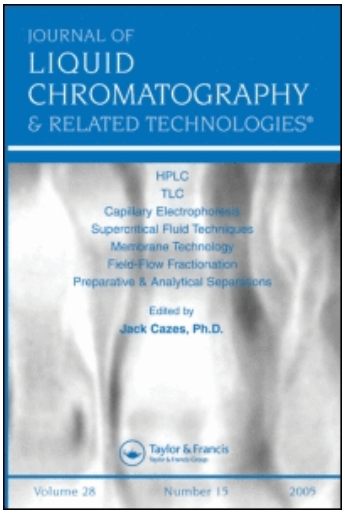

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New Conducting Polymer Affinity Chromatography Stationary Phases

G. G. Wallace[a]; K. E. Maxwell[a]; T. W. Lewis[a]; A. J. Hodgson[b]; M. J. Spencer[b]
[a] Department of Chemistry, University of Wollongong, New South Wales, Australia [b] Polymer Systems Technology Illawarra Technology Centre Northfields Avenue Wollongong, New South Wales, Australia

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NEW CONDUCTING POLYMER AFFINITY CHROMATOGRAPHY STATIONARY PHASES

G. G. WALLACE[1]*, K. E. MAXWELL[1],
T. W. LEWIS[1], A. J. HODGSON[2],
AND M. J. SPENCER[2]
[1]*Department of Chemistry*
University of Wollongong
P. O. Box 1144
Wollongong, New South Wales 2500
Australia
[2]*Polymer Systems Technology*
Illawarra Technology Centre
Northfields Avenue
Wollongong, New South Wales 2500
Australia

ABSTRACT

The use of conducting polymer stationary phases in affinity chromatography has been investigated. The application of applied potential on the antibody-antigen interaction was investigated during the trapping and elution stages.

Amino acid analysis was employed to examine the incorporation of antigen into the stationary phase. Immunoassays were used to examine the activity of the polymer when exposed to Anti-HSA.

*Author to whom correspondence should be addressed

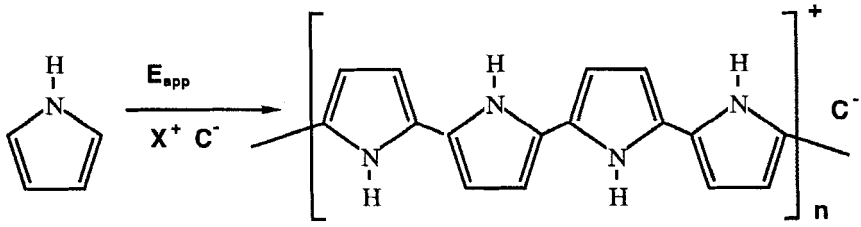
Gel electrophoresis and Immunoassays were used to verify the purity of the Anti-HSA eluted from an antigen containing polymer.

INTRODUCTION

Affinity chromatography is an accepted technique used routinely for the separation and purification of proteins (1-3).

Stationary phases employed for affinity chromatography contain a ligand capable of selectively binding to the antigen or antibody to be retained. In general the ligand is attached to a substrate in such a way that non-specific interactions are minimal and extremely high selectivities are obtained through antigen-antibody interactions. The ability to maintain high specificity is the major thrust of affinity chromatography. A further demanding criterion for affinity stationary phases is that while high affinity is required during the retention stage, elution should preferably be achieved in an eluant mild enough to ensure the proteins are not denatured.

In these laboratories we have developed new stationary phases based on electrically conducting polymers (4,5,6). These materials have been used to preconcentrate and purify analytes prior to analysis (4) and to separate components using chromatographic procedures (5,6). Conducting polymers have several properties which make them attractive for use as stationary phases (7,8). These materials are easily synthesised according to:

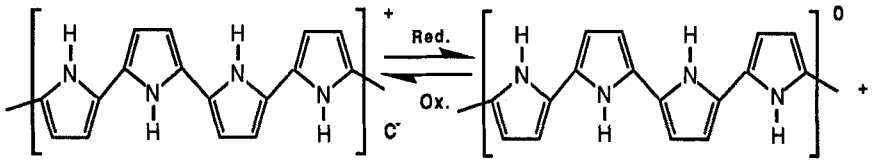


..... (1)

Therefore, retention mechanisms can be engineered into the material by the judicious choice of the monomer and/or the counterion (C^-). For example, hydrophobic or hydrophilic interaction can be encouraged by attaching selected functional groups to the monomer. Alternatively different retention mechanisms may be induced by the use of appropriate counterions (C^-). Selectivity can be encouraged by incorporating species such as metal complexing agents (9,10) or antibodies (11) as the counterion.

