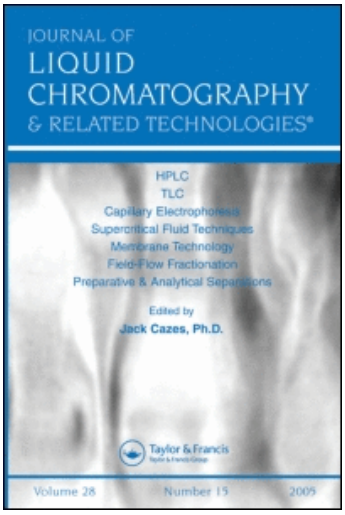

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COMPARISON OF DIALYSIS AND GEL PERMEATION CHROMATOGRAPHY FOR ANTIGEN PURIFICATION

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COMPARISON OF DIALYSIS AND GEL PERMEATION CHROMATOGRAPHY FOR ANTIGEN PURIFICATION

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ABSTRACT

Immunoaffinity chromatography can be applied to the treatment of biological samples. It consists of the separation, extraction, or purification of a molecule (para nitro analine in this paper) from a mixture by a selective interaction with an antibody. The latter is produced by living organisms after their contact with an antigen (modified protein in this case). The production of specific antibodies highly depends on the purity of antigen. This paper describes and compares two methods of antigen purification: dialysis and gel permeation chromatography. This chromatographic method allows one to obtain a pure antigen faster than dialysis but with a slightly larger dilution of the samples. It is not a drawback for the procedure of the antibodies production.

INTRODUCTION

Immuno affinity chromatography[1] can be applied to the treatment of biological samples to isolate active molecules or products inducing allergy (such as Para Nitro Aniline, PNA). It uses a solid phase which retains the product to be isolated by a selective interaction with an antibody.

Antibodies[2] are produced by living organisms (rabbit for example) after their contact with an antigen. This latter is a macromolecule which, injected to a living organism, induces an antibody production. It is not the case of low molecular weight molecules such as PNA. Consequently, it is necessary to couple several PNA molecules with a protein (such as Bovin Serum Albumin, BSA) to yield an antigen. The injection of this modified protein to a rabbit induces a production of antibodies which recognize the PNA molecule.[3] The production of specific antibodies highly depends on the purity of antigen.

The antigen Bovine Serum Albumin – Para Nitro Aniline (BSA - PNA) is synthesized in vitro by reaction of several PNA molecules and aromatic amino acids of BSA. After this reaction, the antigen is in solution with various organic molecules and mineral species. Its purification is, therefore, necessary before its injection into a rabbit. Several methods of protein purification are available and the choice depends on the environment of the protein and on its concentration. Precipitation and aqueous two phase partitioning enable concentration of the protein from a crude extract and electrophoresis and chromatography to purify the protein, taking into account their physical properties.[4]

Dialysis and gel permeation chromatography separate molecules according to their sizes. These two techniques are very interesting when protein is in solution with low molecular weight species, either organic (PNA and its derivatives in this case) or mineral (buffer solution).

This paper describes and compares dialysis and gel permeation chromatography applied to the isolation of a modified serum albumin bovine (antigen) from a reaction mixture, from two points of view which are the purification time and the sample dilution.

EXPERIMENTAL

Reagents

Dialysis was carried out through a cellophane bowel of 10 cm of length and 3.2 cm of diameter (Polylabo, Strasbourg, France). Gel Permeation Chromatography (GPC) columns are pre-packed disposable columns PD-10 containing 9.1 mL of Sephadex G25 M (Pharmacia Biotech, Uppsala, Swenden). Water distillated through a Milli-Q apparatus from Millipore Corporation was used. Para-nitroaniline (PNA) and para-nitrophenol (PNP) (Rectapur quality) were purchased from Prolabo (Paris, France). Sodium nitrite, hydrochloric acid at 35.5% (*w/w*), sodium hydroxide, potassium dihydrogenphosphate, and anhydrous potassium hydrogenophosphate were RP Normapur quality and supplied by Prolabo (Paris, France). Bovine serum albumin (BSA) (Cohn Fraction V powder, 96%, *w/w*) was obtained from Sigma (Saint-Quentin Fallavier, France).

