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# HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY ON SILICA-BOUND CONCANAVALIN A

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## SUMMARY

Concanavalin A was covalently bound to porous silica (100–1000 Å) by three different coupling procedures. The performance of the preparations was evaluated from their ability to resolve a mixture of closely related sugars. The Concanavalin A–silica was used for efficient and rapid (5–20 min) analysis/purification of samples containing the glucoproteins peroxidase and glucose oxidase. Such columns could be used at least 60 times with retained performance.

## INTRODUCTION

Lectins, in particular Concanavalin A (Con A), are well known ligands in (bio)affinity chromatography. Their ability to bind carbohydrates has been utilized for the purification of a large number of compounds such as carbohydrates, glucoproteins, hormones[1,2] and cells[3]. Immobilized Con A has also been used in an analytical context, for example, in the "lectin electrode"[4].

In this paper we report an extension of the use of lectins as ligands in affinity chromatography. By incorporating them into high-performance liquid chromatographic (HPLC) systems, they are rendered more versatile for both analytical and preparative applications. This combination of techniques has been named high-performance liquid affinity chromatography (HPLAC)[5], and has been used for analysis/purification of a number of compounds of biological interest. Examples are the separation of dehydrogenases with silica-bound AMP[5,6], sugars and nucleotides with silica-bound boronic acid[6,7], various enzymes with silica-bound triazine dyes[6,8,9] and proteases with silica-bound inhibitors[10].

In this study Con A is bound to porous silica and used for the separation of closely related sugars and for analysis and purification of the glucoproteins peroxidase and glucose oxidase.

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## MATERIALS AND METHODS

The packing materials LiChrosorb Si 100 (5 μm) and LiChrospher Si 1000 (10 μm) were obtained from E. Merck (Darmstadt, G.F.R.). γ-Glycidoxypropyltrimethoxysilane was purchased from Dow Corning (Midland, MI, U.S.A.), tresyl chloride from Fluka (Buchs, Switzerland) and fungal glucose oxidase (Grade I) from Boehringer (Mannheim, G.F.R.). Concanavalin A, Type IV, *p*-nitrophenyl-α-D-mannoside, *p*-nitrophenyl-α-D-glucoside, *p*-nitrophenyl-β-D-glucoside and horse radish peroxidase (Type II) were obtained from Sigma (St. Louis, MO, U.S.A.).

### *Binding of Con A to silica*

Con A was coupled to porous silica with 100 Å or 1000 Å nominal pore size. Three coupling methods were used (Fig. 1). Details of method A are given in refs. 6 and 7, of method B in ref. 11 and of method C in refs. 5 and 6. The concentrations of reactive groups were as follows: Method A, 2 μmol epoxy groups per $m^2$ (= near maximum); Method B, 0.5 μmol tresyl groups per $m^2$; Method C, 2 μmol aldehyde groups per $m^2$ (= near maximum).

The pH during the coupling of Con A to the activated silica was as follows: Method A, 8.5; Method B, 6; Method C, 5–8.

