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Expanded bed chromatography for one-step purification of mannose binding lectin from tulip bulbs using mannose immobilized on DEAE Streamline

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Abstract

Expanded bed chromatography allows chromatographic separations to be performed starting from unclarified material. Aiming to purify from plants mannose binding lectins, which are valuable reagents, we evaluated expanded bed methodology using immobilized mannose as an affinity chromatography support. Ligand was immobilized on DEAE Streamline, a matrix which was commercially available but obviously primarily designed for ion-exchange chromatography in expanded bed conditions. Nevertheless through adjustments of mobile phase ionic strength, it was easy to perform true biospecific affinity chromatography on this support and a single-step purification procedure for mannose binding lectins from unclarified tulip bulbs extract was devised. The cleaning in place procedure allowed repeated use of the affinity column. This report seems to be the first article on proteins purification from plant material by expanded bed chromatography and gives another example supporting the concept that expanded bed methodology may be used with very selective affinity chromatography supports.

Keywords: Expanded bed chromatography; Stationary phases, LC; Immobilized mannose; Afinity adsorbents; *Galanthus nivalis*; Lectins

1. Introduction

Mannose binding lectin extracted from snowdrop bulbs (*Galanthus nivalis*) is an interesting reagent. Its affinity for high mannose sugar chains explains why it was used for affinity chromatography purification of murine IgM [1] and human α_2-macroglobulin [1,2]. It has also been used to purify in large amounts human immunodeficiency virus (HIV) protein GP120 [3] which is needed for various vaccine development projects. *Galanthus* lectin is an efficient inhibitor of in vitro retroviral infections and of HIV induced syncitium formation [4]. Mannose binding lectins extracted from *Listera ovata, Narcissus pseudonarcissus, Hippeastrum hybrid*, [4], from *Allium cepa, Allium ascalonicum*, and *Allium porrum* [5] also have antiviral activities, while other parent lectins (from e.g. *Allium sativum*) are inactive [5]. *Galanthus* lectin is widely used after immobilization in micro titer plates for sensitive enzyme-linked immunosorbent assay (ELISA) assays of antibodies developed against HIV [6], or against the feline immunodeficiency virus [7]. Moreover *Galanthus* lectin is used in lectin blotting procedures to identify the presence of high mannose sugar chains in glycoproteins [8].

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Galanthus nivalis lectin is usually prepared by affinity chromatography on immobilized mannose [9]. *Galanthus nivalis* bulbs can be purchased from flower growers but the price is quite high and they are not readily available in large quantities. Clearly it might be interesting to evaluate other starting materials to purify lectins with similar specificities and potential uses, bearing in mind that there are differences in the properties of structurally related lectins. Hence one given mannose binding lectin may not be used indiscriminately in place of another with well established properties.

Galanthus nivalis is an Amarylladaceae, and in this family the presence of mannose binding lectin has also been demonstrated in *Narcissus*, *Leucojum*, *Hippeastrum* and *Clivia* [10]. Mannose binding lectins are also present in Araceae, [11] Orchidaceae [12] Liliaceae [13] and Alliaceae [10,13,14] but at widely differing concentrations: while concentration of lectin is reported to be in the range of about 2 to 4 mg/g tissue in Amarylladaceae it drops to 1 mg/g in many studied alliaceae and even to a very low level of 0.5 to 10 μg/g in many varieties of Alliaceae like *Allium cepa* (the edible onion) or *Allium porrum* (the leek) [10].

If lectin is present at high concentrations in the starting material (as in *Galanthus nivalis* bulbs), significant quantities can easily be obtained from a reasonable volume of crude extract by affinity chromatography performed in a sedimented bed column since necessary clarification prior to chromatography is easily performed.

Obviously handling of larger volumes of other starting materials containing lectin at lower concentrations might considerably increase the labor necessary for clarification. An efficient shortcut might be to perform affinity chromatography without prior clarification using expanded bed chromatography methodology. Expanded bed chromatography is performed using upward flow which induces the chromatography support to rise from its settled state. The support expands until an equilibrium is attained depending on a balance between support particles sedimentation velocity and upward flow. So the void volume of the expanded bed is such that unclarified crude extract may be loaded onto the column: no column clogging occurs since particulates present in turbid load may find their way to column outlet passing between support beads. Expanded bed chromatography is better performed using beads with a higher density than e.g. usual agarose beads. Higher density increases sedimentation velocity sufficiently so that conveniently high flow-rates may be used [15–18].

