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Journal of Chromatography B, 849 (2007) 243–250
JOURNAL OF CHROMATOGRAPHY B
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# Solid phase proteomics: Dramatic reinforcement of very weak protein–protein interactions☆

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Received 20 April 2006; accepted 18 September 2006
Available online 10 October 2006

**Abstract**

Very weak protein–protein interactions may play a critical role in cell physiology but they are not easily detectable in "*in vitro*" experiments. To detect these weak interactions, we have developed a strategy that included: (a) design of a rapid and very effective crosslinking of protein–protein complexes with poly-functional reagents; (b) selective adsorption of very large proteins on lowly activated ionic exchangers, based on the need of a multipoint physical adsorption to incorporate the proteins into the matrix; (c) purification by selective adsorption of protein–protein complexes formed by strong protein–protein interactions, via selective adsorption of the complexes on lowly activated ionic exchangers via multi-protein physical adsorption and leaving the non-associated proteins in the solution; (d) reinforcement of very weak protein–protein interactions by selective adsorption of the complex on lowly activated ionic exchange supports via a synergetic cooperation of the weak protein–protein interaction plus the interactions of both proteins with the support enabling the almost full shifting of the equilibrium towards the association position; (e) control of the aggregation state of proteins like BSA, formed by weak protein–protein interactions. In this last case, it seems that the interaction of the protein molecules placed on the borders of the aggregate with the groups on the support partially stabilizes the whole aggregate, although, some molecules of the aggregate cannot interact with the support. The size of the aggregates may be defined by controlling the concentration of ionised groups on the support: the less activated the supports are, the bigger the complexes. In this way, solid-phase proteomics could be a very interesting tool to detect weak protein–protein interactions.


*Keywords:* Multipoint adsorption; Stabilizaition of protein complex; Purification of protein complex

## 1. Introduction

Protein–protein interactions are operative at almost every functional level of the cell including the structure of sub-cellular compartments, the transport machinery across biological membranes, the packaging of DNA into chromatin, the regulation of gene expression, and the transduction of intracellular signals. On the other hand, aberrant protein–protein interactions are implicated in many diseases [1–16]. Therefore, the study of protein complexes has become the object of intense research in many biological disciplines [17–22].

The detection and purification of protein–protein complexes associated via very strong protein–protein interactions, may be efficiently achieved using a variety of methods (such as affinity chromatography, gel filtration, native and two-dimensional electrophoresis) [22–33]. Nevertheless, the development of simple protocols for the detection, concentration and purification of very small traces of these stable protein–protein complexes (contained in a complex mixture of proteins) is still an interesting goal that will be also discussed in this revision.

Protein–protein complexes associated via very weak protein–protein interactions may also play critical roles in cell physiology. These unstable complexes could be stabilized inside certain cell compartments to fulfill their biological function. However, the detection of these very weak protein–protein interactions remains a very difficult task. The concentration of these

☆ This paper is part of a special volume entitled "Analytical Tools for Proteomics", guest edited by Erich Heftmann.

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doi:10.1016/j.jchromb.2006.09.024

"transient" protein–protein complexes formed by these weak interactions may be negligible if compared to the isolated components, even being these in many instances in very low concentration [34–36]. In this way, detection, concentration and purification of very small traces of unstable complexes may become a very exciting tool in Proteomics.

In this review, recent straightforward protocols intended for the detection of such unstable protein–protein complexes are discussed. The final protocol is quite simple but it is based in three interdisciplinary approaches: (a) new protocols for highly efficient cross-linking of protein–protein complexes, (b) design of lowly activated anionic exchanger supports for the selective adsorption of large proteins and protein–protein complexes, (c) studies on the reinforcement of very weak protein–protein interactions when adsorbed on solid surfaces.

Some of the intermediate steps may also be interesting tools in Proteomics, and will also be discussed: detection of protein–protein complexes in solution, concentration of stable protein–protein complexes formed by interaction between traces of a protein and other proteins by selective adsorption on solid supports, etc.

## 2. Crosslinking of protein–protein complexes

The development of rapid and efficient protocols for cross-linking of protein–protein complexes plays a key role in the characterization of very unstable protein–protein complexes [37–40]. In fact; it may be assumed that the cross-linking of the complexes during their transient stabilization by adsorption on solid supports may be strictly necessary. Otherwise, further analysis and characterization of these "weak" complexes would be very difficult because of the almost-complete dissociation of the non-crosslinked unstable protein–protein complexes.

