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Affinity chromatography of plasma proteins (guanidinobenzoatase): use of mimetic matrices and mimetic soluble ligands to prevent the binding of albumin on target affinity matrices

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Abstract

Serum albumin is the most abundant protein in plasma and it has a high capacity to bind many small compounds and macromolecules. In this way, albumin may promote important interferences during affinity chromatography of plasma proteins. Guanidinobenzoatase (GB) is a very relevant plasma protease that seems to be related to tumoral processes. This enzyme may be adsorbed on tailor-made agmatine-amide-agarose (CH-A) supports (e.g., the ones having 2 μmol of guanidino groups per ml of agarose attached to the support, through a 6 C aliphatic chain). Such tailor-made supports containing a very low concentration of ionized groups are hardly able to adsorb any protein by anion-exchange. However, they are able to strongly adsorb albumin. In order to solve this problem new mimetic affinity matrices have been designed: (i) by using the same ligand immobilized through a different chemical linkage [guanidino groups attached via secondary amino bonds, (AEA)] or (ii) by using slightly different ligands (e.g., 1,8-octanediamine containing a primary amino group instead of a guanidino one) also attached to the support via amido bonds (CH-DAO). Albumin adsorbs on the target and on the two mimetic matrices while GB is mainly adsorbed on the target one. Moreover, the adsorption of albumin on the affinity matrix (CH-A) is very strongly inhibited by the presence of low concentrations of soluble ligands (e.g., 1,8-octanediamine containing two ionized primary amino groups). On the contrary, the adsorption of GB on CH-A is hardly inhibited by the presence of such mimetic soluble ligand. In this way, the former offering of crude GB samples to AEA plus the use of mimetic inhibitors during adsorption of the extract on CH-A completely prevent the undesirable adsorption of albumin. In a such way, an extremely selective adsorption of GB can be performed. Such an improved chromatography procedure allows a very easy affinity purification and detection of GB.

Keywords: Mimetic matrices; Mimetic ligands; Enzymes; Guanidinobenzoatase; Albumin

1. Introduction

For the last 30 years, affinity chromatography has become a very powerful tool for protein purification [1–3]. This technique utilizes the high specificity

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between a target protein and an immobilized ligand (e.g., some inhibitor or substrate analogue) to perform an easy separation of the target protein from crude protein extracts. Frequently, mainly working at a laboratory scale, the pure protein is obtained after a ''very selective'' desorption of the previously adsorbed protein with low concentrations of high-affinity soluble inhibitors or substrate analogues. From this point of view, the existence of additional non-specific interactions (ionic, hydrophobic or so on) between other proteins of the crude extract and the affinity matrix could be not very relevant. However, a very high selectivity in the first adsorption of our target protein with complete elimination of such undesirable non specific adsorptions of contaminant proteins may become a very interesting goal. In this way, we could be able to: (i) use much smaller chromatographic beds to purify very high amounts of target proteins, (ii) reduce the interaction between our target protein and some proteases present in the extract, (iii) facilitate the further desorption of the completely pure target protein without risk of co-desorption of contaminant proteins, (iv) use affinity matrices for concentration and detection of very small traces of very relevant proteins (e.g., with biomedical significance), etc.

Most of non-specific adsorptions (ionic, hydrophobic ones) can be strongly reduced by using tailor-made affinity matrices with a low density of ligand moieties on the matrix surface. However, other more complex adsorptions could also occur when a specific immobilized ligand may be recognized, not only by the target protein, but also by other proteins presented in the same extract (e.g., proteins from the same family, isoenzymes, etc). In order to solve this problem, the following logical hypothesis can be assumed: two proteins that have a common ligand, may have very different affinity towards slightly different ligands (mimetic ligands). From this point of view, we propose the search of new mimetic matrices and new mimetic soluble ligands also able to ''recognize'' the contaminant proteins but having a much lower affinity towards our target protein. In this way, mimetic ligands could be used to inhibit the binding of competing protein on our affinity matrix. Also, matrices prepared by immobilizing these mimetic ligands could be used as ''traps'' to retain competing proteins.