The number of described applications of expanded bed chromatography to protein purification seems to be rapidly increasing: purification of recombinant proteins by ion-exchange chromatography starting from *E. coli* or yeast extracts, purification through protein A affinity chromatography of recombinant IgG expressed in mammalian cells have been described [[19] and references cited therein]. Recombinant *Bacillus subtilis* [20], milk [21] and even muscle extract [22] have also been used as a starting material. Immobilized dyes have been shown to be advantageous as ligands grafted to the expanded bed support [15,22,23]. Affinity chromatography has also been performed on immobilized receptors in expanded bed mode [24]. In fact, commercial availability of neutral supports without any grafted ligand or even of preactivated supports should lead to development of various affinity chromatography procedures, with the restriction that if harsh regeneration methods are needed to maintain satisfactory performance of the expanded bed support they may be deleterious to fragile ligands [19,25].

When we began this work we were not aware that neutral supports dedicated to use in expanded bed columns might be commercially available for ligand grafting[1], hence we chose to immobilize mannose on one of the easily available supports, DEAE Streamline, an anion-exchanger. With this matrix, as shown in this report, we were able to devise a single-step, efficient procedure for purification of mannose binding lectin from tulip bulbs.

Interestingly, scouting experiments aimed at optimizing chromatography conditions led us, incidentally, to identify some pitfalls of mobile phases which are in current use for mannose binding lectins purification. This will be also discussed.

[1]Neutral agarose based supports suitable for expanded bed chromatography might have been purchased from UpFront Chromatography (Copenhagen, Danmark) which has been marketing this product since 1995. Such supports were also made commercially available recently from Pharmacia (Uppsala, Sweden).

2. Experimental

2.1. Immobilization of mannose on DEAE Streamline adsorbent and Sepharose 6B

The technique is basically derived from a published procedure [26]. 50 ml of DEAE Streamline, or Sepharose 6B (Pharmacia, Uppsala, Sweden) were rinsed with several volumes of coupling buffer (0.5 *M* Na_2CO_3) then suspended in a final total volume of about 100 ml. 10 ml divinylsulphone were added and the suspension was shaken for 2.5 h at room temperature. Thereafter the gel was rinsed on a fritted disk with 150 ml coupling buffer and transferred to a vessel containing 8 g mannose dissolved in coupling buffer (final volume was about 150 ml). The support was shaken for 18 h at 37°C and then washed with 200 ml coupling buffer, 200 ml 100 m*M* sodium acetate buffer pH 5.4 containing 1 *M* NaCl and 200 ml 20 m*M* *N*-(2-hydroxyethylpiperazine-*N'*-(2-ethanesulfonic acid) (HEPES) pH 6.9 buffer containing 2 *M* NaCl. It was then stored in this buffer.

2.2. Preparation of flower bulb crude extract

Preparation of crude extract in amounts suitable for the preparative purification procedure was as follows: 150 g of flower bulbs were manually freed of the outer sclerotic layer and homogenized with a Waring blender in 800 ml buffer A (20 m*M* HEPES pH 6.9 buffer). The thick suspension was passed through one layer of Miracloth (Calbiochem, La Jolla, CA, USA). The turbid filtrate was diluted to half by adding one volume of buffer A and used as such for purification of lectin by chromatography on expanded bed columns. When it was used for scouting experiments with sedimented bed columns it was clarified by centrifugation, and a salt composition made as indicated in results.

2.3. Scouting of several chromatographic conditions for mannose binding lectin purification using small scale chromatography

First, scouting experiments to test several mobile phase conditions were performed using sedimented column and clarified crude extracts. Elution of lectin was followed by functional assays performed as described below. However, most of the experiments were repeated, or others were directly performed using variable amounts of purified *Galanthus nivalis* lectin, and optical density monitoring of column effluent was used to trace lectin elution. Columns were 0.5 cm internal diameter HR 5/2 Pharmacia columns, packed to a height of 1 or 1.4 cm as indicated below with mannose DEAE Streamline, mannose agarose, or T Gel (prepared as described [27]). Details on mobile phase compositions will be given in the results section.

2.4. Column and setup used for expanded bed chromatography

Some trials have been performed in an original Pharmacia Streamline 25 column but frequent clogging of frits was encountered when bulb extract was pumped into the column. Hence the following modifications of column assembly were performed (see Fig. 1): The glass tube was replaced with a 2.6 cm internal diameter acrylic plastic tube. This plastic tube was fitted with a lateral inlet tube situated near the bottom of the column so that mobile phase or crude bulb extract might be introduced into the column above the original Pharmacia inlet bottom piece. The original axial inlet tubing was maintained. Thus depending on the positions of connected valves (see Fig. 2) mobile phase was pumped to the column in upwards mode either through the lateral inlet or through the axial tubing running into the column under the bottom net. This axial tubing was the only outlet used when the column was operated in descending flow mode. Two lateral column outlets were bored at a height of 80 cm measured from the bottom of the column. Obviously these outlets may be only operative if the position of the Pharmacia plunger is kept above the location of outlets. When the plunger is moved below the location of the outlets, flow into the column (in descending flow) or out of the column (in ascending flow) may only come through the original plunger inlet. The O ring fitted to the mobile plunger was changed so as to give a leak tight seal despite the slightly larger bore (26 mm) of the acrylic tube compared to the original glass tube (25 mm). A small cylindrical magnetic spinbar (6.4×16.5 mm) was put into the column so