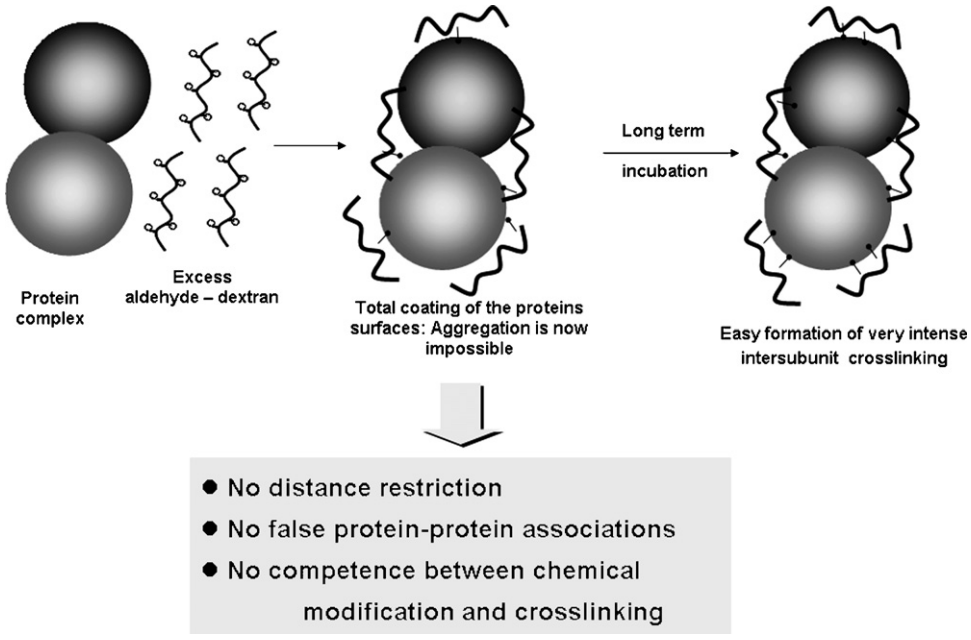
### 2.1. Aldehyde–dextran as a suitable cross-linker

Dextran is a polymer of α-1,6-glucose (with some branches in 1–4) that is commercially available in a wide range of molecular weights. Dextran is easily oxidized by periodate giving a poly-aldehyde-polymer [41–42]. Aldehyde–dextran is a highly hydrophilic random coil polymer able to interact with amino groups on the surface of proteins. This poly-functional polymer has no strict distance requirements to promote cross-linking between amino groups placed on the surfaces of different proteins forming a complex. The surfaces of the proteins forming the complex may be rapidly covered (through regions which are rich in Lys groups) by these large molecules, promoting a minimal chemical modification in the proteins involved: primary amino groups will be transformed into secondary amino groups [43,44]. Then, the multifunctional nature and large size of this reagent may permit the cross-linking between Lys groups placed in two different proteins forming the complex. On the other hand, as the polymer is unable of reacting with itself, the rapid covering of the proteins surfaces will avoid undesirable reactions between any unrelated proteins [45] (Scheme 1).

### 2.2. Aldehyde–dextran cross-linked protein complexes

Penicillin G acylase from *E. coli* (PGA), is a heterodimeric enzyme composed to two subunits of 62 and 23 kDa, respectively. This enzyme has been successfully cross-linked with aldehyde–dextran [45]. The reaction of a PGA solution with aldehyde–dextran permitted to have both PGA subunits cross-linked, while no PGA–PGA artificially formed dimers could be detected in SDS-PAGE experiments of the crosslinked PGA.

Similar, successful cross-linkings were obtained with bovine pancreas trypsin and chymotrypsin, and porcine pancreas lipase.




Scheme 1. Determination of protein–protein interactions through aldehyde–dextran cross-linking.

In all cases, cross-linking of the different associated polypeptide chains was rapidly achieved and undesirable side-reactions were not observed [45].