Purification of plasma proteins (e.g., for diagnostic purposes) may be problematic because of the high capacity of serum albumin (the most abundant protein in plasma) to bind a wide range of substances. Albumin can act as a carrier of many substances (e.g., fat acids, bilirubin, hormones, vitamins, proteins or enzymes) [4–8]. Such ability of albumin to bind very different ligands could favor its nonspecific binding to many immobilized ligands used for purification of plasma proteins by affinity chromatography [4,5,9,10]. Guanidinobenzoatase (GB) is a ''trypsin-like'' protease with a high selectivity for the guanidine groups of homoarginine derivatives, substituted on the terminal amino group. This enzyme seems to be implied in malignity of tumors, suggesting that it may contribute to the destruction of connective elements as fibronectin [11] and gelatin [12] and in invasion, being related to the sloughing off of metastatic cells into the circulation system [13]. This enzyme is also expressed on the surface of some types of cells having high tissue mobility (e.g., leucocytes, keratinocytes, spermatozoa) [14–16].

In previous papers (Guisan et al., submitted), it has been shown that GB could be strongly and selectively adsorbed on agmatine-amido-agarose. The use of tailor-made affinity supports having a low density of ligand moieties strongly improved the affinity adsorption of GB and it also prevented the non specific adsorption of other plasma proteins by ionic exchange. However a high amount of albumin was adsorbed even on such tailor-made affinity matrices.

In this paper, the preparation of new mimetic affinity matrices is reported. Then, the adsorption of albumin and GB on target and mimetic matrices was evaluated. Furthermore, the possible inhibitory effect of mimetic soluble ligands during adsorption of both, albumin and GB, on the target matrix was tested. In such a way, we have tried to design a new protocol to very selectively adsorb GB on the target matrix with no interference of serum albumin.

2. Experimental

2.1. Materials

6% crosslinked agarose beads were generously donated by Hispanagar (Burgos, Spain). The ascitic

fluid of Ehrlich tumor was kindly provided by Dr. J.L. Subiza from San-Carlos University Hospital (Madrid, Spain). Activated CH-Sepharose 4B (containing aminohexanoic acid) and PD-10 columns were purchased from Pharmacia (Uppsala, Sweden). 1,8-Octanediamine (DAO) was from Merck (Darmstadt, Germany). Epichlorohydrin was from Fluka (Buchs, Switzerland). Agmatine, bovine serum albumin (BSA) and *p*-nitrophenyl-*p′*-guanidinobenzoate (*p*-NPGB) were from Sigma (St. Louis, MO, USA). Some of the compounds utilized in this paper are shown in Fig. 1.

2.2. Preparation of agmatine-CH Sepharose 4B (CH-A) and 1,8-octanediamine CH Sepharose (CH-DAO)

Lowly derivatized agmatine and DAO matrices (2 μmol/ml gel) were prepared by incubation of 1 ml of activated CH-Sepharose 4B with 2 ml of coupling buffer (0.1 *M* sodium phosphate–0.5 *M* sodium chloride, pH 6) containing 20 m*M* agmatine or DAO for 30 min at 4°C. The excess of ligand was washed with five gel volumes of coupling buffer. In order to block out the remaining active groups in the matrix, the gel was transferred to 20 ml of 1 *M* ethanolamine, pH 8.0 at 25°C for 2 h at room temperature, under gentle stirring. The resulting gel was thoroughly washed with five volumes of 0.1 *M* acetate buffer, pH 4.0, containing 0.5 *M* sodium chloride followed by 0.1 *M* Tris–HCl buffer, pH 8.0 containing 0.5 *M* sodium chloride (this washing was repeated five times).

