Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 803 (2004) 237–241
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Preparation of high-density Concanavalin A adsorbent and its use for rapid, high-yield purification of peroxidase from horseradish roots

Laura Franco Fraguas [a,*], Francisco Batista-Viera [a], Jan Carlsson [b]

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

Received 25 September 2003; received in revised form 2 December 2003; accepted 29 December 2003

Abstract

Preparation of Concanavalin A-adsorbents by immobilization on Sepharose activated with 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP-reagent) is reported. High immobilization yields of lectin (above 90%) were attained using an optimized CDAP-activating protocol. The effect of ligand density on the performance of the adsorbent for specific binding of glycoproteins was studied using horseradish peroxidase (HRP) as a model. Adsorption yields of pure HRP exceeding 90% were obtained with Con A-derivatives containing not <20 mg of immobilized Con A/ml of packed gel. With lectin content of 2 mg/(ml of packed gel), only 20% of HRP was adsorbed. Purification of peroxidase from horseradish roots extract was successfully accomplished on Con A–Sepharose with high Con A content.

Keywords: Concanavalin A; Peroxidase

1. Introduction

Lectins are proteins which interact reversibly and selectively with specific residues of carbohydrates present in glycoconjugates [1]. Concanavalin A (Con A), the lectin from *Canavalia ensiformis*, binds molecules containing α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. Immobilized Con A has been extensively used for isolation, fractionation, structural characterization and immobilization of glycoproteins and other biologically important glycoconjugates carrying glucose and mannose moieties [2].

Horseradish (*Armoracia rusticana*) peroxidase (HRP, E.C. 1.11.1.7) is an enzyme of great importance in contemporary diagnostics: about 80% of all immunological assays use enzyme–antibody conjugates with peroxidase as the conjugated enzyme [3]. HRP is a glycoprotein consisting of numerous isoenzymes, all of which contain 18–20% (w/w) carbohydrate. Their average composition is 2 mol of *N*-acetylglucosamine, 2.6 mol of mannose and 0.8 mol each of fucose and xylose [4]. The presence of mannose residues within the carbohydrate chains of HRP provides the basis for a purification method using affinity chromatography with immobilized Con A. Brattain et al. [5] described a procedure for the further purification of commercial HRP, but no attempt was made to purify the enzyme from a crude extract.

The CDAP-activation technique represents an interesting alternative for agarose activation, and the subsequent immobilization of amino-containing ligands, due to its described advantages over the traditional CNBr activation procedure [6].

In this paper, we have optimized one critical step in the CDAP-activation protocol for the synthesis of Con A–Sepharose, and we report application of this adsorbent to the purification of peroxidase from horseradish roots extract. It is also demonstrated that immobilized Con A content is of crucial importance for optimum performance of the Con A–Sepharose in this HRP purification procedure.

2. Experimental

Concanavalin A (Con A), Sepharose 4B and PD-10 columns (Sephadex G-25 pre-packed columns) were from Amersham Pharmacia Biotech (Uppsala, Sweden). The 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate

* Corresponding author. Fax: +598-2-9241906.
E-mail address: lfranco@fq.edu.uy (L. Franco Fraguas).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.12.023

(CDAP-reagent), horseradish peroxidase (HRP, Type II), α-D-methylmannoside and centrifugal Ultrafree-20 filter units (cut-off 10 kDa) were from Sigma Chemical Co. (St. Louis, MO). Triethylamine (TEA) was from Fluka (Buchs, Switzerland). Horseradish roots were purchased from a local market. All other chemicals used were of analytical grade.

2.1. Synthesis of Concanavalin A–Sepharose

2.1.1. Activation of Sepharose with CDAP reagent

Sepharose 4B was activated as described in [6], with some modifications. Typically, 3.0 g of suction-dried agarose (Sepharose 4B) was washed on a glass filter with distilled water, then with acetone:water (3:7 (v/v)) and then with 50 ml of acetone:water (6:4 (v/v)), pre-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) dissolved in 2 ml of cold acetone:water (6:4 (v/v)), was added to the gel suspension under vigorous stirring for 3 min at 4 °C; then 360 μl of 200 mM TEA solution was added dropwise over 1–2 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–Sepharose) was equilibrated with 0.1 M sodium hydrogen carbonate ($NaHCO_3$) pH 8.3 (coupling buffer) and immediately used for coupling purposes. It was determined that 1.0 g of dry activated-Sepharose corresponds to 18.2 g of suction dried (drained) gel and to 28.2 ml of packed gel.

