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The Adsorption / Desorption Behaviour of Horseradish Peroxidase and Porcine Thyroglobulin on Concanavalin A-Sepharose with Different Ligand Densities

L. FRANCO-FRAGUAS[a*], J. CARLSSON[b] and F. BATISTA-VIERA[a]

[a]*Cátedra de Bioquímica, Facultad de Química. CC 1157. Gral Flores 2124, Montevideo, Uruguay and* [b]*Center for Surface Biotechnology, BMC, Uppsala University, Uppsala, Sweden*

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A series of Concanavalin A-Sepharose gels containing between 5 and 480 mg lectin/g of dry gel was prepared by varying the amount of Concanavalin A used in the coupling reaction after activation with 1-cyano-4-(dimethyl-amino)-pyridinium tetrafluoroborate (CDAP) in a modified procedure. The influence of the ligand content on the adsorption / desorption of two different glycoproteins, horseradish peroxidase (HRP, Mw 40 KDa) and porcine thyroglobulin (Mw 670 KDa) was studied. The adsorption of HRP was found to be dependent on the ligand density and the largest amount adsorbed was obtained when the molar ratio between immobilized Con A and applied HRP was 9/1. The adsorption of thyroglobulin on the other hand was rather independent of the molar ratio used. The results indicate that highly effective lectin affinity adsorbents can be rationally designed based on the amounts of immobilized lectin.

Keywords: Concanavalin A; lectin; horseradish peroxidase; thyroglobulin; affinity chromatography

INTRODUCTION

The application of immobilized lectins in affinity chromatography is based on their specific interactions with glycoconjugates. When attempting the synthesis of these lectins derivatives, there are several factors influencing their further effectiveness to adsorb glycoproteins [1]. Their synthesis requires suitable matri-

* To whom all correspondence should be addressed. E-mail: bioquim@bilbo.edu.uy

ces and effective coupling methods for attachment of the ligands. Agarose fulfils most demands as a carrier for adsorptive groups. Many protein immobilization techniques for covalent coupling involve the reaction of protein amino groups with electrophilic moieties introduced on a solid support. Some of the most widely used approaches are based on the use of CNBr activation procedures and cyanylating agents such as 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP) [2].

Immobilized Concanavalin A (Con A) has been extensively used for isolation, fractionation, structural characterization and immobilization of glycoproteins and other biologically important glycoconjugates carrying glucose and mannose groups [3]. An example of its application is the further purification of a commercial horseradish peroxidase on Con A-Sepharose [4].

In this work, we have used the CDAP-activation technique (due to its described advantages over the traditional CNBr activation procedure, [2]) with the purpose of preparing lectin based-adsorbents on agarose supports, suitable for the affinity chromatography of glycoproteins. After optimization of the activation step, a number of Con A-Sepharose derivatives with a wide range of ligand densities were prepared. The gels obtained were then studied as adsorbents in biospecific affinity chromatography with one relatively small and one large glycoprotein, namely horseradish peroxidase (HRP, 40 KDa) and porcine thyroglobulin (670 KDa).

MATERIALS AND METHODS

Concanavalin A (Con A), Sepharose 4B, CNBr-activated Sepharose and Con A-Sepharose were from Pharmacia. The 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP) reagent, horseradish peroxidase (HRP, Type II, RZ 1.5 to 2.0) and porcine thyroglobulin were from Sigma Chemical Co. Triethylamine was from Aldrich. All other chemicals used were of analytical grade.

Activation of Sepharose with CDAP reagent

Sepharose 4B was activated as described in [2], with some modifications. In a typical activation experiment, 3.0 g of suction-dried agarose (Sepharose 4B) was washed on a glass filter with distilled water, acetone:water (3:7 v/v) and then with 50 mL of acetone: water (6:4 v/v), previously cooled to 4°C. The gel was drained by mild suction and then transferred to a 10 mL glass beaker and mixed with 3 mL of acetone: water (6:4 v/v) pre-cooled to 4°C. CDAP (75 mg) dis-