2.3. Preparation of agarose-epoxy-agmatine (AEA) gels

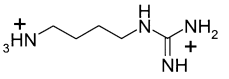
2.3.1. Activation of 6BCL agarose with epoxy groups

6BCL agarose was activated with 10 μmol epoxy groups/ml gel according to the method described by Armisen [17]. 28.5 ml epichlorohydrin (dissolved in 57 ml acetone) was mixed with 150 ml of 1 *M* sodium hydroxide solution containing 6 mg/ml sodium borhydride. Then, 6BCL agarose gel (35 g) was added and left to react for 2 h at 4°C, under a constant gentle stirring. After, a new 28.5 ml volume of epychlorhydrin was added and the reaction continued for 2 h under the same conditions. Then, the gel was extensively washed with distilled water.

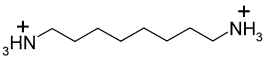
2.3.2. Coupling of agmatine

In order to obtain a derivatization degree of 2 μmol/ml gel, the reaction was carried out in the presence of 20 m*M* agmatine, at pH 6.0, for 30 min at 4°C (Guisan et al., in preparation).

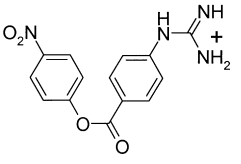
Highly derivatized epoxy-agarose (10 μmol agmatine/ml gel) was obtained by incubating 35 g of epoxy-agarose in 70 ml of 0.1 *M* sodium carbonate buffer–0.5 *M* sodium chloride containing 20 m*M* agmatine at pH 9.0, for 30 min at 4°C, with constant



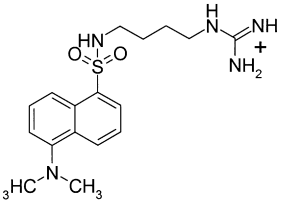
Agmatine



1,8-Octanediamine



P-NPGB



Dansyl-agmatine

Fig. 1. Different related compounds used in GB studies.

gentle stirring. The excess ligand was washed away with five gel volumes of coupling buffer.

2.4. Guanidinobenzoatase purification

In a standard experiment, 10 ml of the ascites fluid of the Ehrlich tumor (diluted 1:10), containing 4.6 mg/ml of protein, was incubated at 37°C with 1 ml agmatine-CH-Sepharose 4B (equilibrated with 10 m*M* sodium phosphate buffer at pH 7.0) for 1 h, under a constant gentle stirring. Thereafter, the mixture was poured into a column. The gel was washed with 10 m*M* sodium phosphate buffer at pH 7.0, until absorbance at 280 nm was zero. Elution was carried out using 5 ml 12 m*M* NPGB [10% dimethylsulfoxide (DMSO)], in 10 m*M* sodium phosphate buffer at pH 7.0.

The soluble GB, containing the hydrolyzed substrate, was submitted to gel filtration through a Sephadex G-25 (PD-10 column) in order to obtain a pure GB solution.

2.5. Study of protein absorption on different supports

A 10-ml volume of 1.2 mg/ml BSA solutions (including different concentrations of NaCl), in 10 m*M* sodium phosphate buffer at pH 7 and 25°C were offered to 0.5 ml of different matrixes for 30 min. GB adsorption was studied using solutions of 0.1 mg of protein per ml. Usually, 3.5 ml of soluble protein was incubated with 0.2 ml of activated matrices, under the conditions described above.

Desorption studies of adsorbed proteins were carried out by incubating, for 30 min, 0.5 ml of a matrix with adsorbed protein under the conditions described above for the adsorption. Concentration of protein was calculated following Bradford's method [19] or by densidometry of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels.

2.6. Guanidinobenzoatase activity

GB activity was assayed spectrophotometrically by measuring the increment in adsorbance at 348 nm promoted by the hydrolysis of 1.2 m*M* *p*-NPGB in 0.1 *M* Tris–1% DMSO, pH 7 at 25°C [18]. One activity unit represents the amount of GB able to release 1 μmol *p*-NPGB per minute, under the conditions of the standard experiment.