2.1.2. Immobilization (coupling) step

Aliquots (3.0 g) of suction-dried CDAP-activated Sepharose equilibrated in coupling buffer were incubated with 3.0 ml of solutions containing increasing amounts of pure Con A dissolved in coupling buffer, and mixed end over end at room temperature for 4 h as described in [9]. The Con A-gel derivatives were washed on a glass filter with coupling buffer, distilled water and finally with 0.1 M acetate buffer pH 6.0 containing 0.5 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ (adsorption buffer).

2.2. Protein determination

The amount of Con A immobilized on Sepharose was determined by total amino acid analysis. Prior to the analyses, the gels were washed thoroughly with water on a glass filter-funnel, suction-dried under vacuum and then dried in a dessicator over P_2O_5 until constant weight was achieved (usually 1 week). The concentration of soluble proteins was determined spectrophotometrically (Shimadzu UV-160A, Shimadzu Co.) at 280 nm ($A_{280}^{1\%} = 11.5$ and 13.1 for Con A and HRP, respectively) [7].

2.3. Adsorption of pure HRP on to Con A–Sepharose derivatives

Con A–Sepharose derivatives with low and high ligand contents, synthesized by the CDAP-activation technique (before and after optimization, respectively) were packed in mini-columns (plastic columns of 5 mm of diameter with a bed volume of 0.75 ml packed gel each). After equilibration with adsorption buffer, 1.6 mg of pure HRP was applied to each mini-column at room temperature. The amount of bound HRP was calculated as the difference between the amount of protein added and the amount of unbound protein, measured spectrophotometrically at 280 nm. Each adsorption experiment was performed at least in triplicate.

2.4. Extraction of HRP from horseradish roots

The extraction from horseradish roots and the fractionated precipitation of peroxidase by ammonium sulphate was performed as described in [8]. The precipitate was dissolved in a minimal volume of adsorption buffer and desalted on a PD-10 column. A final volume of 13 ml of extract in adsorption buffer was thus obtained.

2.5. Affinity chromatography of HRP on Con A–Sepharose

In a typical run a volume of 10 ml of the horseradish roots extract was applied to a column packed with Con A–Sepharose (plastic column of 10 mm of diameter with a bed volume of 3.0 ml packed gel containing 22.2 mg of Con A/ml). Fractions of 3.0 ml were collected at a flow rate of 20 ml/h. The column was washed with adsorption buffer until the absorbance at 280 nm was <0.05. Elution was performed with 0.1 M α-D-methylmannoside dissolved in adsorption buffer. The eluted fractions were tested for HRP activity and SDS–PAGE analysis was performed.

2.6. Peroxidase activity determination

HRP activity was measured spectrophotometrically by recording the increase in absorbance at 460 nm due to the oxidation of *o*-dianisidine [8]. One enzyme unit (E.U.) was defined as the amount of enzyme causing decomposition of 1 μmol of hydrogen peroxide/min at 24 °C at pH 5.4. Enzymatic activity was expressed as E.U./ml and the specific activity (S.A.) was defined as the ratio between the E.U./ml and the A.U. 280 nm/ml. The purification factor was defined as the ratio between the S.A. after, and the S.A. before, affinity chromatography.

2.7. SDS–PAGE analysis

Fractions eluted from the Con A–Sepharose column were pooled and concentrated on a centrifugal Ultrafree-20 filter unit with a molecular weight cut-off of 10 kDa. SDS–PAGE was performed using a combination of PhastSystem

equipment (Pharmacia, Uppsala, Sweden) with ready Phast-Gel Homogeneous 12.5 gels according to the manufacturer instructions. LMW Electrophoresis Calibration Kit (Pharmacia) was used as the marker. Proteins were stained with Coomassie Brilliant Blue.