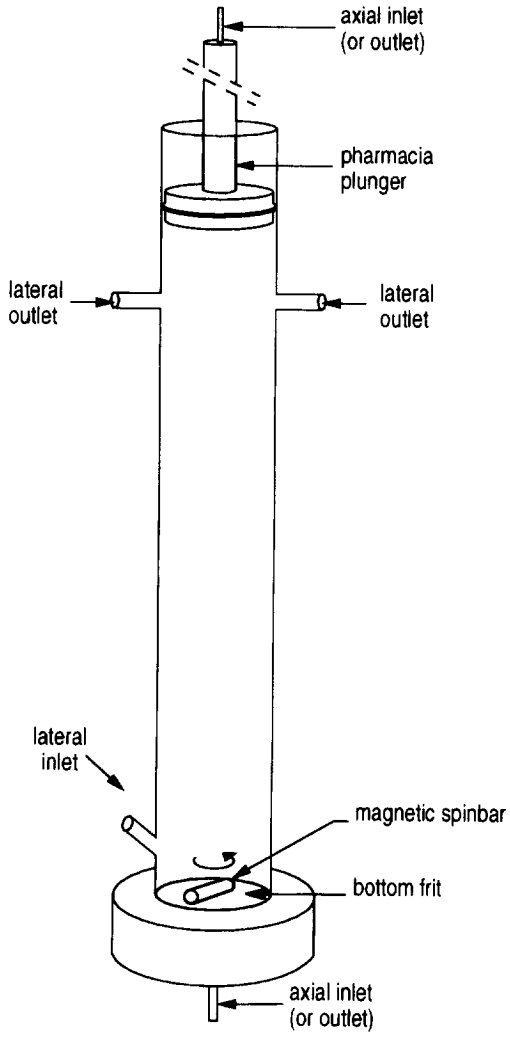

Fig. 1. Column used for the preparative expanded bed chromatography experiments. The glass tube of a Pharmacia Streamline 25 column was replaced with a 2.6 cm internal diameter acrylic tube fitted with a lateral bottom inlet and two supplementary outlet tubes. These outlet tubes are connected to waste and are operational only when the mobile plunger is located *above* outlet ports. The lateral bottom inlet tube is used only in ascending flow mode. The magnetic spinbar is put in rotation only when the column is used in ascending flow mode. See text for other details.

as to allow magnetic stirring of the bed at the column bottom. Magnetic stirring was used only with ascending flow.

The column was connected to a Gilson 302 piston pump through adequate valves (see Fig. 2) so as to

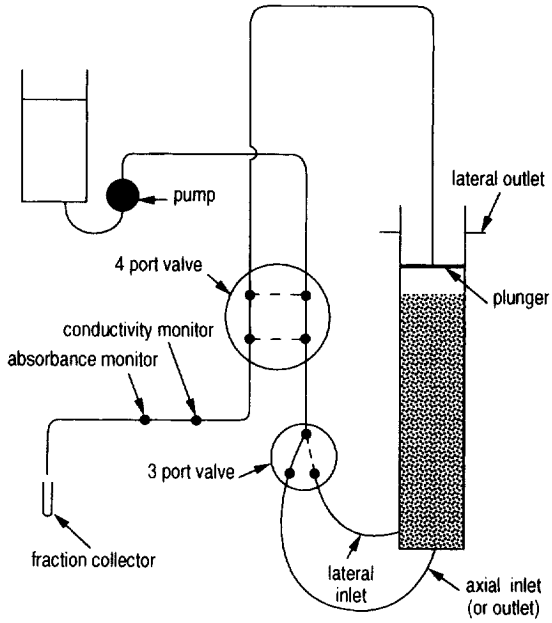

Fig. 2. Flow diagram of the setup used for expanded bed chromatography. Continuous and broken lines drawn through the 3- and 4-port valves show the flow paths realized during the two alternative positions of these valves. The 3-port valve selects which of the lateral inlet or axial bottom inlet/outlet is used. The position of the 4-port valve determines whether the column is used with downward flow (broken line) or upward flow (continuous line).

allow easy selection of the bottom inlet and easy change of flow direction into the column.