The above polymers are readily deposited onto a range of conductive substrates. Conventional electrode materials such as platinum, gold or glassy-carbon have been employed previously. It is also possible to coat the conducting polymer materials on to substrates for separation purposes. For example, in previous work we have coated polymers on to carbon particles (5,6) and also onto silica (9) for high performance liquid chromatography.

An additional feature of conducting polymers is the ability to change their chemical properties by the application of electrical potential. In the extreme case conducting polymers may be reversibly reduced according to:



..... (2)

This process induces marked changes in the chemical and physical properties of the material and in some cases the counterion can be released from the polymer (12,13). This property has been used to advantage in these laboratories in the fine tuning of chemical interactions on conducting polymers for chromatography (6).

In the course of this work conducting polymer stationary phases suitable for use in affinity chromatography have been

developed to determine whether such electrochemical fine tuning of interactions is an advantage with this technique. Several substrates suitable for use in separations technology have been investigated. We have used electrochemical methods, amino acid analysis and immunoassay to examine the chemical and physical properties of the prepared phases.

The ability to control the antibody-antigen interactions during the purification/separation step as well as during the elution process has been investigated. We have chosen human serum albumin (HSA) and an antibody to human serum albumin (AHSA) as a test case since these proteins are readily available.

EXPERIMENTAL

Reagents:

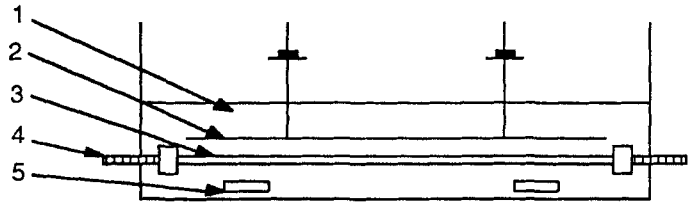
Rabbit anti human serum albumin (AHSA), human serum albumin (HSA), Ovalbumin and Tiron were obtained from the Sigma Chemical Company, U.S.A.. AHSA and HSA were dialysed extensively against distilled water before use. Disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, glycine and constant boiling (6N) HCl (Sequanal grade) were obtained from BDH Chemicals, England. Metallised gold film obtained from Sierracin Intrex was employed.

Preparation of polymer coated metallized film

Metallised films were coated galvanostatically; current densities of 1.00 uA/cm^2 were typically used.

Preparation of polymer coated fibres and column packing

Carbon fibres were stretched between two stainless steel clamps. This acted as the working electrode and was placed in a



1. GROWING SOLUTION
2. AUXILLARY ELECTRODE
3. CARBON FIBRES } WORKING ELECTRODE
4. CLAMP
5. STIRRING BAR

Figure 1 The electrochemical cell used for electropolymerisation on carbon fibres.

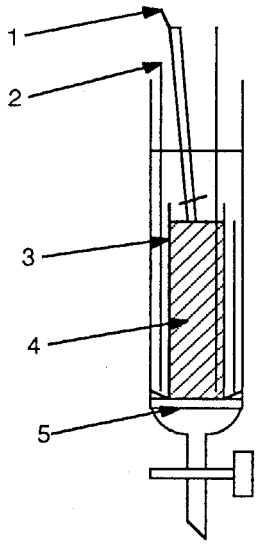
stirred solution with a stainless steel plate (auxillary electrode) suspended above the carbon fibres. A schematic of the system is shown in Figure 1. The carbon fibres were coated with the polymer using galvanostatic control, 1.54g of these coated fibres were cut into 1cm lengths and dry packed into the glass column (length = 100mm, internal diameter = 14mm).

Amino Acid Analysis

Pieces of metallised (gold) film were cut and weighed on an analytical microbalance accurate to 10μg. The polymer was then coated on the foil and it was re-weighed. The coated foil was then placed in a hydrolysis tube and hydrolysed using 6N HCl. The hydrolysate was then analysed for the amino acid content.

Instrumentation

A custom made galvanostat was used for polymer growth. Absorbance readings were obtained using a Shimadzu UV-visible



1. REFERENCE ELECTRODE
2. AUXILLARY ELECTRODE
3. FILTER PAPER
4. WORKING ELECTRODE =STATIONARY PHASE
5. FRIT

Figure 2 The electrochemically controlled chromatography (ECLC) column system

spectrophotometer and amino acid analysis carried out using an ICI Instruments Amino Acid Analyzer . Enzyme linked immunosorbent assays (ELISA) were carried out using polystyrene plates coated with HSA (10 μg/ml). Column fractions or standards (AHSA) were detected with alkaline phosphatase conjugated goat anti rabbit IgG (BioRad, Richmond CA) according to the manufacturers instructions.