3. Results

For the immobilization of Con A, the CDAP-activation technique was chosen, due to its advantages over the traditional CNBr-activation technique [6]. The influence of the post-activation washing step on the immobilization yield of Con A was assessed. Preliminary attempts to prepare Con A–Sepharose adsorbents suitable for purification of HRP, by strictly adhering to reported protocols [6] failed, due to poor ligand immobilization yields. The influence of some experimental conditions monitored during the synthesis of the adsorbents was investigated. As a result, the washing step with 50 mM HCl, performed during the CDAP-activation procedure, was reduced from 15 to 3 min. This washing step is required to hydrolyze the gel-bound pyridinium isourea derivative, formed as a by-product during the activation procedure. According to the authors, the active cyanate groups should not be affected by this treatment. However, it was found that after a short (3 min) washing period the activated agarose formed was able to immobilize almost eight-fold more Con A than after a longer (15 min) washing period (Fig. 1). After this modification was introduced, Con A was coupled to Sepharose in yields exceeding 90% for a wide range of added amount of lectin (3–30 mg of Con A/ml of packed gel). All these adsorbents have been stored at 4 °C for more than 1 year without loosing binding capacity (data not shown).

To analyze the influence of the ligand density of the adsorbent on the adsorption yield of HRP, pure HRP was

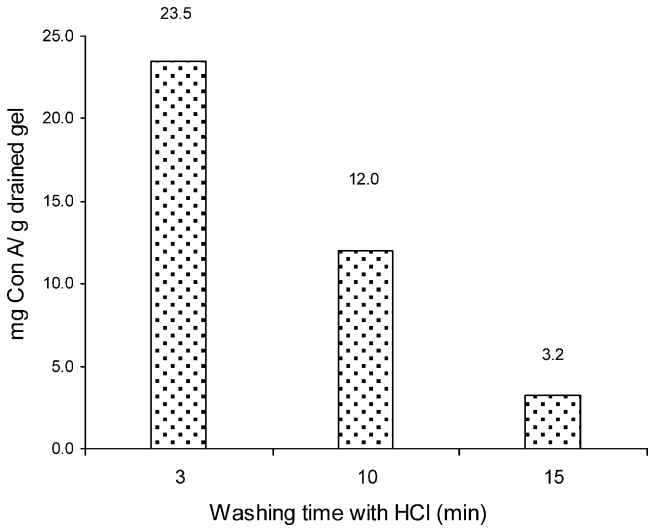

Fig. 1. Amount of immobilized Con A as a function of the incubation time of CDAP-activated Sepharose with HCl during the washing step. The amount of added Con A in all three cases was 25 mg/g of drained gel.

Table 1
Adsorption of pure HRP on to Con A–Sepharose derivatives with high, medium and low ligand densities, prepared by the CDAP activation technique

Immobilized Con A (mg/ml of packed gel)[a]	Molar ratio (Con A/added HRP)	nmol HRP adsorbed[b]	Molar ratio (Con A/bound HRP)
2.4	0.42/1	8.4	2.02/1
7.1	1.25/1	20.0	2.51/1
11.3	1.71/1	32.0	2.14/1
22.2	3.92/1	36.0	4.36/1
26.4	4.67/1	38.0	4.92/1

[a] Determined by total aminoacid analysis.
[b] An amount of 1.6 mg (40 nmol) of pure HRP was added in each case. The results are the average of three runs.

applied onto five columns packed with five different derivatives prepared with low, medium and high Con A densities. Table 1 shows that much more HRP is bound to the adsorbents with higher ligand densities. In parallel, the adsorption of HRP to a Con A-derivative prepared using the traditional CNBr-activation technique was evaluated. For this purpose, a commercial Con A–Sepharose adsorbent was used, with a ligand density (10.0 mg Con A/ml of packed gel) close to the content of one of the synthesized adsorbents (11.3 mg Con A/ml of packed gel). The results for both adsorbents showed the same adsorption percentage (80%) of HRP and similar recoveries (data not shown).