2.5. Preparative procedure for tulip bulb lectins purification by expanded bed affinity chromatography

Mannose DEAE Streamline was used as column packing (height of sedimented bed was 15 cm). Optical density and conductivity were recorded at the column outlet through Pharmacia apparatuses. Buffers which have been used were: buffer A, 20 m*M* HEPES pH 6.9, buffer B, 20 m*M* HEPES pH 6.9 containing 0.2 *M* NaCl, buffer C: 0.5 *M* mannose in buffer B, buffer D, 2 *M* NaCl in 0.1 *M* sodium acetate buffer pH 5.4, buffer E, 0.1 *M* sodium acetate buffer pH 5.4 containing 1% (v/v) Triton X-100.

The routine procedure for tulip bulb lectin purification is as follows: prior to chromatography, the

bed is stabilized in expanded conditions by pumping buffer A through the lateral bottom inlet at an (upward) flow linear velocity of 200 cm/h with magnetic stirring switched on. Then routinely 1400 ml of turbid extract is loaded into the column in upward configuration through the lateral bottom inlet. The position of the mobile plunger is set above the lateral outlets and column effluent directed to waste. After completion of sample loading, the column is rinsed with upward flow by pumping about 1000 ml buffer A through lateral inlet. The effluent is then perfectly clear. Flow is then applied to the column through the axial inlet, the mobile plunger is lowered to about 3 cm above resin level and signals from optical density and conductivity monitors recorded. The column is then developed with buffer B (in upward flow conditions) which elutes an optical density peak. Fractions may be collected. When absorbance at the column outlet returns to baseline, the magnetic stirrer is stopped. The gel bed is allowed to sediment by pumping buffer B downwards at 100 cm/h linear velocity. When the gel is fully sedimented, the pump is stopped and the mobile plunger adjusted to a position slightly above the gel level. Thereafter flow is resumed at 100 cm/h linear (downward) velocity. The column is developed successively with buffer C which elutes lectin and buffer D.

The column is regenerated by pumping successively 2 l buffer D at 200 cm/h velocity (upwards with stirrer on), 600 ml buffer E downwards (at 100 cm/h linear velocity with stirrer off) and finally 2 l Buffer E upwards (at 200 cm/h with stirrer on). The column may be then reequilibrated in buffer A before being used for another purification cycle.

Position of the gel upper level is monitored at regular intervals throughout the purification and cleaning procedure.

Lectin present in buffer C eluted fractions is passed in 5 m*M* ammonium carbonate buffer pH 8.3 on a BIOMAX 10 Millipore membrane fitted in an ultrafiltration cell and lyophilized.

2.6. Functional assay of tulip bulb lectin

Mannose binding lectins are able to induce agglutination of trypsin treated rabbit erythrocytes. Rabbit erythrocytes were washed by centrifugation in phosphate-buffered saline (PBS; 150 m*M* NaCl in 5 m*M* sodium phosphate buffer pH 7.6). They were then treated with bovine trypsin at 37°C for 1 h (one volume of erythrocytes, two volumes of 1 mg/ml trypsin solution in PBS). Then erythrocytes were washed six times with PBS containing 1 mg/ml of bovine serum albumin and 0.1 m*M* phenylmethylsulfonylfluoride (PBS–BSA). 20 μl of solution to be tested and serial dilutions of it (made in PBS–BSA) were mixed in Eppendorf tubes with 20 μl trypsin treated rabbit erythrocytes suspension in PBS–BSA (1% hematocrit). After 10 min incubation at 20°C agglutination was evaluated through gel tests by adding 20 μl of Eppendorf contents to DiaMed-ID micro typing cards (DiaMed, Cressier sur Morat, Switzerland). These cards are marketed for cold agglutinins determination in blood transfusion laboratories.

2.7. Sodium dodecyl sulfate (SDS) gels and amino acids sequence determination

SDS gels were performed in 15% acrylamide minigels according to the Laemmli procedure in a Novex apparatus (San Diego, CA, USA). Gels were run with reduced samples and stained with Coomassie Brilliant Blue. Sequencing of bands blotted onto PVDF membranes [28] was performed by Service de Microsequence de l'Institut Pasteur using an Applied Biosystems 477A sequencer.

2.8. Purification of Galanthus nivalis lectin

Galanthus nivalis lectin was used to aid the evaluation of the influence of mobile phase composition on mannose DEAE Streamline and mannose agarose behaviors. It was purified as follows: clarified snowdrop bulb crude extract in buffer A was prepared as described above and loaded on a mannose agarose column equilibrated in buffer A. After adequate column rinsing with buffer A, lectin was eluted through 0.5 *M* mannose dissolved in buffer A. Purified lectin present in mannose eluted fractions showed one single band on SDS–polyacrylaide gel electrophoresis (PAGE). After dialysis to 5 m*M* ammonium carbonate pH 8.3 lectin was lyophilized.