solved in 2 mL of acetone: water (6:4 v/v), was added to the gel suspension under vigorous stirring at 4°C for 3 min; then, 360 µL of 200 mM TEA solution was added dropwise over 1 to 2 min. After 3 min the entire reaction mixture was quickly added to 50 mL of ice-cold 50 mM HCl. The incubation period of the gel suspension with 50 mM HCl was varied between 3 and 15 min, then it was rapidly transferred to a glass filter funnel, washed with 50 mL of ice-cold water and suction-dried under vacuum. The activated gel (CDAP-agarose) was equilibrated with buffer and immediately used for coupling purposes. We determined that 1.0 gram of dry activated-Sepharose corresponds to 18.2 grams of wet gel and to 28.2 mL of packed gel.

Immobilization (coupling) step

Con A-Sepharose derivatives were synthesized by incubating the CDAP-activated Sepharose (a fixed amount of wet gel) with increasing amounts of pure Con A. In order to achieve a range of different ligand contents (mg of immobilized Con A / gram dry gel) the following amounts of Con A were added: i) for contents between 10–50 mg/ g dry gel, 1–5 mg per g of wet gel; ii) for contents between 100–150 mg/g of dry gel, 10–15mg per g of wet gel and iii) for contents between 200–300 mg / g of dry gel, 20–30mg per g of wet gel. Each coupling experiment was performed as follows: aliquots of 3.0 g of wet activated gel, equilibrated in 100 mM $NaHCO_3$ pH 8.3 (coupling buffer) were incubated with 3 mL of the appropriate solution containing the Con A dissolved in coupling buffer and mixed end over end at room temperature for 4 hours. The gel derivatives were washed on a glass filter with the coupling buffer, distilled water and finally with 100 mM acetate buffer pH 6.0 containing 500 mM NaCl, 1 mM $CaCl_2$ and 1mM $MnCl_2$.

Determination of the amount of bound protein

The amount of Con A immobilized on Sepharose was determined by total amino acid analysis performed at the Aminosyra Analys Centralen, BMC, Uppsala, Sweden. Prior to the analyses, the gels were washed thoroughly with water on a glass filter-funnel, suction-dried under vacuum and dried in a dessicator with P_2O_5 until constant weight.

Adsorption of glycoproteins to Con A-Sepharose

The glycoproteins, HRP and porcine thyroglobulin, were dissolved in 100 mM acetate buffer pH 6.0, containing 500 mM NaCl, 1mM $CaCl_2$ and 1mM $MnCl_2$

(adsorption buffer). The ligand content of the synthesized Con A-Sepharose derivatives as well as the molar ratio between the immobilized ligand (Con A) and the glycoproteins are summarized in Table I. Each column was packed with 0.75 mL of the corresponding Con A-Sepharose gel. The applied amount of HRP was 1.6 mg in each experiment and for the case of thyroglobulin it was 2.0 mg. The amounts of bound glycoproteins were calculated as the difference between the amount of protein added and the amount of unbound protein, measured spectrophotometrically at 280nm ($A^{1\%}_{280} = 13.1$ and 10.5 for the HRP and thyroglobulin, respectively) [5]. When the adsorption was quantitative, elution of the adsorbed material was performed, under different conditions. Each adsorption experiment was performed at least in triplicate.

TABLE I Molar ratio between the immobilized ligand (Con A) and the added glycoproteins

mg Con A / g dry gel[a]	*nmoles Con A / g wet gel*[b]	*Molar Ratio*	
		Con A / added HRP[c]	*Con A / added Thyroglobulin*[d]
4.9	3	0.08 / 1	1 / 1
45.0	25	0.6 / 1	8 / 1
81.4	44	1 / 1	15 / 1
122.3	96	3 / 1	27 / 1
131.8	73	2 / 1	24 / 1
150.8	80	2 / 1	25 / 1
203.0	121	3 / 1	40 / 1
243.1	113	3 / 1	37 / 1
299.1	200	5 / 1	67 / 1
319.0	195	5 / 1	67 / 1
390.2	369	9 / 1	–
403.0	391	10 / 1	–
479.1	452	11 / 1	–

a. Immobilized Con A, determined by total amino acid analysis (prior to the analyses, the gels were washed with water on a glass filter-funnel, suction-dried under vacuum, weighted and dried in a dessicator with P_2O_5 until constant weight).
b. nmoles of immobilized Con A (106 KDa) per gram of wet gel. The ratio between wet and dry gel was calculated for each sample, and used to express the results in terms gram of wet gel.
c. In each experiment, 40 nmoles of HRP was added.
d. In each experiment, 3 nmoles of thyroglobulin was added.