Procedure of the Protein Modification

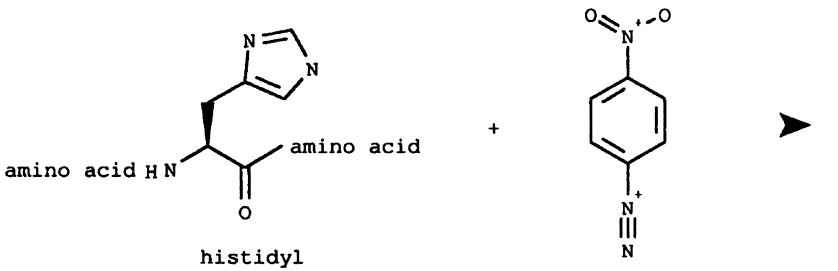
Sixty mg of PNA were dissolved in 5 mL of hydrochloride acid at 1 mol.L^{-1}. 0.5 mL of a sodium nitrite solution at 15 mg. L^{-1} were then added to the acid solution of PNA (to respect stoechiometric conditions). The mixture was stirred at 4°C for half an hour to yield the diazonium salt of PNA. 100 mg of BSA were dissolved in 5 mL of sodium hydroxide solution at 0.1 mol. L^{-1}. The diazonium salt solution was added to the protein solution, drop by drop, at 4°C, while stirring and maintaining a constant pH at 8.0 by addition of a sodium hydroxide solution at 1 mol. L^{-1}. A brown coloration developed in the reaction mixture. The final volume of the reaction mixture containing the antigen was 11 mL and the antigen concentration before purification was 9.09 g/L (100 mg in 11 mL) (assuming the derivatization does not have a significant effect on the molecular weight of the protein).

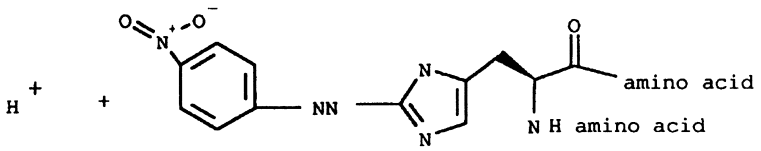
Theoretical Background

Two parameters have been used to evaluate the performances of both methods. The hourly yield (g/h) is defined as the ratio of the recovered quantity to the experimental duration. The volumetric yield (g/l) is defined as the final concentration of the purified antigen solution.

RESULTS AND DISCUSSION

The studied protein proceeded from the reaction between the diazonium salt of PNA and the protein amino acids, such as tyrosyl, tryptophanyl or histidyl groups as shown below:





At the end of the reaction, the modified protein was in an aqueous solution with various species. They are mineral species, such as Cl^-, Na^+, nitrite (necessary to organic reaction), and organic species such as PNA which have not reacted, Para nitrophenol (PNP) which is yielded by hydrolysis of the diazonium salt of PNA did not react with the protein according to:[5]

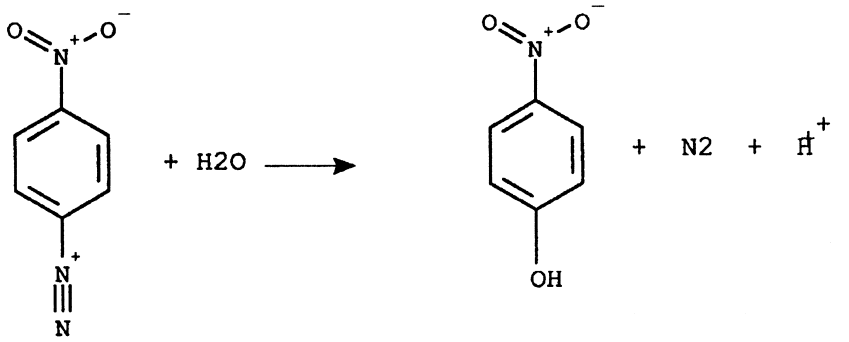


Figure 1 shows the reference UV and visible spectra of PNA (A) and PNP (B) plotted with Pye Unicam SP 8-100 spectrophotometer (Thermo Optek, Trappes, France). The absorption maxima are 386 nm and 340 nm for, respectively, PNA and PNP.