The aldehyde–dextran has also been applied to the stabilization of immuno-conjugates as a model of stable protein–protein complexes. For example, purified anti-horseradish peroxidase (Anti-HRP) and horseradish peroxidase (HRP) were cross-linked with aldehyde–dextran [45]. Previous studies showed that when size exclusion chromatography was performed of a mixture of HRP-Anti-HRP and Anti-HRP in excess at pH 7, the immunocomplex is strongly bound and only one peak corresponding to HRP activity was detected, with a molecular weight of approximately, 200 kDa. When this chromatography was performed at pH 4, there was again only one HRP peak, but in this case with a molecular weight of 33 kDa, corresponding to HRP. This means that under these conditions the immunocomplex was completely dissociated. Using cross-linked samples, the chromatograms were now identical at both pH values (pH 7.0 and pH 4.0). In both cases, the only immunocomplex was detected by chromatography and this means that the antigen–antibody complex was fully stabilized after the treatment with aldehyde–dextran. The cross-linking of HRP (in the absence of antibody) gave a single peak of around 50 kDa, again showing that this treatment promotes a slight increase in molecular weight (the attachment of dextran to the protein) but it did not promote cross-linking of non-associated proteins [45].

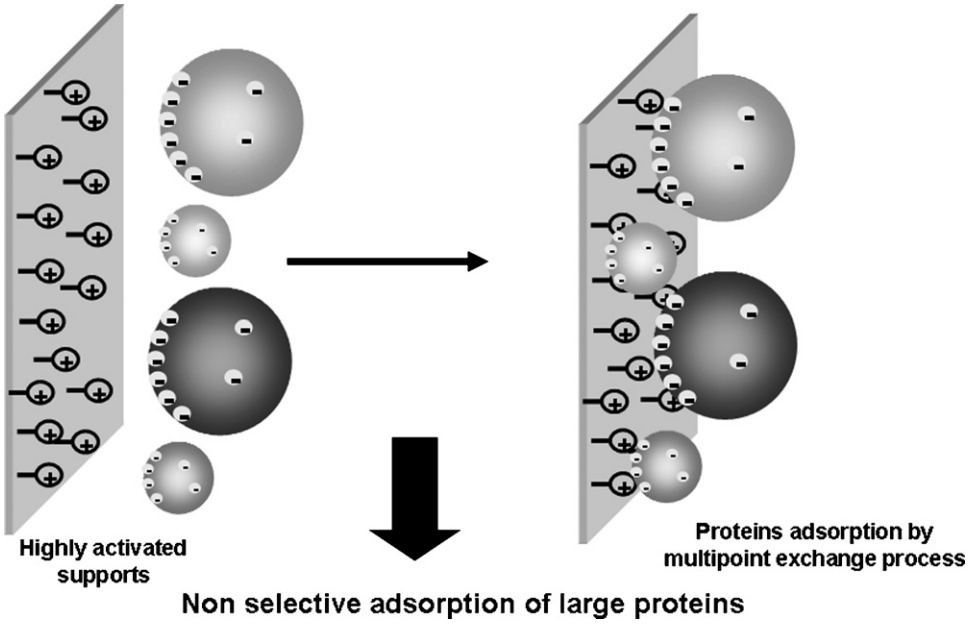
This methodology seems to be very simple and effective to stabilize any protein–protein complex existing in solution with no generation of artificial protein-aggregates. Moreover, if the final adduct is not reduced, dextran may be released from the proteins by boiling in the presence of nucleophiles, leaving unfolded but intact proteins (i.e., permitting their sequencing).

