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ULTRAFILTRATION AFFINITY PURIFICATION

ISOLATION OF CONCANAVALIN A FROM SEEDS OF *CANAVALIA ENSIFORMIS*

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SUMMARY

A new approach to affinity purification is described which combines affinity binding with ultrafiltration separation. In this approach, a macromolecular ligand is retained on one side of a membrane and is allowed to interact with a crude extract. Material specifically binding to the ligand forms a high-molecular-weight complex whereas all other material is washed out through the membrane pores. On addition of free ligands or other dissociation media the complexes dissociate and the formerly bound material is liberated, passed through the membrane and collected in a purified state.

This paper describes the purification of concanavalin A from a crude extract of *Canavalia ensiformis* using the surface structures of heat-killed yeast cells (*Saccharomyces cerevisiae*) as macromolecular ligands. A high capacity for concanavalin A was demonstrated. The yield over the total process was *ca.* 70% and the product was homogenous when analyzed by SDS–polyacrylamide gel electrophoresis.

INTRODUCTION

The introduction of affinity chromatography has revolutionized protein purification from a laborious trial-and-error procedure into one with high resolution and efficiency and relative operational simplicity. In order to set up an affinity chromatographic purification step, a ligand must first be available that can be coupled to a solid matrix; chemical modification to introduce appropriate groups on the ligands is the limiting step.

Affinity techniques are routinely used in many laboratories, but few new technical developments have been presented recently, and thus affinity purification technology is in essence the same today as it was ten years ago.

In modern biotechnology, the ability to produce molecules of unique structure and specific biological activity on a large scale has focused attention on the weakest point in the process –the down-stream processing step. Some efforts have been made to improve the capacity of affinity chromatographic procedures[1] in order to solve this problem, but so far no solution that works sufficiently well has been presented.

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In parallel with the development of affinity chromatography, membrane technology has grown into an established technique for fractionation of molecules based on their molecular size[2]. The present report describes a combination of ultrafiltration technology with affinity purification technology, that results in a purification process with great potential for use in large-scale and in continuous processes.

MATERIALS AND METHODS

Chemicals

Yeast cells, *Saccharomyces cerevisiae*, were obtained from a local source. Finely ground, defatted Jack bean meal and horseradish peroxidase (E.C.1.11.1.7), type II and 2,2'-azino-di-(3-ethylbenzthiazoline sulphonic acid) (ABTS) were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-concanavalin A antiserum was from E-Y Labs. (San Mateco, CA, U.S.A.). All other chemicals used were of analytical grade.

Equipment

The hollow fiber units, pumps, level monitors and pressure meters were generously supplied by Gambro (Lund, Sweden).

Preparation of crude extract of Jack beans

Defatted, ground Jack beans (*Canavalia ensiformis*) (500 g) were suspended in 2.5 l of 0.9% sodium chloride solution, containing 1m*M* each of Ca^{2+}, Mg^{2+}, and Mn^{2+} at pH 7.0. The suspension was stirred overnight at 4°C and then filtered through cheesecloth. The filtrate was stored at 4°C, and the extraction procedure was repeated once on the solid residue. The two filtrates thus obtained were pooled and centrifuged for 1 h at 14,500 *g* at 4°C. The supernatant containing the lectin was stored at 4°C.

Preparation of affinity sorbent material

Cells of *Saccharomyces cerevisiae* were used as affinity sorption material. They were heat-killed before use. Yeast cells (150 g pressed weight) were suspended in 1.0 l of 0.9% sodium chloride solution (9 g/l). The cell suspension was continuously stirred for 40 min in a water bath at 70°C. Soluble impurities were removed at room temperature by continuously recirculating the cell suspension through a hollow fiber unit with a molecular weight cut-off of 1,000,000 daltons. Sodium chloride solution (9 g/l) was continuously added to keep the volume constant. Washing proceeded until the effluent from the outlet of the membrane unit showed no absorbance at 280 nm.