2.7. SDS–PAGE analysis

SDS–PAGE (10% polyacrylamide, 0.1% SDS) was run using a SE 250-MIGHTY SMALL II (Hoefer) mini-gels apparatus according to the Laemmli buffer system [20]. Gels were stained using Coomassie blue stain.

3. Results and discussion

3.1. Adsorption of serum samples from the ascitic fluid on agmatine (AMIDE) agarose (CH-A) matrices

Serum samples from the ascitic fluid of mouse with Ehrich tumor have been offered to CH-A matrix (lowly activated as described in methods). All guanidinobenzoatase (GB) was immobilized on this support. However, Fig. 2 shows that this support was

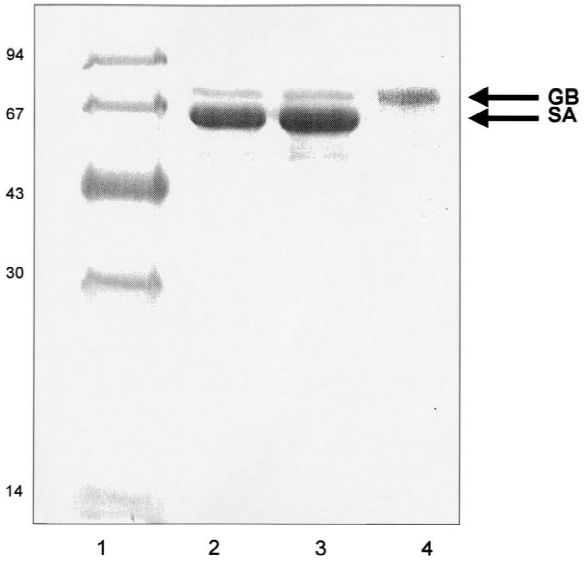


Fig. 2. SDS–PAGE analysis of the protein adsorbed on CH-A. Experiments were performed as described in Experimental. Lane 1: molecular mass markers; lane 2: crude preparation of ascitic fluid of Ehrlich tumor; lane 3: proteins adsorbed on CH-A under standard conditions, lane 4: pure GB.

able to absorb a high amount of plasma albumin. When using a similar support completely blocked with ethanol amine and without agmatine, significant adsorption of any protein could not be detected.

The adsorption of albumin on this support promoted a significant contamination of the final sample, greatly reducing the efficacy of the purification protocol (Table 1). Both albumin and GB could be released from the support by using only 12 m*M* of *p*-NPGB. This suggested a specific adsorption of albumin on the affinity matrix.

3.2. Adsorption of BSA and GB on different matrices

Following the hypothesis proposed in the Introduction, two mimetic supports were prepared and the adsorption of commercial BSA on these supports was studied: agmatine-(secondary amine)-agarose (AEA) and 1,8-octanediamine-(amide)-agarose (CH-DAO). Fig. 3 shows the employed supports and the most significant results.

Using a AEA matrix with 10 μmol agmatine/ml, the extent of binding of the BSA to the support (26%) were much lower than using CH-A gel (90%) or CH-DAO (88%). These results did not seem to be related to the ionic properties of the matrices, because the AEA matrix used had much more charge than the other matrices (Fig. 3). These results suggested that the adsorption of the albumin might proceed via a pocket with a certain affinity for an aliphatic chain ending in a positively charged group. The affinity of the albumin by this type of compounds seems to be reduced but not annulled by the existence of a second charged amino group far from the first group.

However, GB was not adsorbed on AEA matrices, and the adsorption on CH-DAO matrices was only marginal, while it was fully adsorbed on CH-A.

3.3. Use of mimetic matrices to reduce the amount of contaminant proteins

Bearing in mind the behavior of the albumin and GB with the different matrices, we have tried to use AEA to eliminate the maximum amount of proteins from the medium, keeping the GB in the supernatant. Fig. 4 shows that after three cycles of adsorption of serum samples from the ascitic fluid of mouse with Ehrich tumor on AEA, the amount of GB was maintained almost constant, while the total protein concentration dropped to around 30%. Fig. 5 shows that the adsorption of this extract on CHA gave a very improved purification of the GB (Table 1), but still a significant amount of albumin could be detected in the gel.

