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Essential role of the concentration of immobilized ligands in affinity chromatography: Purification of guanidinobenzoatase on an ionized ligand

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Abstract

Guanidinobenzoatase, a plasma protein with possible application as a 'tumor marker', has been fully purified by one-step affinity chromatography. The affinity matrix was prepared by 'controlled' immobilization of an enzyme inhibitor (agmatine) onto commercial agarose gels containing carboxyl moieties activated as *N*-hydroxysuccinimide esters. In this way, agmatine becomes immobilized through an amido bond and preserves an ionized guanidino moiety. Different matrices with different concentration of ligands were prepared in order to evaluate their properties as affinity supports. Interestingly, matrices with a very low concentration of immobilized ligands (2 μmol/ml, corresponding to the modification of only 5% of active groups in the commercial resins) exhibited a low capacity for unspecific adsorption of proteins (as anion-exchange resins) and displayed also a high capacity for specific adsorption of our target protein. On the other hand, when affinity matrices possessed a moderate concentration of agmatine (10 μmol/ml of gel or higher), two undesirable phenomena were observed: (a) the matrix behaves as a very good anionic exchange support able to non-specifically adsorb most of plasma proteins and (b) the specific adsorption of our target protein becomes much lower. The latter phenomenon could be due to steric hindrances promoted by the interaction between each individual immobilized ligand and the corresponding binding pocket in the target protein. These hindrances could also be promoted by the presence of a fairly dense layer of immobilized ligands covering the support surface, thus preventing interactions between immobilized ligands and partially buried protein-binding pockets. In this way, a successful affinity purification (23.5% yield, ×220 purification factor, a unique electrophoretic band) could be achieved by combination of three approaches: (i) the use of affinity matrices possessing a very low density of immobilized ligands, (ii) performing affinity adsorption at high ionic strength and (iii) performing specific desorption with substrates or substrate analogues.

Keywords: Guanidinobenzoatase; Enzymes

1. Introduction

For more than 30 years affinity chromatography has become a powerful and exciting technique for protein purification [1]. The high specificity between a target protein and an immobilized ligand (e.g. an inhibitor or a substrate analogue) on a porous matrix, allows an easy separation of the target protein from other proteins contained in crude extracts.

Most ligands used for affinity chromatography do

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not have a completely inert structure (exhibiting some hydrophobic, ionic features). This may induce the ideal chromatographic process to become highly dependent on the matrix preparation. Affinity matrices that have been prepared via introduction of a high concentration of ligands on the matrix could act as ion-exchange or hydrophobic resins, thereby causing unspecific adsorptions of both contaminant proteins and our target one. Bearing in mind that a high percentage of proteins become adsorbed on anionic exchangers at neutral pH values [2], positively ionizable immobilized-ligands may be a specially interesting problem. In this case, many proteins of a crude extract (negatively charged at a neutral pH), may become ionically adsorbed on the chromatographic resin much faster than affinity recognition could take place. Because target protein is not the only protein adsorbed onto the matrix, the size of the affinity columns (necessary to adsorb all the target protein) should be larger than needed if exclusively the target protein becomes adsorbed.

This massive unspecific adsorption of contaminant proteins onto the affinity matrix could also promote some problems during the desorption process; the target protein may be accompanied by impurities which diminish the purification efficiency. For this reason, it seems that the use of very low density of immobilized ligands might improve the global performance of the affinity chromatography.

On the other hand, the density of immobilized ligands could be an important factor in order to improve the specific bioadsorption. Since the early days of the affinity chromatography, it has been emphasized that the use of spacer arms could be a very efficient method to increase the efficiency of the biorecognition phenomena between the immobilized ligands and the protein binding sites (small binding pockets partially buried inside the inner structure of the protein) [3–6]. Another important variable is the density of immobilized ligands [7–9]. Such density may control the intensity of adsorption of the protein onto the affinity matrix [10,11]. In some cases, in the presence of a very high density of immobilized ligands, even containing fairly long spacer arms, the access of an isolated immobilized ligand to the buried binding sites of the protein may be hindered by the presence of a very dense layer of immobilized ligands (Fig. 1), in a similar way that matrices containing a high loading of antibodies reduce the efficacy of the system [11].