RESULTS

Preparation of Con A adsorbents

In order to examine the influence of the density of immobilized Con A on the binding of HRP and thyroglobulin, experiments were designed to give a number of affinity gel preparations with different amounts of Con A. This was not possible with the traditional CDAP-activation procedure, which regardless of the amount of initially added Con A gave derivatives with a rather low lectin content (between 2.4 and 3.3 mg per mL of packed gel). Moreover, when the adsorption of HRP to these gels was studied, it was found that 80 to 90 % of the initially added protein was recovered in the nonbound material eluted from columns packed with the lectin gels. However, when the HRP was applied to a column packed with a commercial Con A-Sepharose which had a much higher ligand content (9,7 mg per mL of packed gel), 80 % of the initially added HRP activity was adsorbed, which was consistent with the report of Brattain [4]. This indicates that the lack of enzyme adsorption could be related to the lectin concentration on the gel. To be able to prepare Sepharose derivatives with increasing ligand density it was necessary to modify the CDAP-activation procedure as follows. The incubation period for agarose with the CDAP reagent was increased from 30 seconds to 2 min, and the washing step with 50 mM HCl was reduced from 15 min to 3 min. The washing step is required to hydrolyze the gel bound pyridinium isourea derivative, formed as a byproduct in the activation procedure. According to the literature, the active cyanate groups should not be affected by this treatment [2]. However, it was observed that after a short (3 min) washing period the activated agarose formed was able to immobilize almost 8-fold more Con A than after a longer (15 min) washing period. After these modifications, Con A was coupled in yields exceeding 90 % of the added protein. The coupling of Con A with respect to the presence of low molecular weight carbohydrates during immobilization was also studied. The coupling yields obtained (percentage of the immobilized Con A related to the initially added Con A) were 92 % for coupling without mannose and 89 % for coupling in presence of 100 mM mannose. Thus, the presence of the sugar did not significantly affect the yield. During the course of these studies, activation of Sepharose-4B by the conventional CNBr-activation technique was also tried and the resulting activated gel was used for coupling of Con A. However, the highest lectin immobilization yields and degrees of substitution were obtained with the modified CDAP-activation technique (data not shown).

Influence of the solid phase lectin content on the adsorption of glycoproteins

Several Con A-Sepharose derivatives covering a very wide range of ligand content were prepared as described under Methods section. The optimal gel lectin density for the adsorption of HRP (a 40 KDa glycoprotein) was analyzed and compared with the much larger glycoprotein, thyroglobulin, with a molecular weight of 670 KDa. The Con A ligand /HRP (thyroglobulin) molar ratios assayed are given in Table I. The adsorption yield of HRP increased progressively with increasing content of immobilized Con A (Figure 1). For ratios between 0.08/1 and 5/1 (Table I), the enzyme adsorption was low but maximum yields (exceeding 90 % of adsorption) were obtained with immobilized Con A/ added HRP molar ratios equal to or higher than 9/1. In the case of thyroglobulin, adsorption was rather independent of the molar ratio, between 1 / 1 to 67 / 1 of immobilized Con A /thyroglobulin (Figure 1). The amounts of HRP and thyroglobulin used in the experiments were 40 nmoles (1.6 mg) and 3 nmoles (2.0 mg), respectively. For the lowest Con A load (4.9 mg/ g dry gel or 0.16 mg / mL packed gel) the derivative was able to adsorb 55 % of the applied thyroglobulin (1.1 mg of protein) while in the case of HRP only 16 % of the added protein was adsorbed (0.2 mg). Thus the main determinan for the adsorption of the low Mw protein seems to be the immobilized ligand density.