3.4. Use of mimetic soluble ligands to prevent the adsorption of contaminant proteins

Previous results suggested that compounds having two positively charged moieties were able to interact with albumin while having a much weak interaction with GB. Fig. 6 shows the results obtained when albumin and GB was offered to CH-A in the presence of 25 m*M* agmatine or 1,8-octanediamine. Agmatine was able to greatly reduce the adsorption of albumin on the matrix, but also had a marginal

Table 1
Purification of GB following the different procedures described in the text (experiments were carried out as described in Experimental)

Protocol	Volume (ml)	[Protein] (mg/ml)	Total activity	Specific activity	Yield (%)	Purification factor
Crude	10	4.6	0.17	0.0037	100	1
1	3.5	0.5	0.147	0.084	86.4	22
2	3.5	0.2	0.14	0.20	82.3	54
3	3.5	0.04	0.13	0.92	76.5	250

1=Purification of GB by incubating the crude extract in the presence of CH-A gel in 15 m*M* NaCl. See Results.

2=Purification of GB by using AEA trap gel as preliminary step to the affinity chromatography in CH-A performed in 15 m*M* NaCl. See Results.

3=Purification of GB by using AEA trap gel as preliminary step to the affinity chromatography in CH-A performed in the presence of 25 m*M* 1,8-octanediamine. See Results.

GB/ALBUMIN BINDING ABILITY ON AEA, DAO AND CHA GELS.

	PCG (μEq/ml gel)	AB binding (%)	GB binding (%)
AEA	20	26	<1
CH-DAO	2	92	15
CH-A	2	95	>99

PCG: positively charged groups (guanidino or primary amino ones)
AB: albumin
GB: Guanidinobenzoatase.

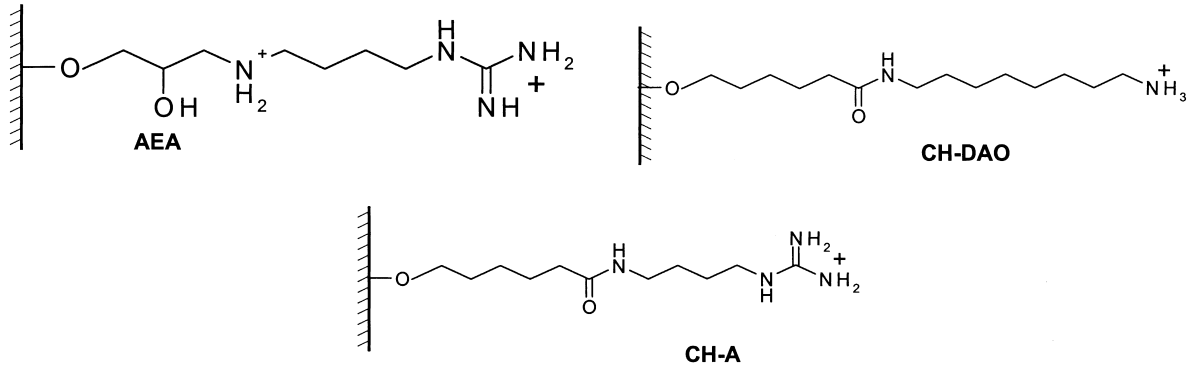


Fig. 3. Adsorption of GB and serum albumin on CH-A and mimetic supports. Experiments were carried out as described in Experimental.