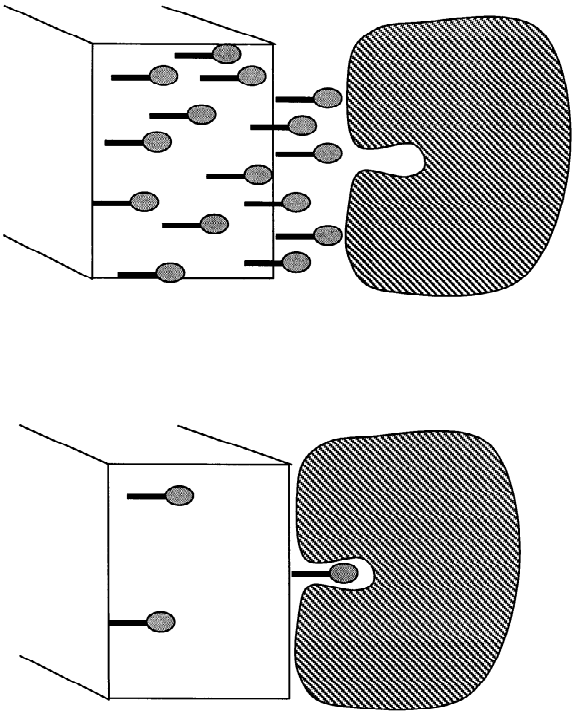

Fig. 1. Possible explanation of the effect of ligand density on the biorecognition of the ligand by the enzyme.

Affinity chromatography matrices are usually prepared by binding ligands (e.g. through amino, carboxyl, hydroxyl groups) onto commercially available pre-activated matrices. These commercial matrices usually contain a relatively high concentration of reactive groups, and so the non-controlled binding of affinity ligands to these pre-activated matrices may yield no optimal affinity chromatography columns.

Guanidinobenzoatase is an interesting enzyme that may be used as tumor marker [12–15]. It may be purified by affinity chromatography on agmatine (a positively charged ligand) attached to agarose matrices through an amido linkage [16–18]. Commercial agarose gels containing a high concentration (15 μmol/ml gel) of *N*-hydroxy succinimide esters can be used to prepare affinity matrices. In this paper, we report the one-step full purification of this enzyme via a fine control of the preparation of tailor-made affinity matrices with special emphasis on the essen-

tial role of the density/concentration of immobilized ligand.

2. Experimental

2.1. Materials

Ehrlich ascites tumor plasma was kindly provided by Dr. J.L. Subiza (San-Carlos University Hospital, Madrid, Spain). Pure guanidinobenzoatase was obtained as previously described [16]. Activated CH-Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). Diaminooctane was from Merck (Darmstadt, Germany). Agmatine and *p*-nitrophenyl-*p′*-guanidinobenzoate (NPGB) were from Sigma (St. Louis, MO, USA).

2.2. Preparation of agmatine–CH Sepharose 4B (CH–A)

Ligand coupling onto the CH Sepharose 4B gel was carried out by reacting, under different conditions, 0.7 g of wet agarose with 2 ml of coupling buffer (0.1 *M* phosphate buffer and 0.5 *M* sodium chloride), containing different concentrations of agamatine. The coupling mixture was agitated end-over-end at 4°C for different reaction periods. Coupling conditions in order to achieve different derivatization degrees (from 0 to 15 μmol/ml) are shown in Fig. 2.