Quantitative determination of concanavalin A

To quantitate concanavalin A (con A) in the various samples, an enzyme-linked immunosorbent assay (ELISA) was set up. The assay was performed in Dynatechs Microelisa microtiter plates, with flat-bottomed wells, which are easily monitored in multiscanning photometers. The wells were coated by pipetting 100 μl of a coating solution containing 5 μg of rabbit anti-concanavalin A antibodies per 1 ml of 0.1 *M* carbonate buffer, pH 9.5 per well. Incubation proceeded for 2 h at 37°C in a moisture chamber. The wells were then thoroughly washed with a solution containing 0.9% sodium chloride and 0.05% Tween 20.

The samples to be assayed for con A were prepared by dilution with a standard

dilution buffer, comprising 0.1 *M* sodium phosphate and 0.05% Tween 20, pH 8.0. A 100-μl volume of diluted sample was added to each well. Plates were subsequently incubated at room temperature for 2 h and washed as described above. Con A present in the sample was bound by the anti-concanavalin A antibodies coupled to the wells, in an amount directly proportional to the amount of con A present in the incubation mixture.

To visualize con A in the wells, incubation with 100 μl of a horseradish peroxidase solution (40 μg per millilitre of standard dilution buffer) was performed as in the previous step. Horseradish peroxidase, a glucoprotein, interacts with con A through a lectin–carbohydrate interaction.

The amount of peroxidase bound was then determined by incubation with a substrate solution composed of 20 ml of 50 m*M* citrate buffer, pH 4.0, 5.33 μl of 30% hydrogen peroxide and 100 μl of ABTS (439 mg dissolved in 20 ml of distilled water). A 200-μl volume of this substrate solution was incubated per well for 15 min, after which the absorbance at 405 nm was read in a photometer (Titertek Multiscan).

Bioassay of concanavalin A

The biological activity of concanavalin A in the various samples was determined by its capability to agglutinate human red blood cells. The hemagglutination test was performed in Titertechs Microtiter plates, with V-shaped wells. Each sample was diluted in serial two-fold dilutions. Diluted sample (100 μl) and a 2% red blood cell suspension (100 μl) were added to each well. The plate was incubated for 1 h at room temperature. The plate was then visually inspected for estimation of the agglutination endpoint of each dilution series.

The affinity purification process for Concanavalin A

The procedure discussed here for the purification of concanavalin A from a crude extract is based on the fact that con A interacts with carbohydrate residues exposed on the surface of *Saccharomyces cerevisiae* cells. The experimental set-up is schematically illustrated in Fig. 1. The total system is built up around a hollow fibre unit with a molecular weight cut-off of 1,000,000 daltons, which is connected to a peristaltic pump (A) that continuously recirculates a stream containing the affinity sorbent material (*i.e.*, the yeast cells) mixed with the material to be purified, through the inner parts of the fibers (flow-rate 500 ml/min). Material that is capable of penetrating the membrane pores leaves the interior of the hollow fibre (containing the cells) and enters the outer part of the membrane unit. The outlet from the outer shell of the membrane unit is connected to a photometer for continuous monitoring of the absorbance at 280 nm in the effluent. By monitoring of the effluent from the membrane unit, the purification process is conveniently followed.

The pump (A) is connected to a pressure meter (B), placed between the outlet of the pump and the inlet of the membrane unit. The pump is controlled by the pressure in this stream. This security system is used to protect the hollow fibre membranes from harmful pressures. The level of liquid in the beaker (C) is controlled by a level sensor controlling a second pump (D).

The subsequent washing and dissociation steps were performed using a similar experimental set-up.