## 3. Selective adsorption of large proteins on lowly activated adsorbents

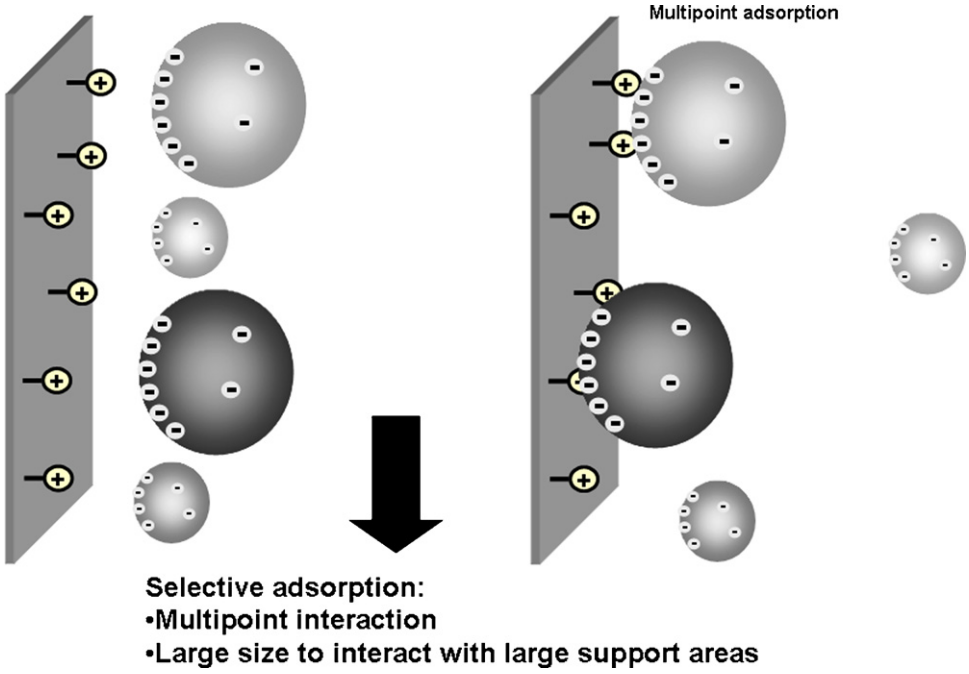
Anion-exchange chromatography, using commercial highly activated anionic-exchangers (DEAE-agarose, Q-agarose, etc.) is a widespread technique used for protein purification [46–55]. It is a simple and rapid method for protein separation, which is usually based on the selective desorption of the proteins that have been adsorbed on the support. Most of the proteins (e.g., 60–70% of the proteins from a crude extract from *E.coli*) become adsorbed on the chromatographic matrix [55–57]. The adsorption on this matrix requires a multipoint interaction between the protein and the supports [55–61]. The different proteins may be desorbed from the support at different ionic strengths, depending on the intensity of the interaction of each protein with the ionic-exchanger.

At first glance, non-commercial lowly activated anionic-exchangers should not be able to promote multipoint physical adsorption of small proteins. Thus, only large proteins might be able to become adsorbed via multipoint physical adsorption on this kind of supports. Large proteins have very large surfaces, which are able to interact with groups in the support that are placed far apart from each other (Schemes 2 and 3).

Crude extracts of proteins from *E. coli* were incubated with supports having 1 μmol of amino groups per gram of support [59]. After 1 h of incubation, less than 5% of the proteins were adsorbed on the support. After desorption of the adsorbed proteins and by a gel filtration experiment it was found that the proteins adsorbed on lowly activated anion-exchangers correspond to the fraction of proteins of the crude extract having the largest molecular size. On the contrary, the proteins adsorbed on highly activated supports have a distribution of sizes that




Scheme 2. Non-selective ionic adsorption on highly activated ionic exchangers.

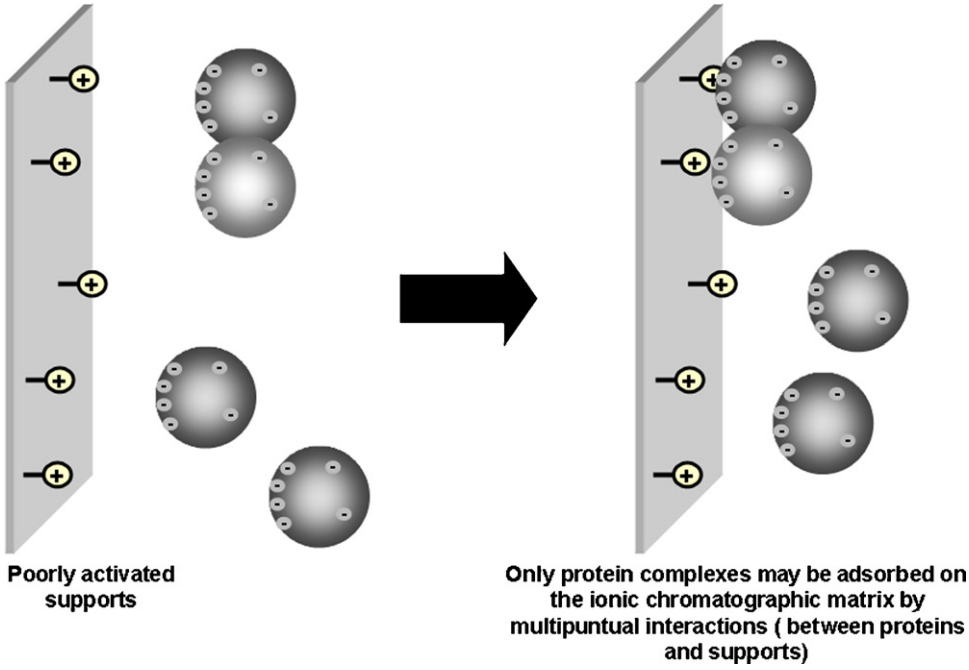



Scheme 3. Selective adsorption of large proteins lowly activated ionic exchangers.