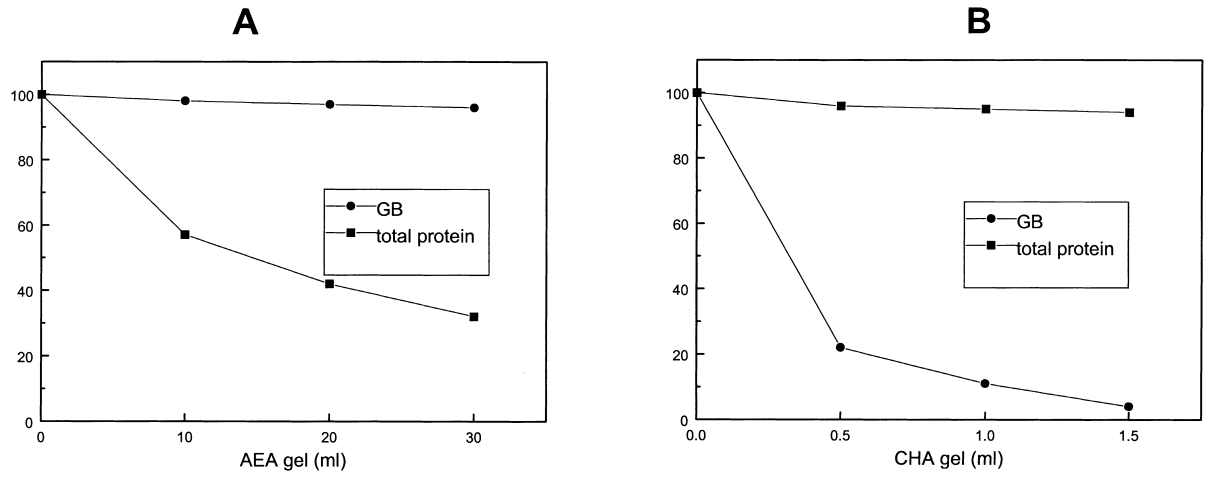


Fig. 4. Effect of mimetic supports and substrates to eliminate BSA from the sample or to prevent the adsorption of BSA on affinity matrices. The figure shows the adsorption of proteins (squares) and GB (circles) on AEA under standard conditions (A) and on CH-A in the presence of 25 m*M* octanediamine (B). Experiments were carried out as described in Experimental.

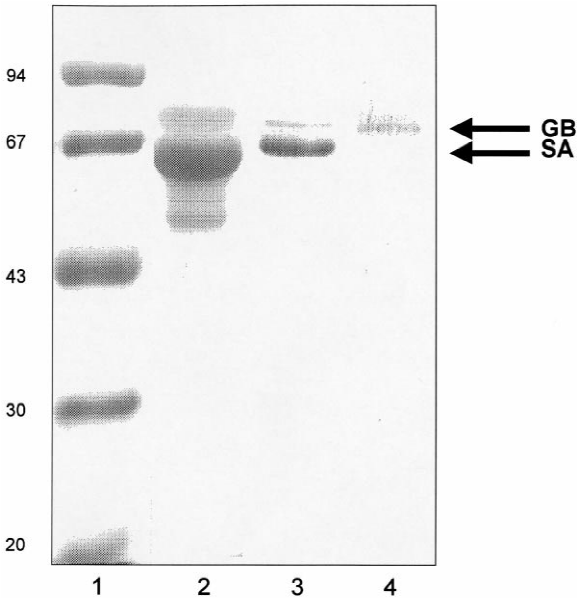


Fig. 5. Purification of GB by using mimetic matrices and mimetic ligands. Experiments and SDS–PAGE analyses were performed as described in Experimental. Lane 1: molecular mass markers, lane 2: crude preparation, lane 3: proteins adsorbed on CH-A under standard conditions after reduction of serum albimin by using trap matrices (see text), lane 4: proteins adsorbed on CH-A in the presence of octanediamine after reduction of serum albumin by using trap matrices (see text).

effect on the adsorption of the GB. However, 1,8-octanediamine had a much higher effect on the adsorption of BSA (less than 5% of BSA was adsorbed on the matrix) and presented a negligible effect on the adsorption of GB on CH-A.