After the purification, the antigen will be alone in solution, separated according to the low molecular weight species mentioned above. As pure antigen is not available commercially, its reference UV and visible spectrum will be plotted after dialysis purification.

Dialysis

After the organic reaction, the 11 mL of the reaction mixture were introduced into a cellophane bowel against distillated water and kept at + 4°C. Then, the first dialysis water was collected. It was an aqueous solution of PNA and its derivatives. A UV and visible spectrum was plotted. Its absorption maximum was 340 nm while that of PNA is 386 nm. As a matter of fact, the spectrum with a maximum at 340 nm corresponds to the one of PNP. The majority of PNA molecules have reacted to yield diazonium salt. The whole of very unstable diazonium salt not reacted with protein have been hydrolyzed by water.

Dialysis water was changed several times per day during five days until its absorbance at 340 nm was lower than 0.01 (detection limit of apparatus). In this case, PNP and the other species of low molecular weight were no longer in the

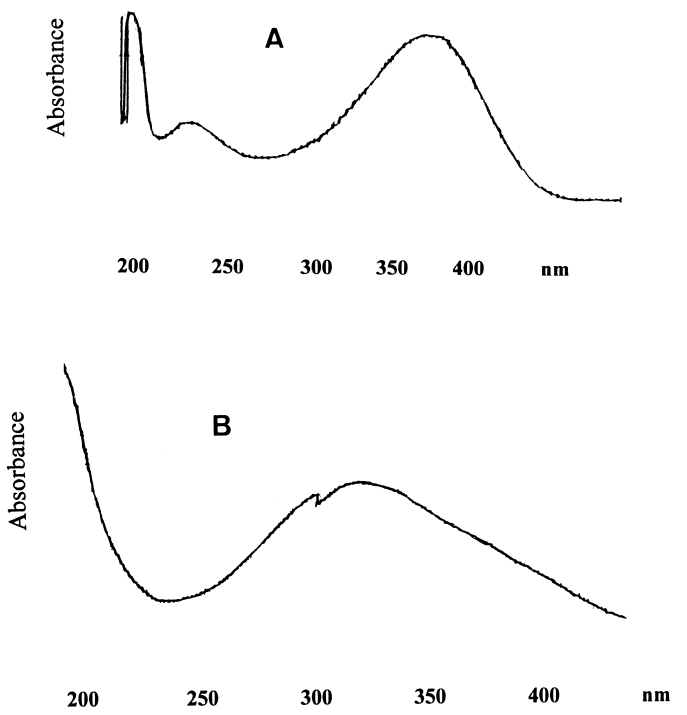


Figure 1. Reference UV and visible spectra of PNA (A) and PNP (B) between 200 and 400 nm, 0.5 AUFS.

protein solution. Then a last dialysis was accomplished against phosphate buffer 0.1 mol. L^{-1} at pH 7.0 because, the pure antigen has to be dissolved in a phosphate buffer at 0.1 mol. L^{-1} before being injected into a rabbit.

Nine point four mL of the dialysis liquid containing antigen in phosphate buffer were collected and centrifuged to eliminate possible solid particles. It is a pure solution of antigen in phosphate buffer which is characterized by its UV and visible spectrum (Figure 2). It exhibits two maxima, one of which is characteristic of the protein at 276 nm.

After the dialysis, the concentration of the antigen and the hourly yield were respectively 10.64 g/L (100mg in 9.4 mL) and 0.83 mg/h (100mg in 5*24 h).