is very similar to that of the crude extract [59]. Similar results were obtained when using other crude protein extracts (e.g., *Acetobacter turbidans*) and other tailor made adsorbents (e.g., supports containing metal chelate moieties) [61]. Furthermore, very large thermophilic enzymes cloned in *E.coli* could be selectively adsorbed on mildly activated anion-exchangers and IMAC-supports, because after a short heating the only multimeric enzymes reaming associated and soluble were the thermophilic ones [61].

## 4. Selective adsorption of stable protein–protein complexes on very lowly activated anion-exchangers

Protein–protein complexes associated via strong non-covalent protein–protein interactions are obviously larger than the individual components [60]. Thus, associated components of the complex may behave like large proteins able to be adsorbed on lowly activated anion-exchangers. It could be possible to find a tailor-made support not able to adsorb individual proteins but




Scheme 4. Selective adsorption of protein complexes on lowly activated ionic exchangers.

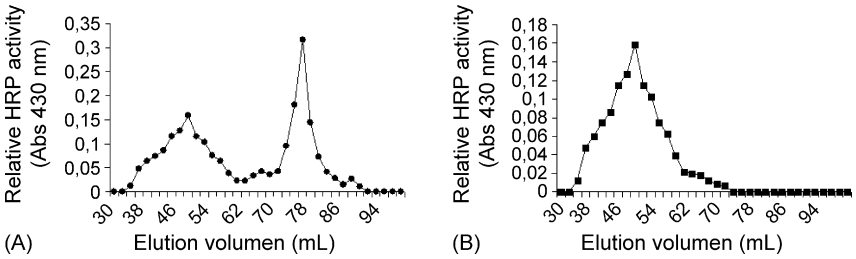



Fig. 1. Gel filtration analysis of adsorbed protein mixtures further cross-liked with aldehyde–dextran. One milliliter of HRP (10 mg) and Anti-HRP (1 mg), in 5 mM sodium phosphate buffer was incubated in the presence of MANAE-agarose activated with 40 (A), or 1 (B) μmol/g at pH 7. Then, the adsorbed proteins were cross-linked using 10 ml of 200 mM sodium phosphate buffer pH 7 containing 15 mg/ml of aldehyde–dextran 20 KDa in the presence of 150 mM trimethylaminoborane during 24 h at 25 °C. Next, the proteins were desorbed and loaded in a 100 mL of agarose 4 BCL glass column. Flow rate was 0.5 mL/min. The proteins were eluted from the column using 100 mM sodium acetate pH 4.

able to adsorb the protein–protein complex (Scheme 4). In this way, protein–protein complexes could be detected, concentrated and purified by selective adsorption on these tailor-made supports.

Immunoglobulin molecules fully adsorb on highly activated supports but the rate of adsorption decreases when using lowly activated supports and it becomes negligible when using supports having 1 μmol of amino groups per wet gram of support [60]. However, the addition of an excess of HRP to the suspension promotes the complete and fast adsorption of Anti-HRP on this support. To effectively, confirm that the proteins adsorbed were only those forming the complex, the immobilized proteins were cross-linked with aldehyde–dextran and further analyzed by gel filtration. Fig. 1 shows that only antigen–antibody complexes have been adsorbed on the anion-exchanger. As expected, non-complexed IgGs were not presented on that very lowly activated support (Fig. 1) [60].

Obviously, when using highly activated supports (e.g., supports containing 40 μmols of amino groups per wet gram), both protein structures (the isolated antibodies and the antibody-antibody complexes) were rapidly adsorbed on the anion-exchanger.