Horseradish roots extract was applied to a column packed with Con A–Sepharose derivative prepared as described (containing 22.2 mg of Con A/ml of packed gel). After the column was thoroughly washed with adsorption buffer, elution was performed with 0.1 M α-D-methylmannoside. The purification of HRP was analyzed by SDS–PAGE (Fig. 2) and by the increase in specific activity (Table 2). Affinity chromatography as described yielded an HRP purification factor of 14.0. The eluted material migrated as a single protein band in the SDS–PAGE analysis with an apparent molecular weight of 42 kDa, which is in agreement with the molecular weight reported [8]. The adsorbent can be easily regenerated by pumping five bed volumes of the adsorption buffer into the column and stored for long periods at 4 °C in this buffer, supplemented with Thimerosal (0.02% (w/v)). The synthesized Con A–Sepharose can be used for at least five cycles of adsorption, washing, elution and regeneration, with no loss in binding capacity.

Table 2
Purification of peroxidase from a crude extract of horseradish roots by affinity chromatography on Con A–Sepharose

Fraction	Volume (ml)	$A.U._{280}$/ml[a]	EU/ml[b]	S.A.	Purification (fold)	Recovery (%)[c]
Applied[d]	10	14.0	17.2	1.2	–	–
Eluted	3	2.4	41.9	17.4	14	73

[a] A.U.: absorbance units at 280 nm.
[b] E.U.: enzyme unit as defined under Section 2.
[c] Percentage of the recovered enzyme activity.
[d] Resuspended ammonium sulphate precipitate of the crude extract.

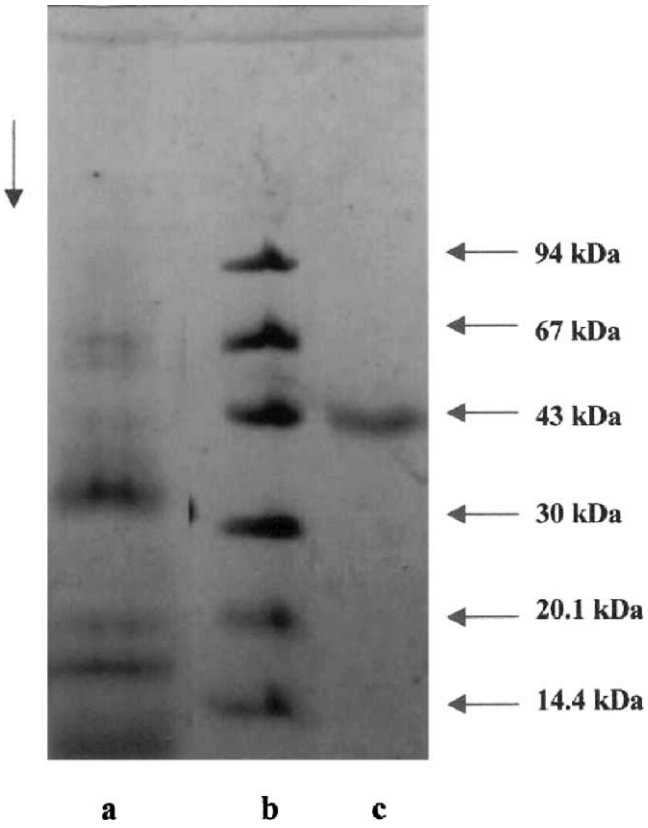

Fig. 2. SDS–PAGE of fractions from the affinity chromatography of HRP on Con A–Sepharose with high ligand content. The polyacrylamide gel (homogeneous, 12.5%) was stained with Coomassie Brilliant Blue. Lane a: crude extract of horseradish roots; lane b: low molecular weight markers; lane c: eluted HRP.

4. Discussion

The HRP interaction with Con A–Sepharose has demonstrated to be highly dependent of the ligand density [9]. The results herein reported indicate that in order to adsorb more than 90% of added HRP, Con A-derivatives must contain not less than 22 mg of immobilized lectin/ml of packed gel. For these adsorbents the molar ratio between the immobilized Con A and the bound HRP suggests multi-point interactions, e.g. one molecule of HRP interacts simultaneously with at least four molecules of Con A (Table 1). Gels containing about one-tenth that density of Con A were able to adsorb only 20% of the added HRP. For this adsorbent the observed molar ratio indicates that one molecule of HRP interacts simultaneously with two molecules of Con A. Besides, the molar ratio between the immobilized Con A and the added HRP indicates excess of ligand (the Con A possesses four combining sites/mol), so the capacity of the column is not responsible for the low adsorption percentages.