### *Determination of Con A content*

Con A–silica (10–50 mg), extensively washed with aqueous 0.5 *M* NaCl, was

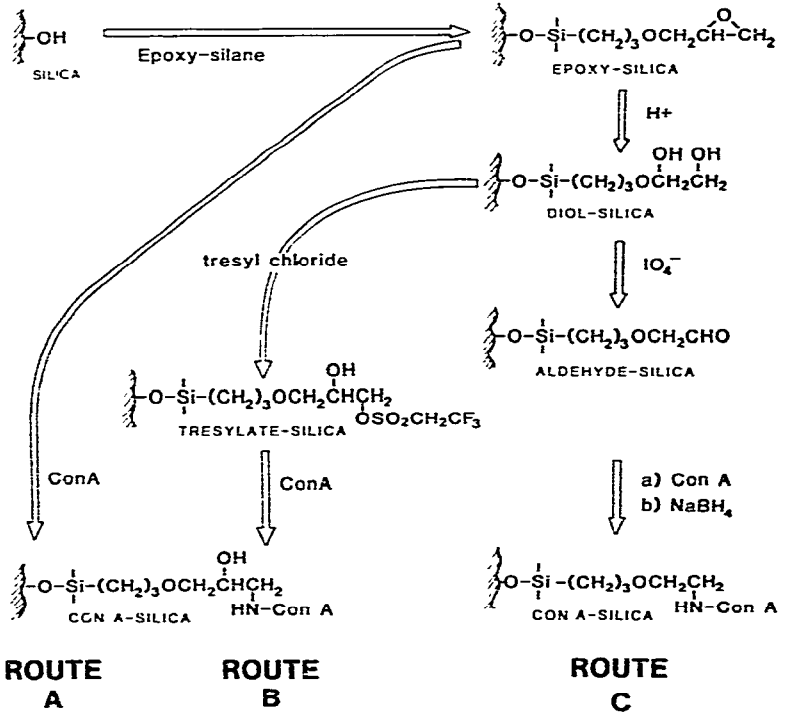


Fig. 1. Three pathways for the preparation of Con A–silica. Diol-silica is commercially available.

suspended in 2.5 ml of a saturated sucrose solution and the UV spectrum recorded using a suspension of Con A-free silica as a reference[6]. The Con A content was calculated from the absorbance at 280 nm and the literature value $A^{1\%}_{1\,cm} = 1.1$ at 280 nm (ref. 12). The sucrose solution has approximately the same refraction index as the silica particles and thus eliminates light scattering[6].

*Column packing and chromatographic conditions*

An HPLC system comprised of a high pressure pump (Altex), an injector fitted with 4.1-ml loop (Altex), one or two UV-visible spectrophotometric detectors (LDC) and a two-channel recorder was used. The separation columns, 5 × 0.5 cm or 10 × 0.5 cm, were packed by the slurry packing method using buffer or 50% sucrose as the suspending medium. The mobile phase contained, besides buffer, NaCl, $MgCl_2$ and $CaCl_2$, proposed stabilizers of Con A.

## RESULTS AND DISCUSSION

*Coupling of Con A to silica*

An obvious first step in this work was to find suitable conditions for coupling Con A to the silica surface. Three coupling procedures, A, B and C according to Fig. 1, were evaluated. Procedures A and B lead to the same spacer between Con A and the silica. Procedure A is very facile, involving the direct reaction between the (amino group-containing) Con A and the epoxy silica. The speed and efficiency of the coupling were, however, comparatively low. Only about 20% of added Con A became bound to silica, even when the Con A density was as low as 2 mg/g silica.

Method B, activation of diol-silica with tresyl chloride, is a straightforward and convenient procedure that has the advantage of high coupling efficiency. A coupling yield of 50–70% was thus obtained at Con A loadings of 40–70 mg/g silica. It should be noted that diol-silica is commercially available.

Method C, involves several steps but allows very high coupling yields; for example, about 100% at Con A loadings below 20 mg/g silica and at least 70% for loadings up to 80 mg/g silica. Most experiments were carried out with Con A–silica prepared according to Method C. The amount of lectin coupled by a given method could be regulated by varying the amount of lectin added.

*Chromatographic evaluation of Con A–silica*

A number of Con A–silica packings were prepared, packed into columns and subsequently evaluated with respect to their ability to resolve a mixture of related sugar derivatives, namely *p*-nitrophenyl derivatives of β-D-glucose, α-D-glucose and α-D-mannose. The nitrophenyl derivatives were chosen as they are conveniently monitored with a UV-detector. In Fig. 2 a typical separation is depicted. The first substance to emerge —the β-D-glucoside— came very close to the solvent front, in accordance with its known weak interaction with Con A[13]. The α-D-glucoside on the other hand showed a more substantial interaction with the bound ligand, the mannoside even more so, again as expected from literature data[13]. The broad peak of the mannoside indicates a poor efficiency for this compound, and plate height calculation confirmed this (plate height approximately 2 mm). The satisfactory separations obtained are thus due to the marked selectivity of the Con A–silica. The reason for the