After all the non-interacting material has been washed out of the system, the

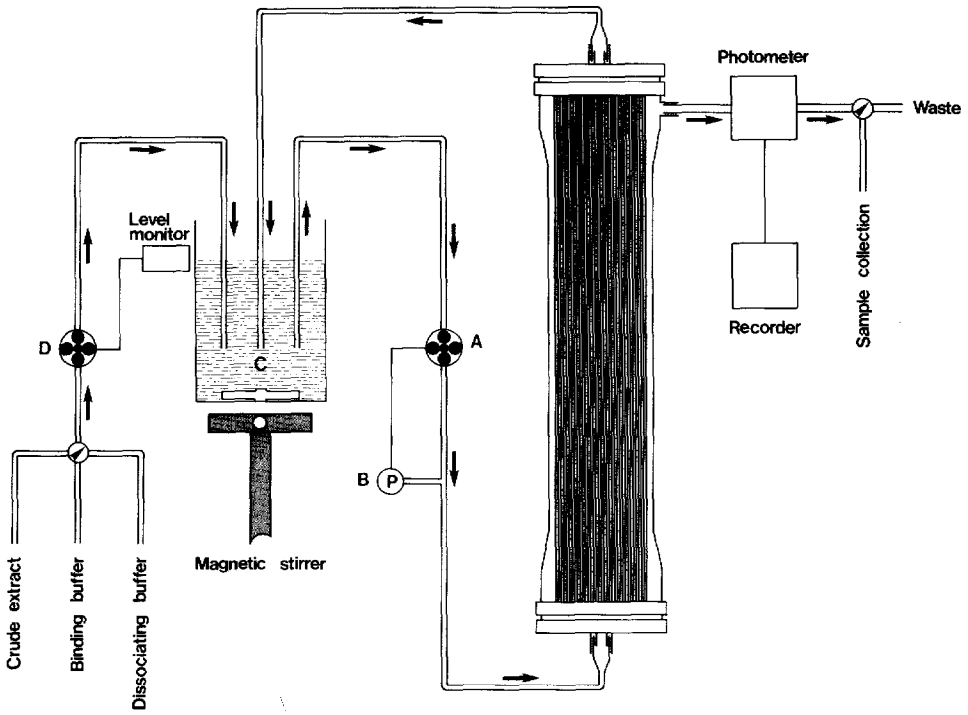

Fig. 1. A schematic representation of the experimental set-up used for the purification of concanavalin A from a crude extract of *Canavalia ensiformis*. The diagram illustrates an ultrafiltration membrane system consisting of a hollow fibre unit with a molecular weight cut-off of 10^6 (long column), a peristaltic pump (A) for the continuous recirculation of the affinity sorbents, a pressure meter (B) and a level sensor placed on the outside of a beaker (C). The level sensor is connected to a second peristaltic pump (D), which keeps the volume of the recirculation stream at a constant, predetermined value.

only materials present in the recirculation stream are the cells with bound con A. Dissociation of this complex is performed by addition of glucose (150 g/l), which competes with the sugar residues on the cell surface for binding on con A. The complex of con A–glucose has a molecular weight below the cut-off of the membrane, and thus leaves the system.

The dissociation process is continued until the absorbance at 280 nm in the effluent returns to zero. The affinity sorbent material is then regenerated by addition of an appropriate buffer.

Determination of purity

The purity of the fractions containing concanavalin A was investigated using sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis.

Samples (80 μg) were applied to a 15% polyacrylamide gel, prepared according to Davis[3]. Electrophoresis was performed for 1 h at 15 mA and 3 h at 30 mA, pH 8.3.

Quantitation of concanavalin A

In order to monitor the purification process, assays for the purified substance had to be set up. An ELISA procedure with rabbit anti-concanavalin A antibodies was