In this way, a very simple protocol to detect, concentrate and purify protein–protein complexes from their individual components has been established [45,60]:

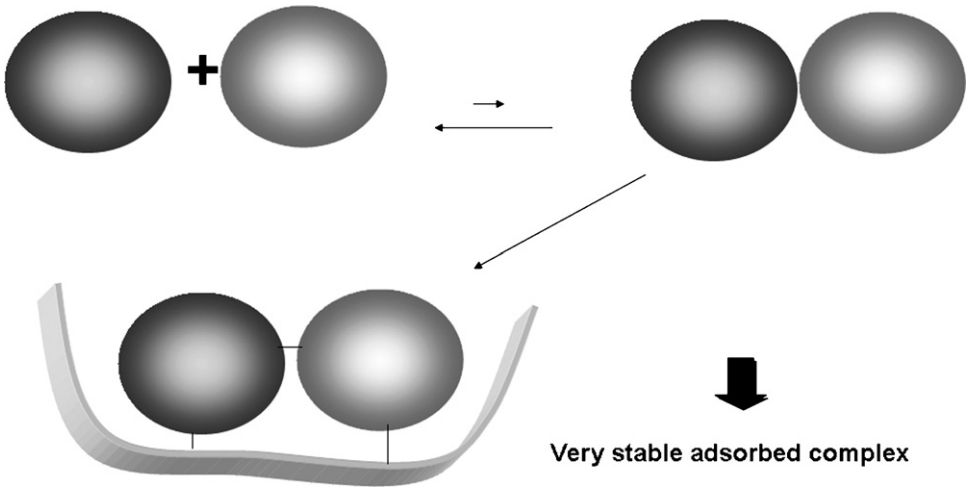
a. The individual components of the complex are offered at low ionic strength to activated supports (e.g., anion-exchangers, metal chelate supports) with different activation degrees, selecting the support having the highest concentration of active groups that is not able to promote the adsorption of any of the individual target proteins.
b. This support is offered to a sample containing the different components of the complex, the protein–protein complexes become selectively adsorbed on the lowly activated supports at low ionic strength. Then, they are extensively washed and they may be desorbed from the support (under very mild conditions). Finally, they may be further analysed by different physico-chemical techniques: gel filtration, mass spectrometry, antibody recognition, etc.
c. If desired, the complexes may be cross-linked with aldehyde–dextran to stabilize the complex under any experimental condition.

## 5. Stabilization of unstable protein–protein complexes by selective adsorption on lowly activated anion-exchangers

Protein–protein complexes associated via very weak protein–protein interactions could be highly stabilized when both proteins become jointly adsorbed on a lowly activated solid support unable to strongly adsorb each individual protein [36]. Now, the adsorbed complex may become much more stable than the soluble one because of the synergy of several simultaneous interactions: the protein–protein interaction plus the interaction/interactions of each of the components of the large protein–protein complex with the groups in the support (Scheme 5). Moreover, the adsorption of the associated proteins in a suitable position should greatly increase the apparent concentration of the proteins forming the complex, further reinforcing the forces keeping the complex together [36]. The dissociation of the soluble complex only requires the breaking of the weak forces that bind the complex. However, the solid-phase protein–protein complex could only be destroyed when a complete desorption of the multi-pointly adsorbed protein–protein complex plus a simultaneous dissociation of the apparently extremely high concentrated complex occurs. Each individual interaction may significantly stabilize the other ones and hence, the adsorbed protein–protein complex could become highly stabilized, and the association equilibrium will be shifted towards the complex formation.

The presence of 15% dioxane promotes the almost complete dissociation of the antigen–antibody complex [36], being in this case an example of complex formed by weak protein–protein interactions. A mixture of horseradish peroxidase and an excess of purified-Anti-HRP in 15% dioxane was treated with aldehyde–dextran in order to cross-link and stabilize all antigen–antibody complexes existing under those conditions. Gel filtration analysis shows that less than 5% HRP was associated with its antibodies under these conditions (Fig. 2).

An almost identical gel filtration chromatogram was observed when HRP and Anti-HRP incubated in 15% dioxane were adsorbed on amino supports having 40 μmols of amino groups per wet gram of anionic-exchanger and cross-linked with aldehyde–dextran. The individual HRP or Anti-HRP molecules existing in solution were rapidly adsorbed on the support and




Scheme 5. A possible mechanism of stabilization of unstable protein–protein complexes. Solid-phase dramatic stabilization via three synergistic weak interactions.

hence, the association/dissociation equilibrium coincide with that that was found in solution.

On the other hand, anti-HRP cannot adsorb on very lowly activated supports (e.g., having 1 μmols of amino groups per wet gram of anionic-exchanger), even in the presence of 15% dioxane. However, in the presence of HRP and 15% dioxane, Anti-HRP may be slowly but almost completely adsorbed on the support. After cross-linking with aldehyde–dextran the analysis of the adsorbed Anti-HRP showed that Anti-HRP was only adsorbed as an immuno-complex [36] in spite of being incubated with the support under unfavourable conditions for the formation of such complexes (Fig. 2).