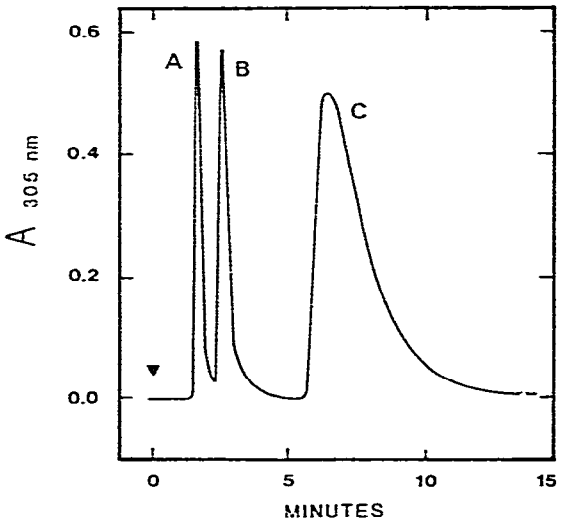


Fig. 2. Separation of glucosides on medium substituted Con A–silica. Column: 0.5 × 10 cm, 25 mg Con A per g silica, 10 μm, nominal pore size 1000 Å. Mobile phase: 0.025 *M* Tris–HCl, 0.25 *M* NaCl, 0.5 m*M* $CaCl_2$, 0.5 m*M* $MnCl_2$; pH 6.8. Flow-rate: 1 ml/min. Sample (injected as indicated by the arrow): 0.3 ml containing 1 μg *p*-nitrophenyl-β-D-glucoside (A), 1.5 μg *p*-nitrophenyl-α-D-glucoside (B) and 8 μg *p*-nitrophenyl-α-D-mannoside (C).

low efficiency for the mannoside is probably a low dissociation rate constant for the complex silica-bound Con A–*p*-nitrophenylmannoside[6,10].

In Table I are listed data for a representative selection of Con A–silica preparations and the corresponding $k'$ values for *p*-nitrophenyl-α-D-mannoside. The $k'$ values for the β-D-glucoside varied up to 0.7 and for the α-D-glucoside up to 3 (not shown in the table).

An immediate conclusion that can be drawn from Table I is that the retention is strongly dependent on the Con A concentration, the extreme examples being preparations 1 and 6 where an increase in Con A concentration from 2 to 60 mg/g

TABLE I

COMPARISON OF CON A–SILICA PREPARATIONS

The $k'$ value, the mass distribution coefficient (column capacity ratio), was calculated from $k' = (V_e - V_0)/V_0$, where $V_e$ is the elution volume of the substance under study and $V_0$ is the elution volume of a non-sorbed substance.

| | *Packing No.* | | | | | |
|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* |
| Nominal porosity (Å) | 100 | 100 | 1000 | 1000 | 1000 | 1000 |
| Coupling method | A | C | C | C | C | B |
| Coupling pH | 8.5 | 5.7 | 5.7 | 5.0 | 6.0 | 6.0 |
| Con A content (mg/g silica) | 2 | 10 | 25 | 60 | 80 | 60 |
| $k'$ for *p*-nitrophenyl-α-D-mannoside | 0.5 | 2.1 | 3.8 | 16 | 12 | 17 |

correspondingly increased the $k'$ value from 0.5 to 17. Apparently, column parameters other than the gross ligand concentration also govern the retention of the chromatographed substances. The pH of coupling seems to be such a factor. Preparation 4 contained 60 mg/g of Con A but retained the glucoside more strongly than did preparation 5, in spite of the higher Con A content of the latter. The reasons for this behaviour remain to be clarified. A possible explanation is that coupling at a higher pH favours multi-point attachment, or attachment via groups involved in the affinity binding. An alternative explanation is that coupling at a low pH could result in silica-bound dimers, which are possibly more effective ligands than the silica-bound tetramers that should be obtained when coupling at a higher pH[11].