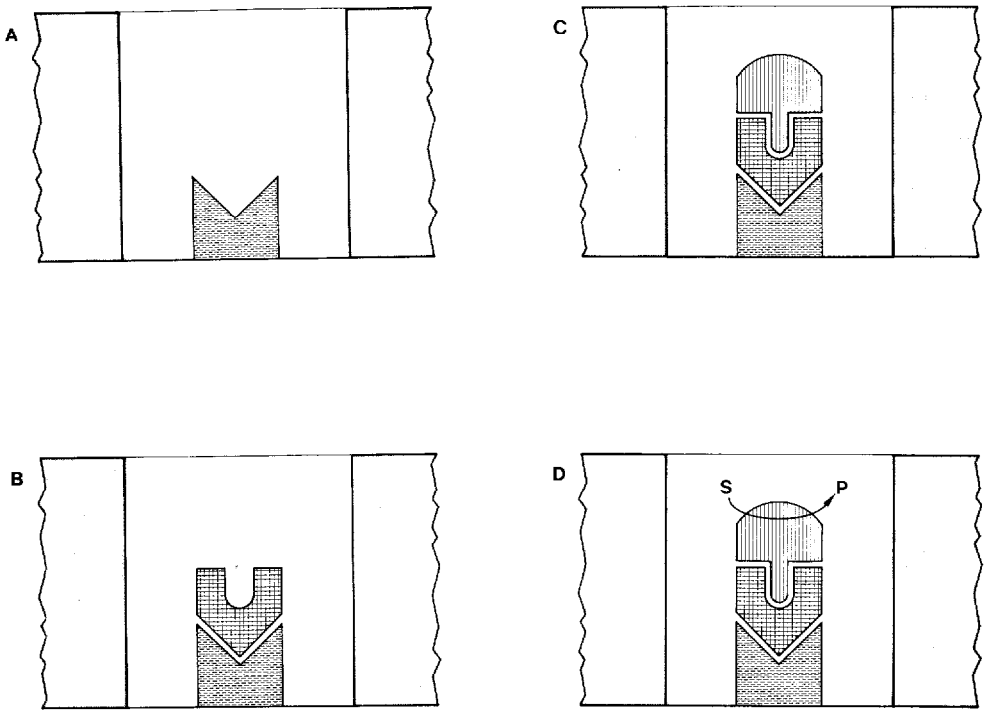

Fig. 2. The principle of the enzyme-linked immuno sorbent assay (ELISA) used in the quantitation of concanavalin A. (A) The wells of a microtiterplate were coated with anticoncanavalin A antibodies. (B) The antibody-coated wells were incubated with samples containing con A. Con A will interact with anti-concanavalin A antibodies. (C) Peroxidase was then incubated in the wells. The glucoprotein peroxidase will interact with con A, bound to the anti-concanavalin A antibodies. (D) Incubation of an enzyme substrate (S), which on enzymatic catalysis forms a coloured product (P), thus giving an estimate of the amount of peroxidase present in the wells, as well as an indirect measure of the amount of con A bound to the wells. This product formation can then be correlated to the amount of con A present in the incubation mixture. Experimental details are given in the text.

used, in which bound concanavalin A was visualized by addition of a glycoprotein, horseradish peroxidase, which forms coloured products on catalysis (Fig. 2).

A calibration curve (Fig. 3) for quantitation of con A was obtained. The biological activity, expressed as red blood cell clotting activity was also tested.

RESULTS

Batch-use ultrafiltration affinity purification

When using this concept of ultrafiltration affinity purification, elution profiles such as those illustrated in Fig. 4 are obtained. As stated earlier, the principle behind ultrafiltration affinity purification is to use membranes with a pore size distribution, that will pass some substances in the extract, but will retain the substance of interest by interaction with a macromolecular ligand that is large enough to be included in the unit.

Material with a molecular weight below the cut-off limit is forced through the

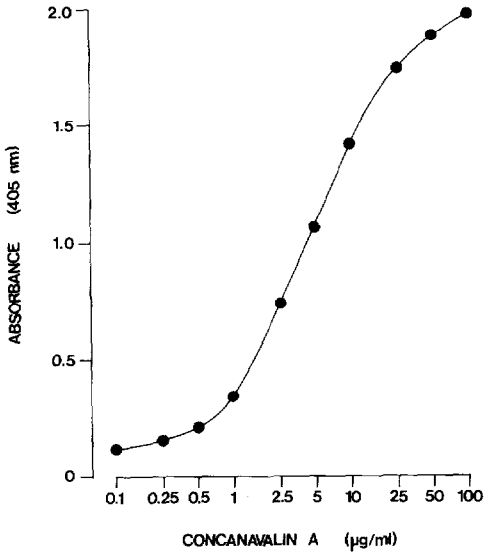

Fig. 3. A calibration curve for concanavalin A obtained in the enzyme-linked immuno sorbent assay (ELISA). Experimental details are given in the text.