In this way, a quite simple protocol to detect, stabilize, concentrate and purify very unstable protein–protein complexes (associated through very weak protein–protein interactions) can be established [36,45,60]:

a. The individual components of the complex are offered at low ionic strength to activated supports (e.g., anion-exchangers, metal chelate supports) with different activation degrees, selecting the support which has the highest concentration of active groups that is not able to promote the adsorption of any of the individual target proteins.
b. The possible unstable protein–protein complexes become adsorbed on the lowly activated supports at low ionic strength. Then, they are cross-linked with a very high concentration of aldehyde–dextran and extensively washed with buffer. As commented before, intense cross-linking with aldehyde–dextran was unable to form false positives of aggregation on soluble proteins. Using immobilized and fully dispersed proteins (the amount of adsorbed protein must be very low), "false" aggregates are even much more difficult to be formed by crosslink with aldehyde–dextran. This way, the different proteins complexes or molecules will be quite far from each other. Thus, only immobilized and associated proteins can be cross-linked with aldehyde–dextran.
c. The adsorbed and cross-linked protein–protein complexes are desorbed from the support under very mild conditions. If the complex is really maintained by very weak forces, without the previous cross-linking, the proteins would be desorbed in a non-associated form. Finally, the cross-linked protein–protein complexes may be further analysed by different physic-chemical techniques: gel filtration, mass spectrometry, antibody recognition, etc.

The comparison of the adsorption on a battery of supports (having progressively lower activation degrees) of a sample in absence and in the presence of a target protein, could also indicate which proteins in the sample are able to interact with the target protein: any band that is only adsorbed on a support in presence of the target protein might be adsorbed as a complex. Parallel experiments, performed with or without aldehyde–dextran cross-linking, and may also allow us to distinguish between samples proteins that are strongly associated to our target protein and

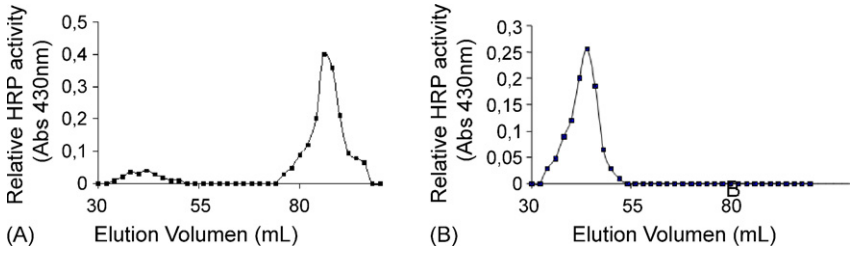



Fig. 2. Gel filtration analysis of analysis of HRP/Anti-HRP mixtures adsorbed in the presence of 15% dioxane on MANAE-agarose activated with 40 (A), or 1 (B) μmol/g. One milliliter of HRP (10 mg) and Anti-HRP (1 mg), in 5 mM sodium phosphate/15% dioxane was incubated in the presence of MANAE-agarose at pH 7. Then, the adsorbed proteins were cross-linked and analyzed as described in Fig. 1.

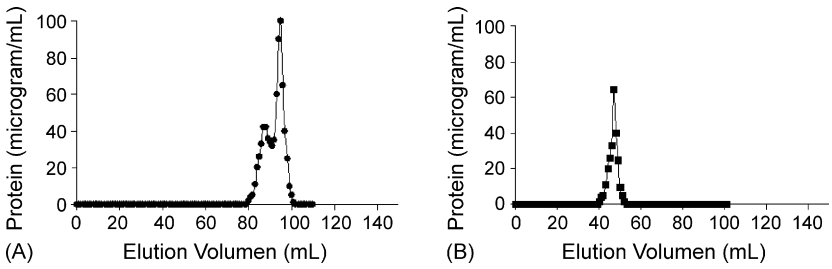



Fig. 3. Gel filtration analysis of cross-linked adsorbed BSA on different activated supports. One milliliter of 0.1 mg/ml of BSA in 5 mM sodium phosphate buffer was incubated in the presence of MANAE-agarose activated with 40 (A), or 1 (B) μmol/g at pH 7. Then, the adsorbed proteins were cross-linked and analyzed as described in Fig. 1.

sample proteins that are very weakly associated to our target protein.