An open column chromatography system amenable to electrochemical control (EC) was employed (Figure 2). Electrochemical control of the column was achieved using a custom made potentiostat. All potentials readings carried out in this work were versus a Ag/AgCl electrode. The auxillary electrode (= carbon foil) was placed around the walls of the glass column. Several layers of filter paper (Whatman 541) was used to isolate the auxillary from the working electrode. Tantalum wire was used to contact both the auxillary and working electrode to the potentiostat.

Electrophoresis was carried out using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a Pharmacia PHAST system. Silver and Coomassie brilliant blue staining were used to stain the protein fractions as described by the manufacturer. Gels were scanned using a laser densitometer (Ultrascan, LKB) .

RESULTS AND DISCUSSION

Preliminary investigations using galvanostatic growth indicated that the incorporation of HSA rather than AHSA into polypyrrole was more readily achieved. Consequently the development of HSA containing polymers to be used for the purification of AHSA was considered in detail.

1. Incorporation of antigen into conducting polymers:

Initial experiments concerned with the incorporation of antigen into the conducting polymer were carried out on metallised gold film according to the procedure outlined in the Experimental Section. The polymer forms according to equation (1) with the antigen (HSA) being incorporated as a counterion. Chronopotentiograms recorded during growth indicated that a conducting polymer was produced since the potential remained constant throughout the polymerisation. The use of the electrocatalyst Tiron had a marked affect reducing the polymerisation potential from ~ +4.00V to ~ +1.30V. Cyclic voltammograms recorded using the polymer coated film as the working electrode also indicated that a conducting material was obtained, with the electrochemical transition from the oxidised to the reduced state (equation 2) being well defined.

Amino acid analysis revealed that protein was incorporated during the electropolymerisation process. A typical amino acid analysis chromatogram is shown in Figure 3.

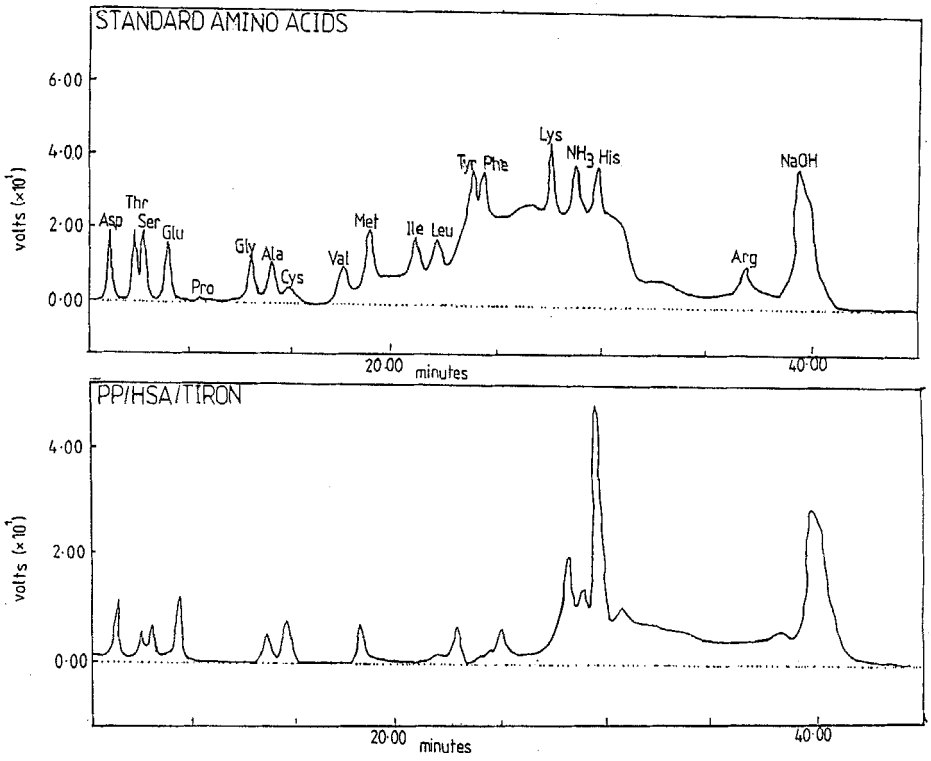


Figure 3 Amino acid analysis chromatogram for (a) the standard and (b) obtained after acid hydrolysis of the protein containing polymer grown using the following conditions.
Current density: 1 mA/cm^2
Time: 12 minutes

The variation of amino acid content with antigen concentration employed in the polymerisation process was investigated.