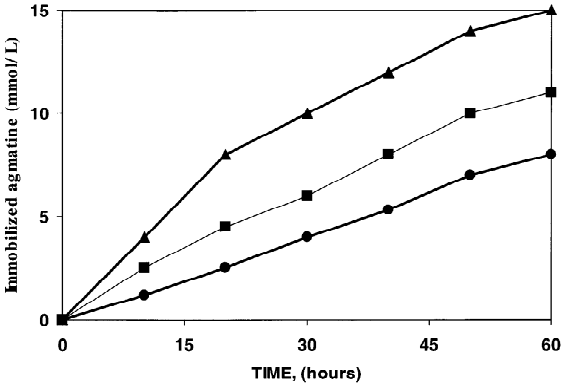

Fig. 2. Course of the immobilization of agmatine on CH–A at different pH values. Experiments were carried out as described in Experimental. pH 7, (triangles), pH 8 (squares), pH 9 (circles).

The excess of ligand was washed away with 50 gel volumes of coupling buffer. In order to block out the remaining active groups, the gel was transferred to 5 volumes of 1 *M* ethanolamine, pH 8.0, for 2 h at room temperature, with gentle stirring. The gel was thoroughly washed with five cycles of alternating pH, each cycle consisting of five volumes of 0.1 *M* acetate buffer at pH 4.0, containing 0.5 *M* sodium chloride followed by five volumes of 0.1 *M* Tris–HCl buffer, pH 8.0, containing 0.5 *M* sodium chloride.

2.3. Adsorption of proteins on CH–A

2.3.1. Influence of the matrix ligand concentration and ionic strength on the unspecific protein adsorption

A protein solution (4.6 mg/ml) was obtained by mixing a crude protein extract of *Escherichia coli* with the necessary amount of 10 m*M* sodium phosphate, pH 7.0, containing different concentrations of sodium chloride. The matrices were equilibrated with 10 m*M* sodium phosphate buffer, pH 7.0 containing the corresponding concentrations of sodium chloride as for the protein solution.

Subsequently, 3.5 g (vacuum dried-wet matrices) of the different agarose gels were added to 10 ml of the protein solution containing 15 or 150 m*M* of NaCl, at 25°C. The suspension was paddle-agitated for ~20 min. Samples of the supernatant were withdrawn and subsequently analyzed by SDS–PAGE and assayed for guanidinobenzoatase activity. Afterwards, the following sequence of procedures was followed: the suspension was filtered through a sintered glass filter, the matrix was washed with 250 ml of the corresponding buffer to eliminate the non-adsorbed proteins) and the water inter-particles was eliminated by vacuum filtration. The protein content of samples of these matrices was analyzed by SDS–PAGE (see below).

2.3.2. Influence of the matrix ligand concentration on the GB adsorption

A 3.5-g amount (vacuum dried-wet matrices) of the agarose gels activated with different amounts of agmatine groups (0, 2, 8 and 15 μmol of agmatine groups/ml gel) was added to a solution $1 \cdot 10^{-5}$ *M* purified GB in 5 m*M* sodium phosphate–5 m*M* NaCl

at pH 7 and 25°C. The adsorption mixture was paddle−agitated for 20 min, the suspensions were filtered, the matrices were washed with five volumes of 5 m*M* sodium phosphate–5 m*M* NaCl, pH 7, and finally vacuum dried. Samples of the matrix containing the adsorbed protein were assayed for GB activity and further analyzed for protein profile via SDS–PAGE.

2.4. Elution of GB using the substrate NPGB

A 3.5-g amount of agmatine–agarose with GB adsorbed was suspended for 1 h in 12.5 ml of 10 m*M* NPGB in 0.1 *M* Tris buffer containing 10% (v/v) dimethyl sulfoxide (DMSO), at pH 7.0 under gentle stirring. Released GB was then recovered by filtration through a sintered glass filter. Once purified, the enzyme solution containing NPGB was applied onto a G-25 Sephadex column (30×2.5 cm, PD-10 column), in order to remove the hydrolysis products.

2.5. GB activity assays

GB activity was assayed spectrophotometrically by measuring the increment in absorbance at 348 nm promoted by the hydrolysis of 1.2 m*M* *p*-NPGB in 0.1 *M* Tris–1% DMSO at pH 7 and 25°C. One activity unit represents the amount of GB able to release 1 μmol *p*-nitrophenol per minute under the standard conditions. When using GB adsorbed on the CH–A matrix, 20 mg of dried-vacuum matrix were added to the enzyme assay.