Immobilization of the lectin Con A can be performed in several ways, using different functional groups in the amino acid side chains involved in the coupling onto the matrix. The most common groups involved in the immobilization of a protein to a solid support are in decreasing order: amino (lysine), sulphydryl (cysteine), hydroxyl (serine), and imidazole (histidine) groups. Gel activation based on the CNBr method leads to the introduction of cyanate ester and imidocarbonate groups into the matrix; the bonds established between the protein ligand and the activated matrix are of isourea- and N-substituted imidocarbonate-types, respectively. N-substituted carbamates also occur when the ligand reacts with cyclic carbonates, which means that the immobilization of amino-containing ligands is a heterogeneous process. In spite of being one of the most widely used activation techniques, however, the CNBr method has the additional serious disadvantage of posing health risks for workers. Because of this and its high vapor pressure, all work with CNBr must be done in strictly controlled and ventilated environments (e.g. fume hoods).

Several cyano transfer complexes have been described as coupling reagents and one of the most useful is 1-cyano-4-(dimethylamino)-pyridinium-tetrafluoroborate (CDAP). This cyanylating agent is a slightly hygroscopic, non-volatile solid, that can be stored at room temperature for extended periods and, most importantly, can be easily handled in laboratories without undue health risks. Furthermore, the reagent introduces cyanate ester structures into the matrix and, as a result, immobilization is a more homogeneous process since virtually all the linkages are of the isourea type. In the optimized CDAP-activation protocol herein reported, the amount of immobilized lectin can be regulated by adjusting the amount of added lectin, since the immobilization yields exceeded 90%. In addition, this protocol allows the preparation of gel derivatives with very high lectin ligand density, i.e. more than 20 mg of lectin/ml of packed gel. Commercial pre-activated matrices very often only permit the immobilization of a predetermined, fixed amount of protein.

High-density Con A–Sepharose adsorbents thus prepared can be usefully applied for the purification of HRP from crude roots extract (Table 2). As indicated by SDS–PAGE, highly purified HRP can be obtained by the two simple steps of ammonium sulphate precipitation and affinity chromatography (Fig. 2).

The experimental work reported here illustrates that the effectiveness and efficiency of the Con A-adsorbent for the purification of HRP depends on the density of Con A coupled to Sepharose, as well as the specific affinity of the lectin for defined sugar residues. Con A ligand density is, in turn, a function of the method and conditions chosen for the immobilization procedure.

Acknowledgements

We are grateful for financial support from the International Program in Chemical Sciences (IPICS, Project URU:02), Uppsala University, Sweden; the Program for Development of Basic Sciences (PEDECIBA), Uruguay; and the International Foundation for Science (IFS, Project

2834/3). We thank Dr. Valerie Dee for English language revision.

References

[1] I.J. Goldstein, R.D. Poretz, in: I. Liener, N. Sharon, I.J. Goldstein (Eds.), The Lectins. Properties, Functions and Applications in Biology and Medicine, 1986, p. 51.
[2] J. Carlsson, J.-C. Janson, M. Sparrman, in: J.-C. Janson, L. Ryden (Eds.), Protein Purification, Principles, High Resolution and Applications, 1998, p. 375.
[3] Biozyme Laboratories: Enzymes and Biochemicals, Biozyme Laboratories Ltd., Peroxidase Product Information, Blaenavon, South Wales, UK, 2001, p. 1.
[4] B.Y. Yang, J.S. Gray, R. Montgomery, Carbohydr. Res. 287 (1996) 203.
[5] M.G. Brattain, M.E. Marks, T.G. Pretlow II, Anal. Biochem. 72 (1976) 346.
[6] J. Kohn, M. Wilchek, Appl. Biochem. Biotechnol. 9 (1984) 285.
[7] H.A. Sober (Ed.), Handbook of Biochemistry, 2nd ed., CRC Press, 1970, Chapter 71.
[8] L.M. Shannon, E. Kay, J.Y. Lew, J. Biol. Chem. 241 (1966) 2166.
[9] L. Franco Fraguas, J. Carlsson, F. Batista-Viera, Int. J. Biochromatogr. 5 (2000) 255.