The Table clearly shows that it was necessary to have a rather high concentration of Con A ($\geq$ 25 mg/g silica) to obtain appreciable retention of the chromatographed sugars. This is partly a reflection of the macromolecular properties of Con A (MW 110,000[11]). The high lectin content of preparation 5, 80 mg/g silica, thus is equivalent to 0.7 $\mu$mol Con A (tetramer) per g or 2.8 $\mu$mol binding sites per g, a rather modest ligand density. From this it follows that care must be exercised in analytical applications to avoid overloading. If the maximum loading is set to 1%, less than 0.01 mg sugar should be injected on a 10 $\times$ 0.5 cm column. It should be noted that it is practically impossible to achieve Con A densities much higher than 80 mg/g. Thus, at a Con A density of around 100 mg/g the silica surface (1000-Å silica; 20 $m^2$/g) will be completely covered by the lectin.

The influence of some chromatographic parameters on the $k'$ value was also briefly investigated. It was found that the same $k'$ value was obtained regardless of the buffers (Tris–HCl, sodium phosphate or sodium acetate) chosen. The pH of the mobile phase, on the other hand, had a slight influence. On going from pH 7 to pH 5, the $k'$ values (mannoside) increased by about 10%, in line with the behaviour of other substances on Con A–Sepharose columns[14].

*Separation of glucoproteins*

The potential of Con A–silica as an absorbent for proteins was probed with peroxidase and glucose oxidase, both enzymes being glucoproteins known to interact with Con A[15]. Furthermore, peroxidase contains a protohemin group which absorbs strongly at 405 nm, whereas glucose oxidase contains flavin–adenine dinucleotide groups and thus has strong absorbance at 460 nm. This allowed for a convenient, semi-specific detection of the enzymes. The column effluent was monitored at 280 nm to obtain a measure of the total protein contents and at 405 nm to detect and quantify the peroxidase or glucose oxidase.

Figs. 3 and 4 show two examples of the chromatography of peroxidase on Con A–silica, a pulse elution and a gradient elution, respectively. The sample applied to the columns was a commercial enzyme preparation. Immediately upon injection (1 min) a broad peak appeared containing 45% of the injected protein but less than 2% of the peroxidase. The broadness of the peak is not primarily due to any appreciable affinity of the protein for the Con A–silica but to the large injection volume (4.1 ml). A pulse (Fig. 3) or a gradient (Fig. 4) of $\alpha$-methylglucoside, a competing compound, was subsequently applied to the columns. As indicated in the figures, the remaining 55% of the protein was now eluted. It consisted of pure peroxidase as judged from the absorbance at 405 nm. The figures thus illustrate two ways of quickly and efficiently purifying a glucoprotein. In the present case, where a commercial, allegedly pure, preparation was used as starting material, the separation forms a convenient last stage purification of the enzyme preparation. Besides the two-fold purification, the enzyme was also concentrated in the process, especially with pulse elution (90% of the

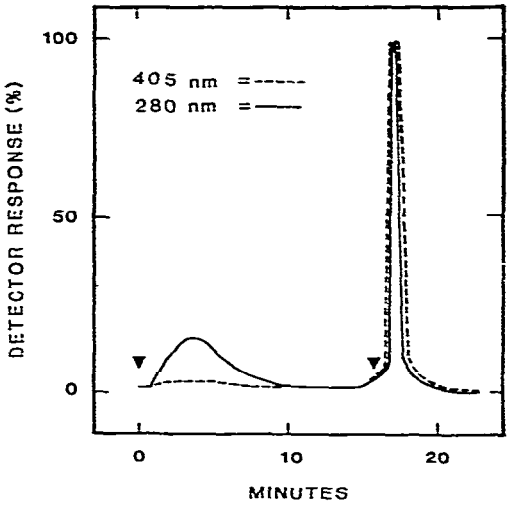


Fig. 3. Chromatography of peroxidase on highly substituted Con A–silica. Column: 0.5 × 5 cm, 60 mg Con A per g silica, 10 μm, nominal pore size 1000 Å. Mobile phase: 0.05 *M* sodium acetate, 0.5 *M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$; pH 5.1. Flow-rate: 1 ml/min. Sample (injected as indicated by the first arrow): 4.1 ml, 1 mg protein per ml. At the second arrow, 4.1 ml of 25 m*M* α-methyl-D-glucoside were injected. A detector response of 100% corresponds to 2.0 absorbance units for $A_{280\ nm}$ and 1.28 for $A_{405\ nm}$.

enzyme could be collected in four times its original concentration). From a purification standpoint, however, a gradient elution is preferable as it also allows for resolution of glucoproteins with approximately the same affinity for the Con A–silica.