2.6. SDS–PAGE analysis

SDS–PAGE (10% polyacrylamide mini-gels with 0.1% SDS) was run using a SE 250-Mighty Small II (Hoefer) apparatus at 150 V according to the Laemmli buffer system [19]. Samples of both solutions and gels were loaded after boiling them for 3 min in the presence of 1% SDS and mercaptoethanol. This treatment promotes the desorption of all proteins adsorbed onto the matrix [20]. SDS–PAGE gels were then stained using Coomassie Blue stain.

3. Results and discussion

3.1. Influence of the matrix ligand concentration and ionic strength on the unspecific protein adsorption on CH–A

Fig. 3 shows an SDS–PAGE gel of the protein (from a crude preparation of *E. coli*) adsorbed on CH–A matrices activated with different ligand concentrations at different NaCl concentrations. The protein adsorption increased with the concentration of ligand on the matrix surface and with decreasing ionic strength (Fig. 3). A high density of coupled ligands seemed to convert the CH-affinity matrix into an anionic exchanger (the guanidino group of agmatine has a p*K* value around 13). This observation is quite relevant, since at neutral pH and low ionic strength most proteins are negatively charged, and therefore may adsorb on the support [2]. Therefore, a very weak derivatization combined with a suffi-

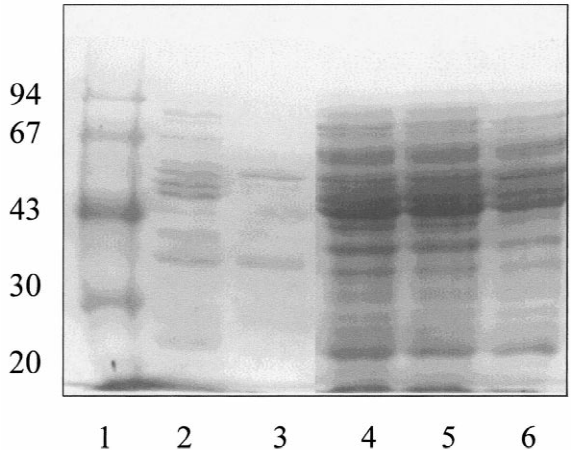

Fig. 3. Effect of concentration of ligand in the matrix and ionic strength on the adsorption of a crude extract from *Escherichia coli* on CH–A. SDS–PAGE was performed on the supernatants obtained after boiling the gels in SDS as described in Experimental. Lanes: 1=molecular mass markers; 2=proteins adsorbed on CH-activated with 2 μmol agmatine/ml gel at 15 m*M* NaCl; 3=proteins adsorbed on CH-activated with 2 μmol agmatine/ml gel at 150 m*M* NaCl; 4=proteins adsorbed on CH-activated with 15 μmol agmatine/ml gel at 15 m*M* NaCl); 5=proteins adsorbed on CH-activated with 15 μmol agmatine/ml gel at 50 m*M* NaCl); 6=proteins adsorbed on CH-activated with 15 μmol agmatine/ml gel at 150 m*M* NaCl.

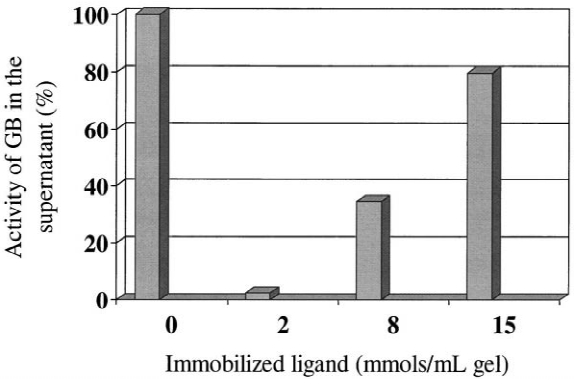

Fig. 4. Influence of immobilized agmatine concentration on the GB adsorption on CH–A. Purified enzyme was incubated with CH–A activated with 0, 2, 8 and 15 μmol agmatine/ml gel. Experiments were performed as described in Experimental.

ciently high ionic strength should minimize the unspecific protein absorption. In fact, the lowest protein adsorption on the CH–A was obtained using a matrix activated with a very low agmatine concentration (2 μmol/ml gel) and adsorbing the proteins at a moderately high ionic strength (150 m*M* NaCl) (see Fig. 3). Proteins were not significantly adsorbed on just blocked (not modified with agmatine) supports.