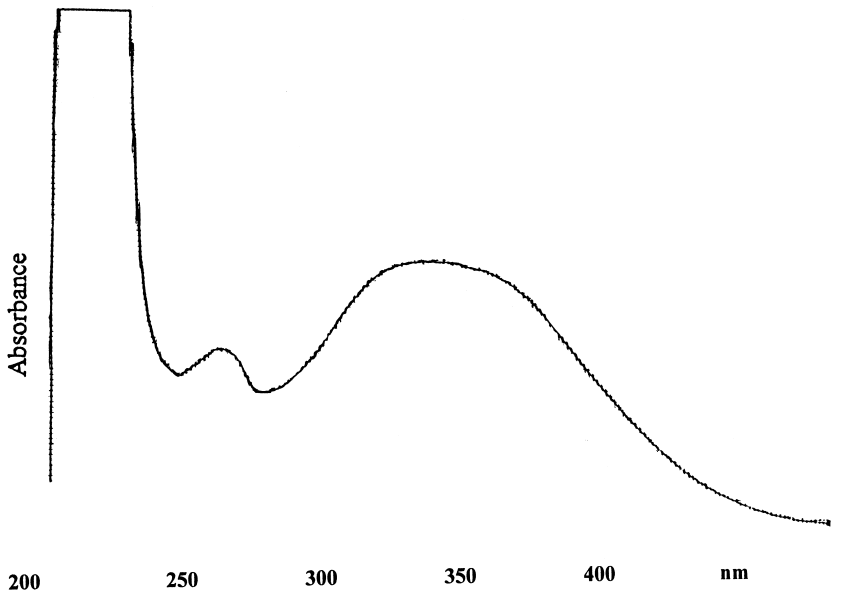


Figure 2. UV and visible spectrum of liquid contained in the bowel after dialysis between 200 and 400 nm, 1 AUFS.

Gel Permeation Chromatography

Antigen can be isolated by using gel permeation chromatography. Indeed, the species in the reaction mixture present very different molecular weights: 138 Dalton for PNP and 700000 Dalton for the modified protein.

Sephadex 25 M excludes solutes with molecular weights higher than 5000 D, which are eluted at hold up volume. It elutes solutes without separation at the total permeation volume if their molecular weights are lower than 1000 D. The modified protein should consequently be eluted first at the hold up volume, while PNA and the various salts should be eluted at the total permeation volume.

The dead volume of these columns is 2.5 mL. Therefore, the protein could be purified only if the sample volume did not exceed this value. The volume of the sample used for one purification by GPC was consequently four times smaller than the one used in the dialysis case.

At the end of the experiment the antigen has to be in a phosphate buffer at 0.1 mol. L^{-1} at pH 7.0, so that the column of gel permeation was equilibrated by

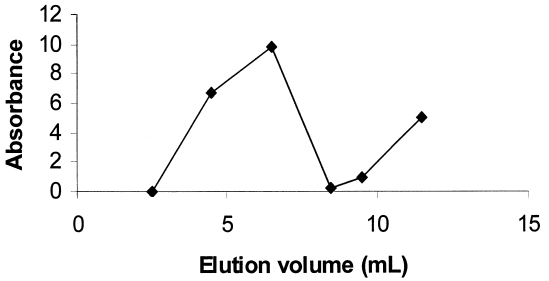


Figure 3. Reconstituted chromatogram after the analysis of fractions collected during the gel permeation chromatography. Absorbance at 276 nm versus the elution volume (mL).

25 mL of phosphate buffer solution before the application of 2.5 mL of the reaction mixture containing the antigen at 9.09 g/L.

Effluent was collected in fractions of 2 mL. The absorbance of each fraction was measured at 276 nm using adequate dilution if necessary. Figure 3 shows the rebuilt chromatogram.

It shows two peaks. UV and visible spectra of each fraction were plotted. The second, third and fourth fractions only contain the antigen and the fifth and sixth ones only para nitro phenol. The antigen is eluted between the 2.5 mL retention volume and the 6.5 mL one. Hence, the pure antigen was contained in a 4 mL volume of the mobile phase.

After the GPC, the concentration of the purified antigen was 6.25 g/L (25 mg in 4 mL), which is lower than the one obtained by dialysis. That is not a drawback for the procedure of the antibodies production, because the protein solution injected into the rabbit has to present a concentration of the same order of amount. Moreover, the antigen has been purified in less than one hour, contrarily to dialysis which required five days of experiment. In this case, the hourly yield was up to 25 mg/h.

In this experiment, GPC has shown its superiority compared with dialysis because it really allowed a much faster antigen purification at a concentration required for the antibodies production.

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