It was found that by increasing the concentration of antigen in the polymerisation solution up to 25 mg/mL, the amino acid

Table 1

Amino Acid Analysis (1) Data

	Reference amino acid	Mass HSA (g)	% w/w HSA/Sample
PP/HSA	glycine	6.13×10^{-6}	2.0
PP/HSA	isoleucine	7.70×10^{-6}	2.6
PP/HSA/Tiron	glycine	4.20×10^{-5}	9.3
PP/HSA/Tiron	isoleucine	4.02×10^{-5}	8.9

(1) Carried out as outlined in the EXPERIMENTAL SECTION

content of the polymer was increased. However, at levels higher than 25 mg/mL the potential required to effect polymerisation increased markedly indicating formation of a non-conducting polymer.

Using 25 mg/mL HSA in the polymerisation solution the effect of adding an electrocatalyst (Tiron) was considered. As stated previously, it was found that the potential required to induce polymerisation decreased while the amino acid content increased. Consequently Tiron was employed in all subsequent polymerisations. Amino acid analysis results are summarised in Table 1. Glycine and isoleucine were employed as markers to calculate an indication of the amount of HSA incorporated.

An ELISA was carried out on the polymer after synthesis. These assays were carried out using polymer coated metallised film in place of antigen coated polystyrene in the microtiter plate. It was shown (Table 2) that the use of Tiron increases the interaction of antibody with the HSA in the polymer.

Table 2

ELISA Data (1) - Units of Activity

	Initial Activity of AHSA Solution (U)	Final Activity per Electrode (U/electrode)	Final Activity per cm^2 of electrode (U/cm^3)
PP/HSA	2	4.0×10^{-2}	2.0×10^{-2}
PP/HSA/Tiron	2	5.3×10^{-2}	2.7×10^{-2}

(1) Carried out as outlined in the EXPERIMENTAL SECTION

Table 3

The Use of Polypyrrole-HSA for Affinity Purification

Load 1ml of 5mg/mL AHSA				
Mobile Phase	Total Units Activity (UA) Eluted(1)	Total mg protein Eluted (2)	Activity Yield (%) (3)	Specific Activity (UA/mg protein)
1. Phosphate (20 mL)	1780	3.8	36	468
2. Glycine (20 mL)	2366	1.0	47	2366

(1) Calculated from ELISA data

(2) Calculated from the absorbance reading at λ = 280 nm assuming 1 absorbance unit (AU) for 1 cm light path = 0.74 mg/mL protein.

(3) Calculated assuming initial activity = 5000 Units

Table 4

Affect of Cathodic Potential (to -0.10V vs Ag/AgCl) on the Preconcentration Stage

Load 1ml of 5 mg/mL AHSA				
Mobile Phase	Total Units Activity (UA) Eluted (1)	Total mg protein Eluted (2)	Activity Yield (%) (3)	Specific Activity (UA/mg protein)
1. Phosphate (20 mL)	2094	3.5	42	598
2. Glycine (20 mL)	1606	1.4	32	1147

(1), (2), (3) see Table 3

2. Chromatography on Conducting Polymer Coated Fibres

Polymer coated fibres were prepared and packed into an open column as described in the Experimental Section.

In order to mask non-specific chromatographic interactions the packing material was saturated with ovalbumin by soaking in a solution of 5 mg/mL and rinsed before loading the AHSA sample.

Following this masking procedure, 1mL of 5 mg/mL antibody solution was loaded onto the column with no potential applied. Eluant fractions (1.5mL) were collected and monitored using UV-

Table 5

Affect of Anodic Potential (to +0.10V vs Ag/AgCl) on the Preconcentration Stage

Load 1ml of 5 mg/mL AHSA				
Mobile Phase	Total Units Activity (UA) Eluted (1)	Total mg protein Eluted (2)	Activity Yield (%) (3)	Specific Activity (UA/mg protein)
1. Phosphate (20 mL)	1313	3.9	26	337
2. Glycine (20 mL)	3563	1.5	71	3563

(1), (2), (3) See Table 3

visible spectrophotometry (λ = 280 nm) to determine the total protein content. In addition the ELISA was used to determine antibody activities in the fractions collected. Following the loading of the antibody and washing of the column elutions were carried out using glycine-HCl buffer (pH = 2.1). It was found that 36% of the original AHSA activity was eluted during the loading stage (see Table 3) with 3.8 mg of protein. Glycine then eluted 47% of the original antibody activity with only 1 mg of protein. Specific activity of the glycine eluted fraction was 2366 compared with an initial value of 1000. This indicated that the antigen containing conducting polymer stationary phase, acted as an affinity support and that purification could indeed be achieved. Recovery of the starting material was 85% during the purification procedure.