The elution with competing counter ligands obviously leaves the enzyme contaminated with the counter ligand. If this is undesirable, a dialysis step or a size exclusion chromatographic step may be included. An alternative is depicted in Fig. 5. Here the peroxidase sample was applied to a column with a lower Con A content (25 mg/g). The contaminating protein leaves the column unretarded and shortly afterwards the peroxidase emerges as a broad peak without any addition of counter ligands to the mobile phase. The drawbacks of this procedure are that the peaks are

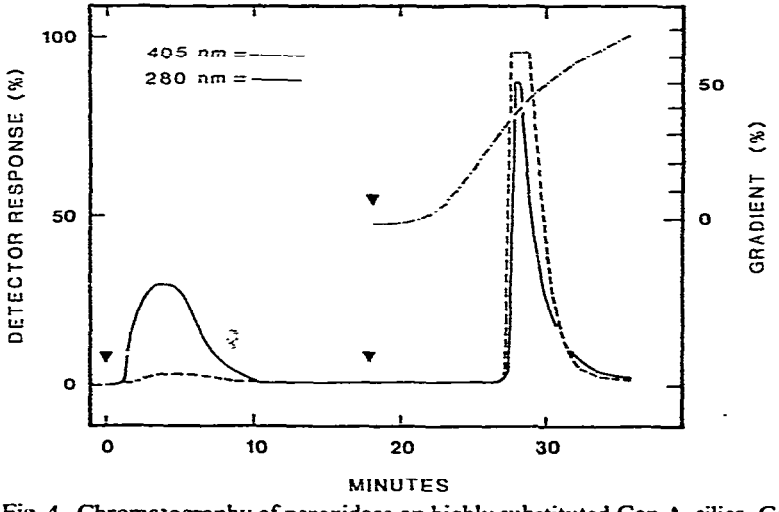


Fig. 4. Chromatography of peroxidase on highly substituted Con A–silica. Conditions as in Fig. 3. At the second arrow a gradient of α-methyl-D-glucoside was applied; 100% = 20 m*M* glucoside. A detector response of 100% corresponds to 1.0 absorbance unit for $A_{280\ nm}$ and 0.64 for $A_{405\ nm}$.

slightly overlapping and that the enzymes are obtained in a dilute form. Elution without addition of counter ligands was also observed with columns having a high ligand density if the columns were overloaded with enzymes. In this way partial purification was achieved and the separated enzymes were not diluted unnecessarily.

In this context it is interesting to consider the capacity of the Con A–silica. It was found that a column containing 1 g of Con A–silica (60 mg Con A per g silica) had a capacity of about 12 mg peroxidase (frontal analysis). This means that 54% of the Con A molecules (or 14% of the Con A subunits) were bound to a peroxidase molecule, indicating a very satisfactory accessibility of the binding sites, especially considering the dense packing of the Con A molecules on the silica surface.

Glucose oxidase was found to bind very strongly to Con A–silica. Addition of high concentrations (0.5 *M*) of the counter ligand α-methylglucoside to the mobile phase did not elute the enzyme. Whether this was due to a very strong lectin–carbohydrate interaction, slow dissociation of the complex or "non-specific" interaction between Con A–silica and enzyme surface structures was not elucidated. A pragmatic solution to the problem is shown in Fig. 6. The glucose oxidase (a commercial preparation) was injected on the column. Protein without affinity for the column appeared after 1 min as a broad peak. By supplying the mobile phase with a pulse of 0.2 *M* glycine-hydrochloride, pH 2.8, 90% of the applied glucose oxidase activity was eluted. Such a low pH will abolish carbohydrate–lectin (bio)affinity and will also suppress the effect of various non-specific interactions. If the method is considered for preparative purposes, the eluent used must obviously not harm the enzyme to be