3.2. Influence of the matrix ligand concentration on the GB adsorption onto CH–A

Fig. 4 shows that the adsorption of pure GB on the affinity gel strongly depends on the concentration of agmatine coupled to the matrix. After the incubation of GB with the gel activated with 15 μmol coupled ligand/ml gel, 85% of the initial enzyme activity observed in the crude extract remained in the supernatant. On the contrary, when using 2 μmol agmatine/ml gel, less than 10% of the initial enzyme activity remained in the supernatant, indicating that under these conditions, GB was efficiently immobilized on the affinity support (see Fig. 4). This could have originated by the promotion of steric hindrances when using a high superficial density of ligands on the support surface which probably makes the recognition of the ligand by the enzyme active center (usually an internal pocket) difficult (Fig. 1).

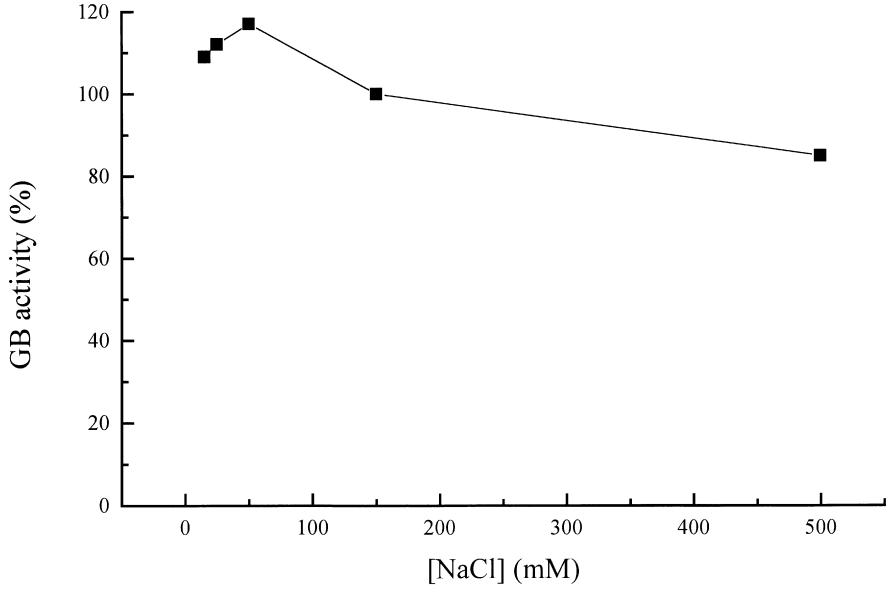

Fig. 5. Influence of the ionic strength on the GB activity. Activity of pure GB was measured at different NaCl concentrations. GB activity was determined as described in Experimental.

3.3. Adsorption of GB from ascitic fluid extract under optimal conditions

Bearing in mind the results presented, the use of a matrix possessing a very low density of ligands at moderately high ionic strength, not only seemed to minimize the unspecific adsorption of the contaminant proteins present in the crude extract, but also strongly facilitates the ligand recognition by the active center of GB. A first requirement for the use of the ionic strength suggested for GB purification, is that the enzyme preserves its ability to recognize the ligand under these conditions. It was found that ionic strength did not have a significant influence on GB activity (Fig. 5); therefore, these conditions can be used in the purification of this enzyme.

The adsorption of GB on CH–A activated with 2 μmol/ml at 150 m*M* NaCl enabled the specific adsorption of GB (accounting for 35% of the GB activity) on the affinity matrix (with only some traces of albumin) (Fig. 6).