Fig. 5 shows than when the supernatant obtained in the previous point was offered to the CH-A in the presence of 25 m*M* 1,8-octanediamine, the GB activity was absorbed on the support in a completely pure form as showed by SDS–PAGE (Fig. 2). The molecular mass of this protein corresponded to the one described by Steven et al. [14] for the monomer of GB. Also, this protein could be submitted to new cycles of adsorption–desorption on CH-A and could be completely associated to the guanidinebenzoatase activity. Therefore, we can assume that this unique protein band detected by SDS–PAGE should correspond to the protein responsible of the guanidinobenzoatase activity.

4. Conclusion

The use of mimetic ligands both in soluble form (to prevent the adsorption of the contaminant proteins) or in an immobilized fashion (to adsorb the

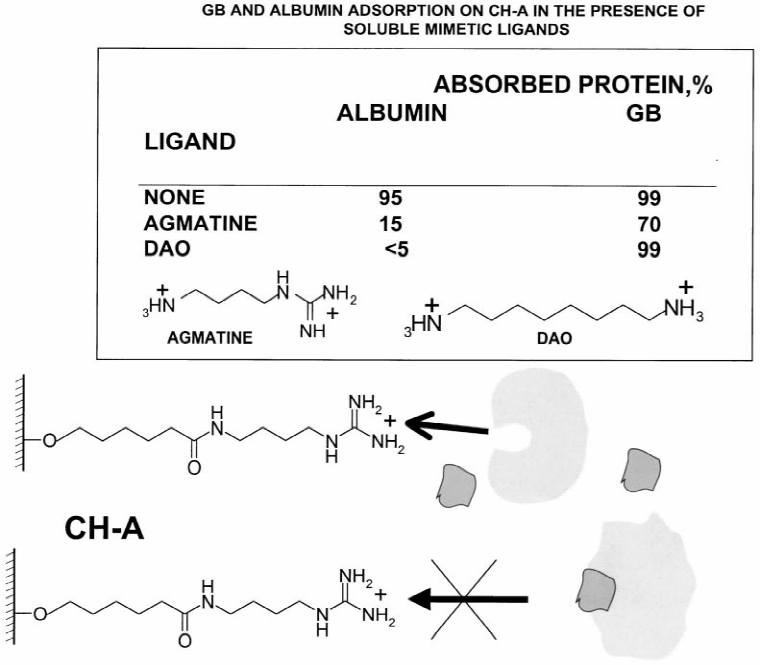


Fig. 6. Effect of different mimetic ligands in the adsorption of albumin and GB on the affinity matrix. Concentration of mimetic ligands was 25 m*M*. Experiments were performed as described in Experimental.

contaminant proteins leaving the target protein in the supernatant) is a powerful tool to improve the affinity chromatography, reducing the problems generated by the presence of low-specific enzymes or proteins that may be adsorbed on the affinity matrix together to our target protein. In general, it is possible to consider that even when trying to separate very similar enzymes or isoenzymes, the recognition of different substrates by both proteins may be quite different, giving the possibility of using the strategies proposed in this paper to separate one enzyme from the other one.

Serum albumin is a protein with many biological functions and become a serious problem when purifying plasmatic proteins because it may adsorb on many affinity matrices by a specific recognition of the ligand. However, its ability to recognize many substances promote that the areas implied in the adsorption may be involved in the recognition of many other substances. Therefore, by studying different compounds with similar structure, it is possible to find some ones that may selectively recognize the albumin, having negligible effects on the target protein. The combined use of mimetic matrices (AEA) to eliminate albumin and the adsorption on the affinity matrix (CH-A) in the presence of 1,8-octanediamine to prevent the adsorption on the affinity matrix of the albumin still present in the solution, allowed the almost fully selective adsorption of GB on the matrices, with a very high yield.

Bearing in mind the implication of this enzyme in tumor development, this selective adsorption of the GB on these tailor-made supports and conditions may enable the development of very simple diagnostic protocols.

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