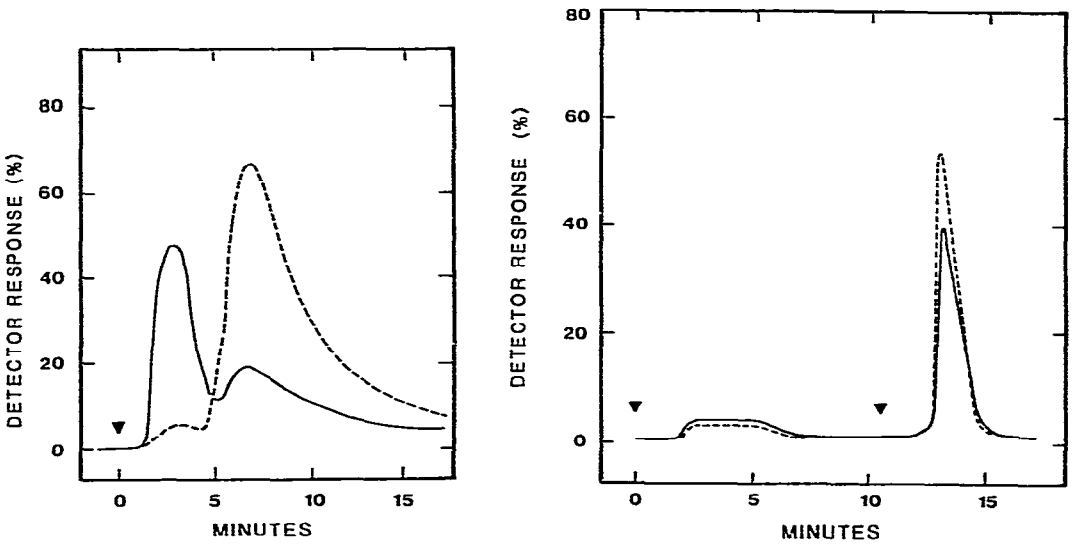


Fig. 5. Chromatography of peroxidase on medium substituted Con A–silica. Column: 10.0 × 0.5 cm, 25 mg Con A per g silica, 10 μm, nominal pore size 1000 Å. Mobile phase: 0.05 *M* sodium acetate, 0.5 *M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$; pH 5.1. Flow-rate: 1 ml/min. Sample (injected as indicated by the arrow): 2 ml, 1 mg/ml. Detector response = 100% corresponds to 0.5 absorbance units for $A_{280\,nm}$ (——) and to 0.32 for $A_{405\,nm}$ (– – –).

Fig. 6. Chromatography of glucose oxidase on highly substituted Con A–silica. Column: 0.5 × 10 cm, 60 mg Con A per g silica, 10 μm, nominal pore size 1000 Å. Mobile phase and flow-rate as in Fig. 5. Sample (injected as indicated by the arrow): 4.1 ml, 1.0 mg protein per ml. At the second arrow 4.1 ml of 0.2 *M* glycine hydrochloride, pH 2.8 were injected. Detector response = 100% corresponds to 2.0 absorbance units for $A_{280\,nm}$ (——) and 0.64 for $A_{460\,nm}$ (– – –).

purified. In a separate experiment it was confirmed that temporary exposure to glycine-hydrochloride, pH 2.8, had little effect on glucose oxidase.

From the above data for peroxidase and glucose oxidase, it is obvious that these enzymes behave quite differently on a Con A–silica column. Consequently, a mixture of the enzymes should be very easy to resolve. This indeed turned out to be the case. A mixture of the two enzymes was resolved as three peaks, one containing contaminating proteins, one containing peroxidase (eluted with α-methylglucoside) and one containing glucose oxidase (eluted with glycine hydrochloride).

*Stability*

The Con A–silica columns can be used at room temperature for long periods without deterioration. Thus, one column was used 60 times without any noticeable change of $k'$ values for the chromatographed substances.

## CONCLUSIONS

Con A–silica in combination with the HPLC technique unifies the attractive features of HPLC and affinity chromatography; namely, sensitivity, speed of operation and specificity. Here, HPLAC separation with Con A–silica has been used in an analytical and semi-preparative mode. By using larger columns, very rapid purification procedures on a preparative scale should be possible. The rapid separations (5–30 min) should be particularly advantageous when purifying unstable compounds or compounds prone to proteolytic degradation by enzymes present in the mixture to be resolved.

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