3.4. Elution of GB with the substrate NPGB from CH–A

The desorption of the enzyme in the presence of low concentrations of enzyme substrate or competitive inhibitors from the matrix may give some indication on the real adsorption mechanism of the enzyme on this matrix. In the case of this enzyme, the preparation of commercial inhibitors is quite complicated, therefore we decided to use the substrate (NPGB) to release the enzyme from the support. The use of a substrate also enabled the study of the catalytic behavior of the enzyme adsorbed on the affinity ligand. Fig. 7 shows that the GB absorbed on CH–A has initially a very low activity (suggesting that GB may had its active center blocked), which increased as time elapsed until reaching the theoretical value calculated from the adsorbed enzyme. Studies of the supernatant showed that this increment in activity was correlated with the progressive accumulation of enzyme in the supernatant, therefore confirming that the enzyme was effectively released from the matrix into the supernatant in the presence of NPGB (leaving free the active center and allowing the enzyme to exhibit full activity).

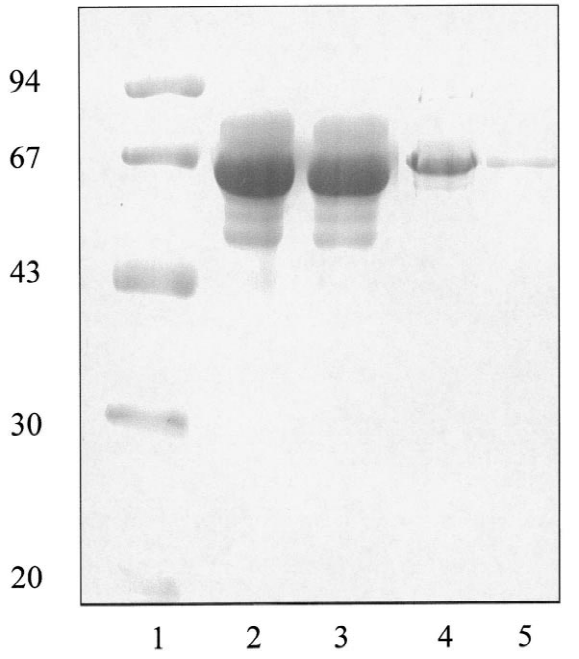

Fig. 6. Purification of GB from ascitic fluid of Ehrlich tumor. SDS–PAGE analysis of the proteins from ascitic fluid of Ehrlich tumor adsorbed on CH–A. Experiments were performed as described in Experimental. Lanes: 1=molecular mass markers; 2=crude preparation of ascitic fluid of Ehrlich tumor; 3=proteins in the supernatant after adsorption on CH–A at 15 m*M* NaCl; 4=proteins adsorbed on CH–A at 150 m*M* NaCl; 5=GB desorbed in the presence of enzyme substrate (NPGB).

The adsorption of GB from ascitic fluid on a tailor-made matrix (2 μmol/ml) in 150 m*M* NaCl and further desorption in the presence of 12 m*M* NPGB yielded a pure GB. Even the small traces of albumin adsorbed on the gel resulted now undetectable in the supernatant after desorption under these very mild conditions. After gel permeation, to eliminate the products of the hydrolysis of the NPGB, this protocol yielded a purification degree of 220 with a fairly good yield (23.5%) (Table 1).

4. Conclusions

The concentration of ligand on the matrix surface plays an essential role for a good performance of affinity chromatography. A high density of not fully inert ligands may promote unspecific adsorption of