3. Results and discussion

3.1. Insight into retention mechanism of mannose binding lectins on mannose DEAE Streamline

Mannose agarose was largely used for mannose binding lectin purification. Operating conditions described in the literature vary somewhat, but most often purification procedures involve loading on the column equilibrated in ammonium sulfate containing buffer [9,10,13,14]. Hence the first trials used similar mobile phases to operate mannose DEAE Streamline.

However it was rapidly established, as will be discussed below, that (i) mannose DEAE Streamline displays biospecific affinity interactions with mannose binding lectins, (ii) as anticipated, mannose DEAE Streamline may retain solutes by ionic interactions, (iii) lastly, when mobile phases contain ammonium sulfate (a water structure forming salt), lectins (and also other solutes in crude extract) are retained by mannose DEAE Streamline and also by mannose agarose, probably, as detailed below, through T-gel like interactions [26].

3.1.1. Biospecific affinity interactions of mannose binding lectins with mannose DEAE Streamline.

In Fig. 3 it is shown that when 0.5 mg, 1 mg and 5 mg purified lectin in 20 m*M* HEPES buffer pH 6.9 were loaded on a mannose DEAE Streamline 0.28 ml column, an optical density peak was generated by changing the mobile phase to 0.5 *M* NaCl in 20 m*M* HEPES buffer pH 6.9 corresponding to elution of some lectin. After return to baseline of the absorbance signal, further development of the column with 0.5 *M* mannose in this buffer induced elution of the remaining part of retained lectin. The eluting effect of mannose proves the onset of biospecific interactions between protein and its immobilized ligand.

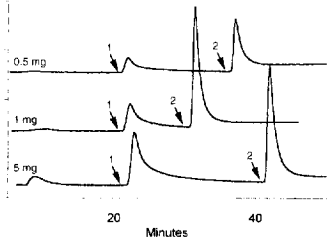

Fig. 3. Mannose DEAE Streamline retains lectin by both ionic interactions and a biospecific affinity mechanism. The column was 0.5 cm internal diameter and 1.4 cm high. Linear velocity was 100 cm/h. Each time the column was equilibrated in 20 m*M* HEPES pH 6.9 buffer, 0.5 mg, 1 mg and 5 mg were loaded onto the column as indicated. After loading, the column was rinsed with equilibration buffer and developed with 0.5 *M* NaCl in 20 m*M* HEPES pH 6.9 buffer (arrows 1) and 0.5 *M* mannose in 0.5 *M* NaCl 20 m*M* HEPES pH 6.9 buffer (arrows 2). Settings of the absorbance monitor were identical for the three traces.

3.1.2. Mannose DEAE Streamline may also retain solutes by ionic interactions

In Fig. 3 it is shown that lectin binds to mannose DEAE Streamline by ionic interactions: some lectin was eluted by an increase of ionic strength (meaning also, by the way, that loads which have been used exceeded the capacity of the column for lectin through biospecific interactions). Interestingly, if 0.5 *M* mannose in low ionic strength buffer was applied on a column loaded with 1 mg lectin, protein was not eluted (not shown) meaning that even if biospecific interactions were disrupted through mannose addition to mobile phase, lectin still stuck to the column through electrostatic interactions.

3.1.3. Ammonium sulfate containing mobile phases promote retention of lectin by mannose agarose and mannose DEAE Streamline

Mannose agarose expectedly behaved as an affinity chromatography support for lectin as shown on Fig. 4 trace A: lectin dissolved in 20 m*M* HEPES buffer pH 6.9 was retained by the column equilibrated in the same buffer and eluted by mannose dissolved in the same buffer. Trace B illustrates the large increase in capacity for purified lectin of a mannose agarose column equilibrated in 20 m*M* HEPES buffer pH 6.9 containing 1 *M* ammonium sulfate: 20 mg lectin dissolved in this buffer were retained by the column and no lectin appeared as unretained material while a lower load induced a breakthrough peak when lectin was dissolved in the same buffer but without ammonium sulfate (trace A).