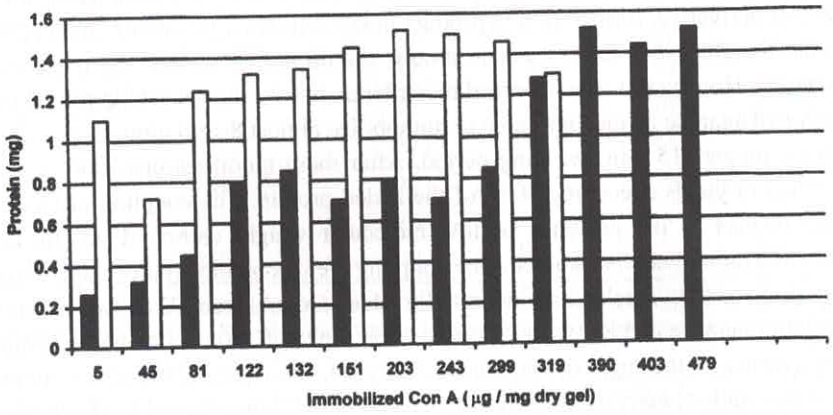

FIGURE 1 Comparative analysis of the amounts of adsorbed glycoproteins: thyroglobulin (□) and HRP (■), on Con A-Sepharose derivatives with different ligand contents. In each experiment, 40 nmoles (1.6 mg) of HRP or 3 nmoles (2.0 mg) of thyroglobulin were added to 0.75 mL of Con A-Sepharose derivative packed in a column. Results are the average of at least three independent experiments

The adsorption/desorption behavior of thyroglobulin and HRP on Con A-Sepharose derivatives was also analyzed (Figures 2 and 3, respectively). Figure 2 shows the behavior for porcine thyroglobulin. In all cases the amounts of protein recovered after elution, were lower than those applied, in spite of the use of different elution approaches (100 mM and 500 mM methyl-mannoside, 1 M glucose, low-pH or boric acid containing buffers). The same behavior was observed with the commercial Con A-Sepharose (containing 273.4 mg of lectin per g of dry gel, or 9.7 mg / ml packed gel). Figure 3 shows the behavior for HRP. In this case, the protein recovery from gels (containing more than 390.0 mg of Con A per gram of dry gel) reached percentages of 90 % of the applied protein (Table II) and the recovery of the adsorbed material exceeded 90 %, for those gels with very high lectin density.

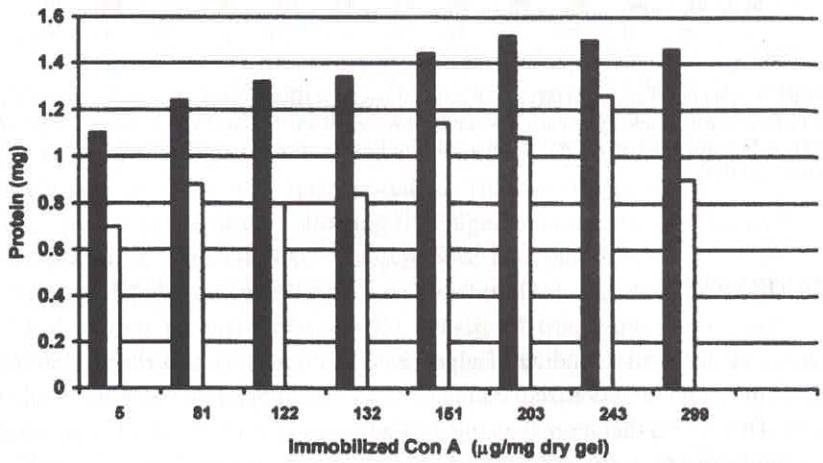

FIGURE 2 Amounts of adsorbed (■) and desorbed/eluted (□) thyroglobulin on Con A-Sepharose derivatives with different ligand contents. In each experiment, 2.0 mg (3 nmoles) of thyroglobulin was added to 0.75 mL of Con A-Sepharose derivative packed in a column. Results are the average of at least three independent experiments

TABLE II Adsorption of HRP onto Con A Sepharose with high degrees of substitution

Con A-derivatives	*Immobilized Con A (mg /mL packed gel)*	*Amounts of HRP adsorbed*[a]	
		(mg)	*%*
Con A -Sepharose ®	**9.7**	1.3	80
	21.5	1.5	95
Con A immobilized on	**22.2**	1.4	90
Sepharose activated with CDAP	**26.4**	1.5	95

a. Columns packed with 0.75 mL of packed gel.