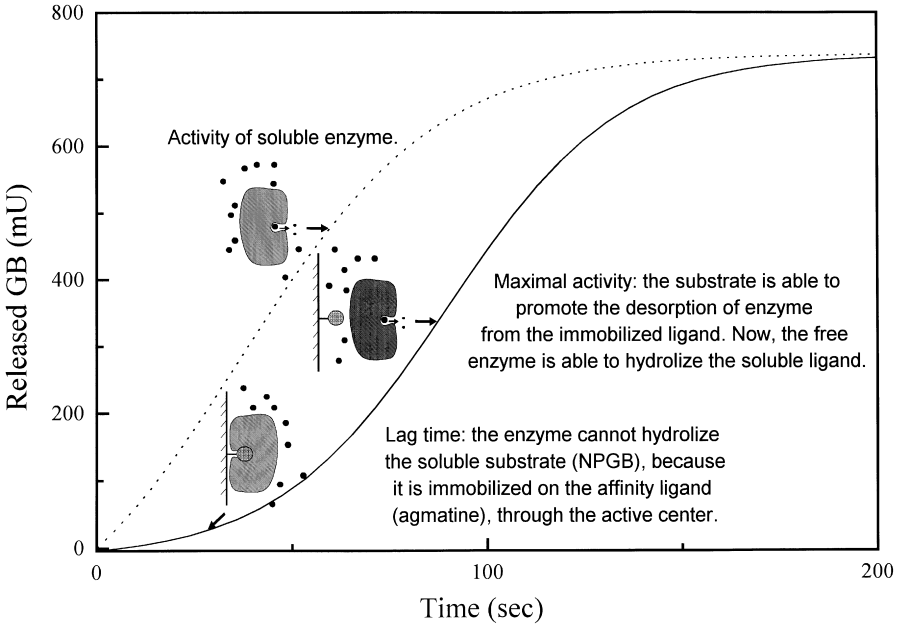

Fig. 7. Reaction course of the hydrolysis of NPGB using adsorbed GB on CH–A (solid line), compared with the activity of an equivalent amount of free GB (dotted line). The assay was carried out as described in Experimental.

proteins. It may also promote steric hindrances for the biorecognition of the ligand by the target protein. Therefore, preparation of affinity matrices with a very low concentration of immobilized ligands constitutes an essential factor for a good performance of affinity chromatography protocols on positively ionized ligands and very likely on any other type of non-completely inert ligand. (a) When ligands are positively charged even at moderate concentrations (e.g. 15 μmol/ml), the support is able to promote the ionic adsorption of most proteins contained in protein extracts (e.g. a crude extract from *E. coli* or an extract of plasma proteins). (b) The biological recognition between GB and immobilized agmatine is facilitated by using low concentrations of ligand in the matrix (2 μmol/ml of support). Contrary to the expected results, the adsorption of GB on the matrices decreased when increasing the concentration of ligands on the matrix. Commercial supports may have up to 20–40 μmol/ml of reactive groups (N-hydroxy succinimide esters) which are able to immobilize agmatine via amido linkages. Therefore, a non-controlled immobilization of agmatine on this commercial agarose should generate affinity matrices with a very poor performance in affinity chromatography. On the other hand, a controlled modification of only 5–10% of the reactive groups in pre-activated agarose is strictly necessary to obtain very accurate affinity matrices. (c) GB is able to recognize the guanidino group of its substrates and inhibitors

Table 1
GB purification degree by affinity chromatography[a]

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	0.014	0.04	0.816	23.5	220

[a] 1=Purification of GB by incubating the crude extract in the presence of CH–A gel in 150 m*M* NaCl. See Results.

even at high ionic strength (e.g. 0.15 *M* NaCl). Hence, combining the use of affinity matrices with a very low density of immobilized ligands and the use of moderately high ionic strength during bioadsorption allows one to get a highly selective adsorption of guanidinobenzoatase on agmatine–agarose. Furthermore, selective desorption with one enzyme substrate allows a full purification of this interesting enzyme (a possible tumor marker) [12–16] via a single-step affinity chromatography. The GB adsorption at low ionic strength on these matrices enhances the adsorption yield, but is also accompanied by a massive pseudo-specific adsorption of albumin on the gel that greatly reduces the purification factor. Elimination of such unwanted side effects will be the subject of a forthcoming paper [16].

5. Abbreviations

GB	guanidinobenzoatase
NPGB	*p*-nitrophenyl-*p′*-guanidinobenzoate
CH–A	Agmatine–CH-Sepharose 4B gel
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
DMSO	dimethyl sulfoxide

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