Increase in capacity is due to the recruitment of new sites on the gel beads distinct from the biospecific affinity sites. Lectin bound to the chromatography support when equilibrating buffer and buffer

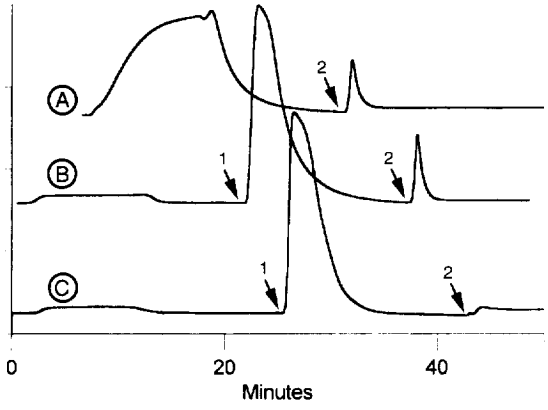

Fig. 4. Effect of ammonium sulfate on capacity for mannose binding lectin of a mannose agarose column. Chromatography of purified lectin on T-gel. The column dimensions were 0.5 cm internal diameter and 1 cm high. Linear velocity was 100 cm/h. Trace A was obtained by loading 5 mg purified lectin dissolved in 20 m*M* HEPES pH 6.9 buffer on the mannose agarose column equilibrated in same buffer. After sample loading column was rinsed with equilibration buffer, at arrow 2 mobile phase was changed to 0.5 *M* mannose in 20 m*M* HEPES pH 6.9 buffer. Trace B was obtained by injecting 20 mg purified lectin dissolved in 1 *M* ammonium sulfate, 20 m*M* HEPES pH 6.9 buffer on the mannose agarose column equilibrated in the same mobile phase, after loading, the column was rinsed with the same buffer. Arrows 1 and 2 indicate changes to 20 m*M* HEPES pH 6.9 buffer and to 0.5 mannose in the same buffer. Trace C was obtained by injecting 20 mg purified lectin dissolved in 1 *M* ammonium sulfate, 20 m*M* HEPES pH 6.9 buffer on a T-gel column equilibrated in same mobile phase. After loading, the column was rinsed with the same buffer. Arrows 1 and 2 indicate changes to respectively 20 m*M* HEPES pH 6.9 buffer and 0.5 *M* mannose in 20 m*M* HEPES pH 6.9 buffer.

used to dissolve lectin contained both 0.5 *M* mannose and 1 *M* ammonium sulfate (not shown).

Ammonium sulfate is used to promote protein retention on hydrophobic interaction chromatography supports [29]. It is known that addition of ammonium sulfate to mobile phases may induce protein retention even on plain unmodified agarose, (in so called salting out chromatography [30]). No significant retention of lectin on unmodified Sepharose 6B was observed when lectin dissolved in 20 m*M* HEPES pH 6.9 containing 1 *M* ammonium sulfate was loaded on a column equilibrated in the same buffer (not shown), this rules out the possibility that a salting out chromatography mechanism might explain increase of mannose agarose capacity for lectin induced by ammonium sulfate.

It has been stressed by others that divinylsulfone activation of agarose gels might impart properties of T-gel supports to these gels [31,32]. T-gel properties are modulated by ammonium sulfate addition to mobile phase. Hence we have tested whether T-gel retains lectin. Trace C on Fig. 4 clearly shows that T-gel retains lectin dissolved in a solution containing ammonium sulfate, lectin was eluted through buffer without ammonium sulfate.

From a practical point of view, the choice of ammonium sulfate containing mobile phases for mannose binding lectin purification on mannose agarose, even if it is widely used, does not seem to be logical since it may increase column capacity for lectin at the expense of selectivity (other proteins are likely to be also retained by the column). We only used divinyl sulfone activated agarose and results might have been different with other activating agents.

A similar increase of capacity induced by ammonium sulfate has been observed with mannose DEAE Streamline (Fig. 5, traces A and B). However, increase in capacity for lectin was not as important as that observed on mannose agarose, as judged from optical density signal recorded at the column outlet during sample loading (compare traces B, Figs. 5 and 4). As anticipated the nature of the salt was important to the onset of the hypothesized T gel like interactions of lectin with mannose DEAE Streamline matrix. Chromatogram C on Fig. 5 exemplifies this: in this experiment 20 mg of lectin was dissolved in 3 *M* NaCl 20 m*M* HEPES pH 6.9 buffer and loaded onto the column equilibrated in the same buffer. A large amount of lectin was eluted during sample loading.