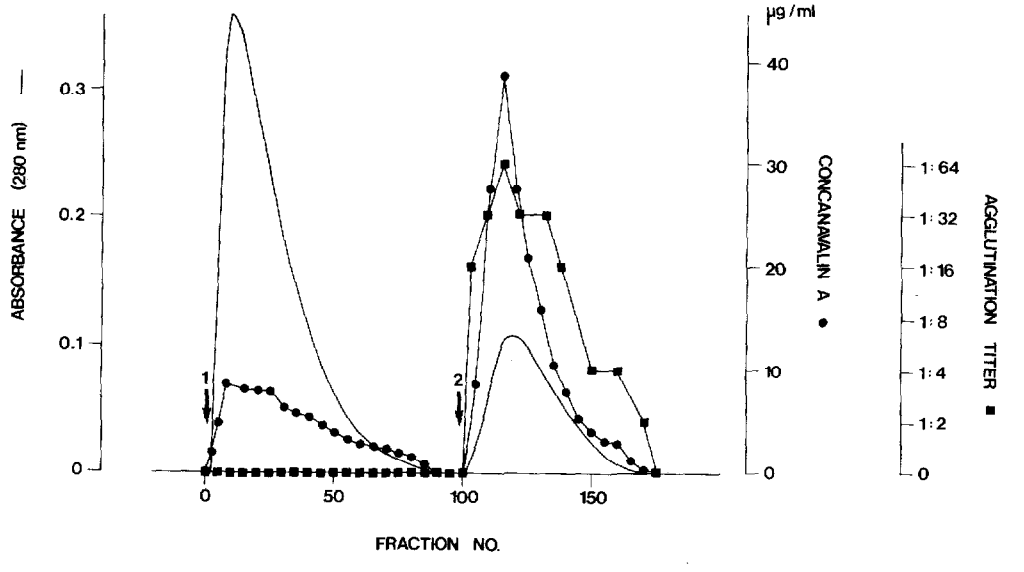

Fig. 4. Results from an ultrafiltration affinity purification experiment. Concanavalin A was purified in a hollow fibre membrane system using surface structures on heat-killed yeast cells as adsorbents. Fractions (10 ml) were collected once a minute. The solid line shows the elution profile obtained when monitoring the effluent at 280 nm. The first peak results after the application of 500 ml of crude extract of *Canavalia ensiformis* and contains non-specifically binding material. The second peak follows the change from a binding solution to a dissociating solution containing glucose. There con A is eluted from the affinity ligand (yeast cells). Results from the ELISA performed on the collected fractions are shown by the line containing circles (●). Results from the hemagglutination study of the collected and dialysed fractions are given by the line containing squares (■). Further experimental details are given in the text.

membrane pores and leaves the recirculating stream containing the ligand. This flow of liquid is passed through a photometer that monitors its absorbance at 280 nm.

When all the impurities have been washed out and a stable baseline is obtained from the photometer, the elution may be started. This is done by introducing a dissociating medium into the recirculation stream. This medium should contain molecules that compete with the ligand residues for the binding of the substance of interest. New complexes will thus be formed, with a molecular weight below the cut-off of the membrane pores, allowing the complex to leave the recirculation stream.

The second peak monitored at 280 nm in the effluent is due to material thus leaving the membrane unit.

The eluted material is collected in a large vessel. Fractions are also collected during the entire elution process for later examination with the analytical tests described earlier. The eluate is dialysed in a similar experimental set-up using a hollow fibre unit with a cut-off of 1000 daltons. This unit allows removal of dissociating ligand (*i.e.* glucose) from the substance of interest (con A), with simultaneous concentration of this final product.