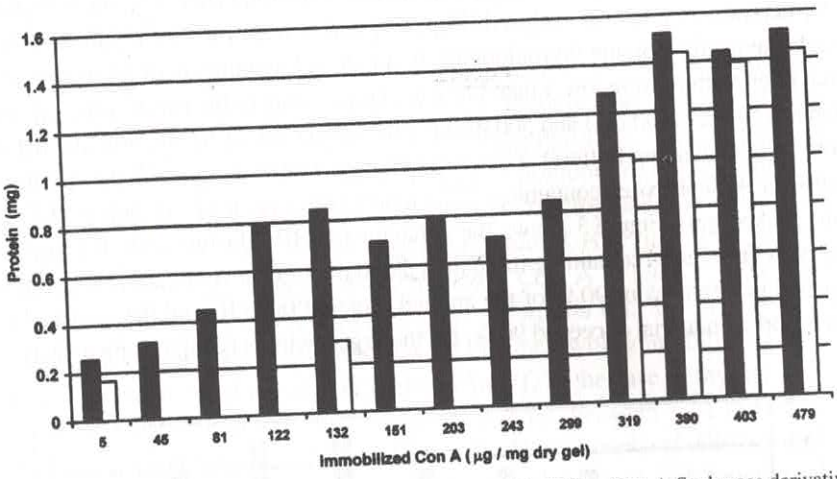

FIGURE 3 Amounts of adsorbed (■) and desorbed/eluted (□) HRP on Con A-Sepharose derivatives with different ligand contents. In each experiment, 1.6 mg (40 nmoles) of HRP was added to 0.75 mL of Con A-Sepharose derivative packed in a column. Results are the average of at least three independent experiments

DISCUSSION

There is evidence that binding of glycoproteins on affinity adsorbents (interactions between the immobilized lectin and the glycocompounds) is of multivalent nature. This means that more than one lectin molecule might be involved in binding a molecule of a glycocompound. It is generally accepted that the binding capacity of the affinity gel increases with the concentration of the immobilized ligand [6]. This parameter seems to be of major importance is the interaction of horseradish peroxidase (glycoprotein) and Concanavalin A (lectin). This lectin from *Canavalia ensiformis*, binds molecules containing α-D- mannopyranosyl, α-D-glucopyranosyl and sterically related residues. At pH 7.0 it is composed of four carbohydrate-free identical subunits with a mass of 26.5 KDa. The association of subunits is pH-dependent. It possesses four combining sites, is a metallo-protein and each subunit contains one Ca^{+2} and one Mn^{+2} ion [7].

We found that the concentration of the immobilized ligand (Con A) has to be carefully optimized, to reach a desirable degree of adsorption of the peroxidase from horseradish (*Armoracia rusticana*). This is a glycoprotein that contains N-linked oligosaccharide chains. It consists of several isoenzymes which all contain 18–20% (w/w) of carbohydrates, in the form of oligosaccharide chains [8]. The average composition is 2 moles of N-acetylglucosamine (GlcNAc), 2.6

moles mannose (Man) and 0.8 mole each of fucose (Fuc) and xylose (Xyl). The following oligosaccharides account for 80 % of the total carbohydrate content:

Man α 1→6 (Man α 1→3) (Xyl β 1→2) Man β 1→4GlcNAc β 1→ 4 (Fuc a 1→3) GlcNAc [9,10]. The largest amount of HRP adsorbed on the prepared Con A derivatives were obtained when the molar ratio of immobilized Con A / applied HRP was 9/1, i.e one mole of added HRP per 9 moles of immobilized Con A. This suggests multi-point interactions between the ligand (Con A) and the counterligand (the HRP), e.g. one molecule of HRP interacts simultaneously with several molecules of Con A, through different carbohydrate residues on the enzyme surface. The probability that this situation occurs is, of course, higher for the derivatives with a high degree of substitution.