These experiments established the rationale for devising a procedure for lectin purification on mannose DEAE Streamline. Obviously, sodium chloride was to be preferred over ammonium sulfate to increase mobile phases ionic strengths: after extract loading in low ionic strength conditions (buffer A: 20 m*M* HEPES pH 6.9), mobile phase ionic strength was increased to elute unwanted ionically retained solutes (buffer B: 0.2 *M* NaCl in buffer A) and thereafter lectin was specifically eluted by adding a competing sugar to this mobile phase (buffer C: 0.5 *M* mannose in buffer B). Finally the column was developed with high ionic strength and a lower pH

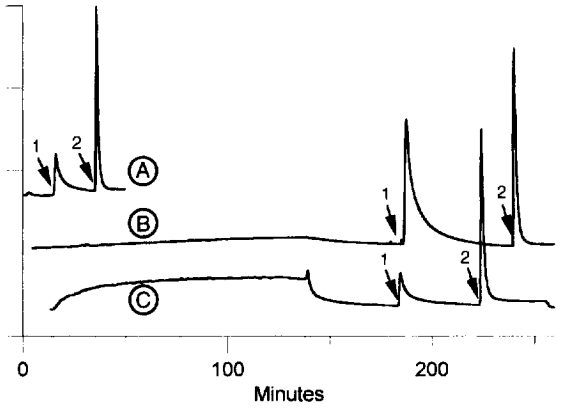

Fig. 5. Increase in capacity for lectin of mannose DEAE Streamline induced by ammonium sulfate addition to sample and equilibrating mobile phase. Column was 0.5 cm internal diameter and 1.4 cm high. Linear velocity rate was 100 cm/h. Trace A was obtained by loading 5 mg purified lectin dissolved in 20 m*M* HEPES pH 6.9 buffer on the column equilibrated in the same buffer. Trace B was obtained by loading 20 mg purified lectin dissolved in 1 *M* ammonium sulfate, 20 m*M* HEPES pH 6.9 buffer on the column equilibrated in the same buffer. Trace C was obtained by loading 20 mg purified lectin dissolved in 3 *M* NaCl, 20 m*M* HEPES pH 6.9 buffer on the column equilibrated in the same buffer. In each case the column was rinsed after completion of sample loading with the respective equilibration buffer before being developed with 0.5 *M* NaCl in 20 m*M* HEPES pH 6.9 and 0.5 *M* mannose in the latter buffer as indicated by arrows 1 and 2 respectively.

mobile phase in order to elute tightly bound proteins (buffer D: 2 *M* NaCl in 0.1 *M* sodium acetate buffer pH 5.4).

3.2. Tulip bulb lectins purification by expanded bed affinity chromatography on mannose DEAE Streamline and characterization of purified product

A chromatogram, obtained as described in the Section 2 is shown in Fig. 6. The height of the expanded bed before loading is usually 2.3 times the height of the sedimented gel bed. The highest expansion ratios are usually observed during sample loading (from 2.8 to 3.4) but after rinsing of the column and clarification of the effluent they return to values observed before sample loading. At the end of the regeneration procedure and reequilibration of the column, the same 2.3 expansion ratio is routinely obtained.

Functional assays performed on aliquots of eluted

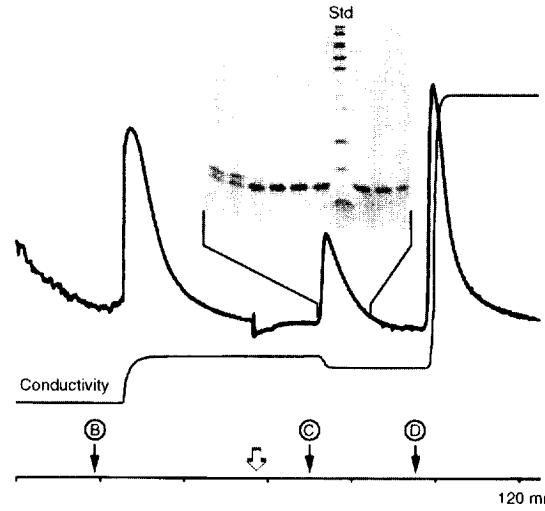

Fig. 6. Preparative chromatography on mannose DEAE Streamline. The internal diameter of the column was 2.6 cm. The sedimented bed height was 15 cm. 1400 ml extract was loaded. Optical density and conductivity tracings were recorded from the moment when the plunger was lowered to about 3 cm from the expanded bed level (at that moment, buffer A was being pumped upwards into the column at 200 cm/h through the axial inlet, and magnetic stirring was on. See text and Figs. 1 and 2). The mobile phase was changed to buffer B as indicated by the arrow, still at 200 cm/h (upward) linear velocity. The white arrow indicates that mobile phase was pumped downwards into the column, by changing the 4-port valve position (see Fig. 2), the magnetic stirrer was stopped, the linear velocity lowered to 100 cm/h, and the position of the plunger adjusted to the top of sedimented gel bed. The mobile phase was changed to buffer C and D as indicated by respective arrows. Insert shows SDS gels of aliquots of fractions eluted by buffer C. Lane marked Std was loaded with molecular mass standards (from top to bottom: M_r 200 000, 116 300, 97 400, 66 300, 55 400, 36 500, 31 000, 21 500, 14 400, 6000).