In our process, 10-ml fractions, which were collected during both elution processes, were assayed with respect to the immunological reactivity as well as the biological activity of the con A.

Fig. 4 shows the recordings from the photometer (solid line) during the above mentioned experiment. Two peaks were obtained. The first of these peaks represents the non-interacting material and the second peak material eluted from the cells following addition of glucose. The results from the ELISA tests of the fractions are also shown (dotted line). The first peak was shown to contain some immunologically reactive concanavalin A whereas the major part of the con A was detected in the second peak. The first peak, however, gave no observable agglutination of red blood cells (line with squares). Thus, these con A molecules that do not interact with the cells are immunologically detectable although they are unable to agglutinate red blood cells. The

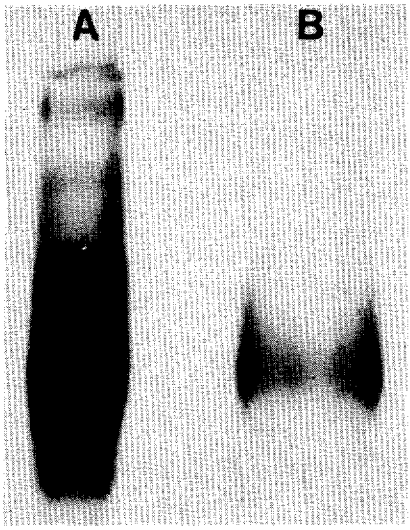

Fig. 5. Results from the SDS–polyacrylamide gel electrophoresis. (A) The crude extract. (B) The purified con A.

second peak, representing the glucose eluted material, was capable of agglutinating red blood cells.

In an experiment lasting for a total of 5 h, 3.4 g of con A were purified. The results from SDS–polyacrylamide gel electrophoresis on the purified material are shown in Fig. 5. The con A preparation was shown to contain one single band when analyzed with gel electrophoresis. The yield in the total purification process was 70%.

DISCUSSION

The technique as described here is based on the same inherent properties of the binding protein as those utilized in conventional affinity chromatography. The unique feature of the technique discussed here is that the different operations, application, washing, elution and reconditioning, can take place at different locations in the room, whereas in conventional procedures all steps must be performed consecutively in the same column.

By using membrane technology it is possible to design a system where the ligand is transported between different membrane units where different operations take place. It is then possible to operate such a process continuously, with the ligand kept and recirculated in a closed volume. The results described in this report indicate that each individual step operates smoothly. Attemps to operate the whole reaction sequence in an integrated manner are currently in progress.

When setting up an ultrafiltration affinity purification system, it is very important to use the correct membranes. This was initially a limiting factor in our efforts because we operated with pore sizes large enough to pass a protein of with a molecular weight of 10^6, and such membranes are at present rarely available on the market. The ligand used had to be either coupled to high-molecular-weight dextran (mol. wt. $2.0 \cdot 10^6$) or, as in the case studied here, to small particles (dead yeast cells).

The total molecular weight of the macromolecular ligand must be such that it shows no tendency to leak from the membrane units. It is thus recommended that, before a ligand is coupled to the macromolecular structure, the latter is extensively prefractionated in a membrane unit to eliminate all low-molecular-weight material. The same approach holds when small particles, *e.g.* cells, are used as supports. In these cases fragments of cell surface structures may ruin the purification process. To minimize these risks, dead cells and even cells treated with cross-linking reagents may be used.

An efficient ultrafiltration affinity purification process demands certain characteristics of the membranes used. Well defined molecular weight cut-offs are a must for the membranes used in the binding, washing and elution steps, whereas in the reconditioning steps membrane units with a low molecular cut-off can be used.

Important criteria for a new purification process are purity and yield of the product. In the system discussed here, the purity of the con A was tested with SDS–polyacrylamide gel electrophoresis and was found to be high. One single band was obtained. The yield in the overall process was 70%.

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