## 6. Stabilization of bovine serum albumin (BSA) aggregates via selective adsorption on very lowly activated adsorbents

Gel filtration experiments of 10 mg/mL bovine serum albumin reveals that BSA is able to form aggregates that are composed by 2, 3 or 4 protein units. However, at 0.1 mg/mL, only monomeric and a small percentage of dimeric protein is detected by gel filtration and mass spectroscopy analysis [62–65]. These very dilute BSA solutions were offered to anionic-exchangers having different concentrations of amino groups per wet gram of support, even only 0.1 μmol/g. In all cases, BSA becomes adsorbed on the supports: completely (100%) and very rapidly (1 h) on highly activated ones or only partially and much more slowly (48 h) on very lowly activated ones [66]. The analysis of the proteins adsorbed here showed that very large aggregates of BSA (not detected using soluble BSA) was adsorbed on the matrix, and that this was larger when the activation degree was smaller (e.g., 500 KDa using supports having only 0.1 micromol/g). Fig. 3 shows the results achieved using supports activated with 2 μmols/wet gram, where dimeric BSA cannot be adsorbed. In fact, only tetramers become adsorbed on these supports, this way it was possible to transform almost 100% of the BSA molecules on tetramer structures. These BSA molecules were almost negligible even when very high concentrations of soluble BSA were cross-linked with aldehyde–dextran. Using supports with a significantly lower activation degree, although not all BSA molecules could be adsorbed on the support, a significant percentage of the offered BSA could become adsorbed. The analysis of these aggregates reveals a progressive growing in the size of the aggregate. Apparently, the BSA aggregate needs to grow to a size that may permit that the BSA molecules placed at the end of the aggregate to interact with groups on the support, placed quite far apart. This is a situation different to the one previously described, where each individual molecule forming the complex was interacting with the support, now some of the units of the aggregate are fixed in their position only by the interaction with other BSA molecules. However, the breaking of this association should promote the full breakage of the aggregate and desorption of the BSA molecules that are interacting with the support. Thus, the synergism among many weak interactions, that individually are not enough to give relevant concentrations of the complex, permits to shift the equilibrium. However, the adsorption of the subunits at the end of the aggregate must compensate and stabilize the whole complex, and in this case the yield of the adsorbed protein did not reach 100% of the BSA molecules. Nevertheless, there are only aggregates on the support because they are the only proteins that can be adsorbed. Moreover, the size of the aggregate is controlled by the distance among the groups in the support: addition of new BSA molecules after those that are interacting with the support should only be stabilized by the weak BSA–BSA interaction, the breakage or formation of this interaction will have no effect on the adsorption of the BSA aggregates to the support. Therefore, if these aggregates are not detectable in solution, will be neither detected on the final suspension, although they had been described for BSA.

These results suggest that very weak protein–$(protein)_n$–protein interactions, so weak that have not been identified to date, may be playing a critical role in the cellular metabolism: the adsorption of complexes of proteins on specific receptors unable to recognize individual molecules. In fact, this new advance (using a battery of supports with a decrease in the activation degree), may permit the study of very complex interactions.

The results presented in this manuscript suggest that the "solid phase proteomics" may be a really powerful tool in the study of protein–protein interactions. The simplicity of this strategy may permit a qualitative jump in proteomics, to permit the detection of very weak protein–protein interactions.

## 7. Conclusions

Solid-phase Proteomics may open new and interesting opportunities to detect protein–protein interactions. A quite simple protocol to detect, concentrate and purify very unstable protein–protein complexes has been described here. This protocol has been set-up on the basis of three main points:

a. The establishment of an interesting procedure for effective and correct cross-link of protein–protein complexes.
b. The discovery of a highly selective adsorption of very large proteins or of stable protein–protein complexes on very lowly activated support.
c. The hypothesis of a possible very positive synergy among protein–protein and protein-adsorbent interactions.

Two very different experimental results strongly support this third point: (a) very unstable antigen–antibody complexes (in the presence of dioxane) were dramatically stabilized after adsorption on very lowly activated supports, (b) BSA aggregates were also stabilized after adsorption on very lowly activated supports.

Similar synergies among protein–protein and protein–surface interactions could be the responsible of the critical role of weak protein–protein interactions in physiology.

## Acknowledgements

This work was supported by "Ramon y Cajal" Spanish contract, and a EC Project (MATINOES G5RD-CT-2002-00752). We thank Dr. Ángel Berenguer for his help during the writing of this paper.

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