fractions revealed the presence of agglutinating activity only in buffer C eluted fractions. SDS–PAGE analysis of reduced aliquots of collected fractions are shown in Fig. 6. Two bands with apparent molecular masses of 10 500 and 8500 are obvious in fractions eluted through buffer C. The bands were sequenced after transfer onto PVDF membranes. The sequence of the 8500 apparent molecular mass band was KPCTPNNV. This sequence corresponds exactly to a sequence published by Van Damme et al. [12] for a mannose specific tulip bulb lectin (called TxLMII for the second mannose binding Tulipa hybrid lectin). The sequence

of the M_r 10 500 band was DRALPCTPN. This sequence is homologous with the former one but does not match it exactly and hence, probably corresponds to a heretofore undescribed isoform of tulip lectin.

Other lectins have been isolated from tulip bulbs. One known as TxLC I (for the first *Tulipa* hybrid lectin with complex specificity) is formed from 28 000 apparent molecular mass subunits. It contains binding sites for GalNac and mannose, however binding of mannose seems to be weak and affinity for mannose agarose can be observed only in the presence of ammonium sulfate [12]. It is therefore not surprising that no M_r 28 000 band was observed in our purified lectin preparation. Another tulip mannose binding lectin has been isolated by chromatography on immobilized mannan by Oda et al. [33]. This lectin called TxLMI [12] (for the first mannose binding Tulipa hybrid lectin) is composed of 17 000 apparent molecular mass subunits. It has not been observed by others who used chromatography on mannose agarose to purify TxLMII [12].

The procedure described in this report completed by a diafiltration lyophilisation step, allows to recover 16 mg purified lectin preparation to be recovered from 100 g starting material.

Until now 15 chromatograms have been made using the same gel, cleaning in place procedure is clearly adequate to maintain constant expansion characteristics of the gel bed. The lifetime of the gel will be established on an ongoing basis by monitoring expansion parameters and purification results. We want to stress that the use of lateral inlet and outlets entirely solved problems associated with frequent frits clogging experienced with the original Pharmacia Streamline column design. Such problems were also experienced by others using common material for column loading (i.e. bacterial extracts [34]). Gentle magnetic stirring does not appear to induce obvious damage to the beads, nor does the presence of the immobile magnetic flea during column elution seem to significantly influence peak shape. Present modification of the bottom part of the column is very similar to that devised and patented by UpFront Chromatography [35].

It may be stressed that viscosity of the crude extract is an important parameter to deal with when using expanded bed chromatography for purification, since terminal velocity of the adsorbent bead is inversely proportional to feed viscosity [15]. Viscosities of extracts from flower bulbs clearly depend on the ratio of bulb mass to volume of extraction buffer, but also noticeably on composition, molecular masses and nature of the storage saccharides present in bulbs.

4. General comments and conclusion

The choice of an ion-exchanger for ligand immobilization did not cause any problem, since interference of ion-exchange groups could be easily coped with by ionic strength adjustments of the mobile phase. It is indeed not the first time that ion-exchangers have been used for immobilization of affinity chromatography ligands: the first silica based supports used for affinity chromatography of proteins on an industrial scale were prepared by coating silica with DEAE dextran [36]. Dextran coverage allowed easy grafting of ligand but DEAE groups, which helped to ensure adequate coverage of silica matrix by making ion pairs with silanols, obviously also gave ion-exchange properties to the support.

When we prepared an affinity chromatography support starting from an ion-exchanger we knew that we would get a mixed mode support able to retain proteins by both ion-exchange and true biospecific interactions. We were amused to discover incidentally that supposed archetypal affinity chromatography supports based on plain agarose could also be mixed mode supports when mobile phase contained water structure forming salts.

This report seems to be the first article on proteins purification from plant material by expanded bed chromatography, but unpublished experiments performed elsewhere dealt with capture of numerous lectins from ground vegetal tissues using expanded bed methodology and small aromatic, ionic mixed mode ligands [37] (these ligands however did not allow complete purification in one step). Expanded bed methodology allowed us to start from very turbid material. The full interest of the technique will become obvious when volumes of treated starting material are increased to values which preclude easy clarification. Use of an immobilized affinity ligand allowed pure protein to be obtained in virtually one

step. This may be added to the growing body of evidence [14,18,21–24] that expanded bed methodology may not be reserved to coarse capture chromatography supports but be applied to very selective affinity chromatography supports.

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