The influence of conditions for synthesis of Con A-Sepharose regarding the capacity for the specific binding of another glycoprotein, porcine thyroglobulin, was also analyzed. Thyroglobulin is the major glycoprotein synthesized by the thyroid gland. The carbohydrate moiety of this glycoprotein has been extensively investigated. It consists of both high-mannose-type (unit A-type) and complex-type (unit B-type) oligosaccharides, which are linked to asparagine residues in the peptide chain in all species studied. The unit A-type consists of more or less equal amounts of the following five high-mannose glycans: $Man_5GlcNAc$, $Man_6GlcNAc$, $Man_7GlcNAc$, $Man_8GlcNAc$ and $Man_9GlcNAc$. All contain a $(Man)_5$ $(GlcNAc)_2$-Asn unit as a core structure [11]. The unit B-type oligosaccharides from porcine thyroglobulin consist of triantennary and biantennary complex-type oligosaccharides as acidic sugar chains[12]. Con A-Sepharose has been found to be extremely useful for the separation of biantennary oligosaccharides from triantennary oligosaccharides, with strong affinity for the trimannosidic core substituted by two N-acetyl-β-glucosaminyl residues. It has been demonstrated that the most active part of a glycan structure towards Con A is not the α-mannose residues in a terminal position but the disaccharide N-acetyl β-D-glucosaminyl -(1→2)-α-D-mannoside [13]. In fact, Con A shows a strong affinity for the trimannosidic core substituted by two N-acetyl-β-glucosaminyl residues, like those present in thyroglobulin.

It was not possible to recover all the initially adsorbed thyroglobulin from the affinity column by elution with an excess of the monosaccharide competitor, in any of the adsorption experiments that we performed with this protein. The desorption was not significantly improved by using eluants with low-pH or boric acid-containing buffers. Glycoproteins containing a high density of the trimannosidic core substituted by two N-acetyl-β-glucosaminyl residues and high mannose-type glycans bind to Con A-Sepharose very tightly, and the resulting polyvalent interaction cannot be easily disrupted by a monovalent sugar such as methylmannoside or glucose, even at very high concentrations. Similar results have been reported for

glycophorin with Wheat Germ Agglutinin-Sepharose [14] and for the interaction between alkaline phosphatase and its antibody immobilized on Sepharose [15]. Occasionally, Con A-glycoprotein complexes are further stabilized by nonspecific hydrophobic interactions. Dissociation of these complexes often requires organic solvent (ethylene-glycol). The interaction between a glycoprotein and a lectin is governed by the binding affinity of each subunit for the glycoconjugate, but if a single molecule of a glycoconjugate possesses several carbohydrate units bound by a lectin, then the avidity of the lectin for the glycoconjugate may be enhanced dramatically [1]. All these facts can contribute to explain why it was difficult to dissociate thyroglobulin from the immobilized Con A.

CONCLUSIONS

The results show that the conditions for agarose activation influence the lectin ligand density which in turn affects the binding of glycoproteins to the affinity gel. The optimized activation and coupling protocol allowed the preparation of derivatives with a wide range of Con A densities.

There are several factors that should be considered during the affinity chromatography of glycoproteins on columns packed with immobilized lectins. One of the most important is the binding constant of the lectin for the type of carbohydrate moiety involved. If the binding affinity is low, one may try to increase the density of the lectin on the adsorbent. Another parameter is thus the amount of lectin immobilized on the support. Our results indicate that highly effective lectin affinity adsorbents can be rationally designed based on the amounts of immobilized lectin. Lectin-based adsorbents with a very high degree of substitution could be used for instance, to purify low molecular weight glycoproteins, especially those with low binding constants, where multipoint interactions become of capital importance. It should also be possible to use derivatives with a low degree of ligand substitution to separate glycoproteins of different molecular weights but with similar binding specificities.

Acknowledgements

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