 mg) in the presence of growing concentrations of soluble anti-HRP. Experiments were performed in 200 mM sodium phosphate at pH 7 and 25 °C as described in Section 2.2.

4. Conclusions

This simple strategy permits the easy and selective adsorption of proteins forming protein–protein complexes, leaving in the supernatant the excess of proteins that are no associated, and concentrating in the support the complexes. This selective adsorption may have some advantages compared to the use of immobilized protein to detect a target one. For example, it the one protein has a strong interaction, similar to the ones showed in this manuscript, it is not easy that the protein may interact with the immobilized protein. Thus, this strategy may present some advantages for the identification of proteins associated with a target one.

The only requirement for the application of this strategy is that there are a significant increment in the size of the larger protein by the association with the other proteins, and that the proteins must be able to become adsorbed on the matrix used, whose only requirement is that adsorb proteins by multi-point interaction.

Acknowledgments

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