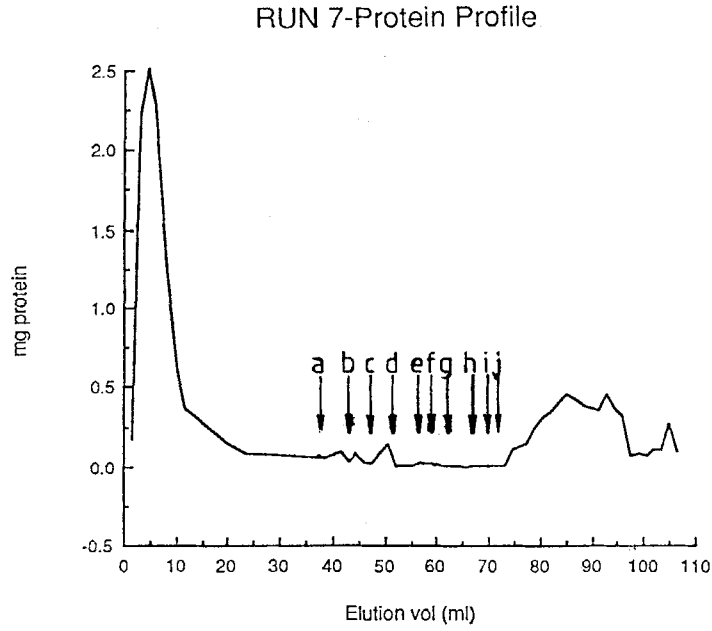
Table 6

Affect of Applied Potential (Eapp) on Antibody Elution

Load 1ml of 20 mg/mL AHSA				
Mobile Phase	Total Units Activity (1)	Total mg protein Eluted (2)	Activity Yield (%) (3)	Specific Activity (UA/mg protein)
Phosphate (20 mL)	8906	12.1	45	736
Eapp = -0.02V (5 mL)	4.5	0.04	< 1	113
Eapp = -0.10V (5 mL)	-	0.4	-	-
Eapp = -0.04V (5 mL)	4.1	0.01	< 1	410
Glycine (20 mL)	5151	5.8	26	888

(1), (2), (3) See Table 3

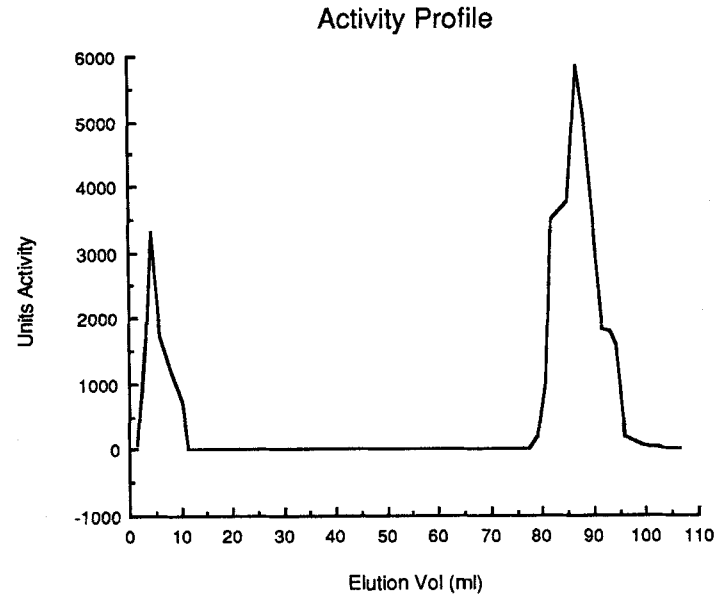
The effect of applied potential on the protein retention stage was then investigated. Application of a cathodic potential (Table 4) had little effect on total protein retention and a detrimental effect on the purification, decreasing both the total activity yield and the specific activity of the antibody. At these potentials polypyrrole is less porous and more hydrophobic (7). The effect of applying more negative potentials than -0.10V vs Ag/AgCl was not investigated in this part of the work.



a= 0.00V
b= -0.02V
c= -0.04V
d= -0.06V
e= -0.08V
f= -0.1V
g= -0.04V
h= 0.00V
i= OFF
j= 0.1M glycine-HCl pH 2.1

Figure 4 Elution profile obtained with ECLC elution [Details Table 6]

(continued)



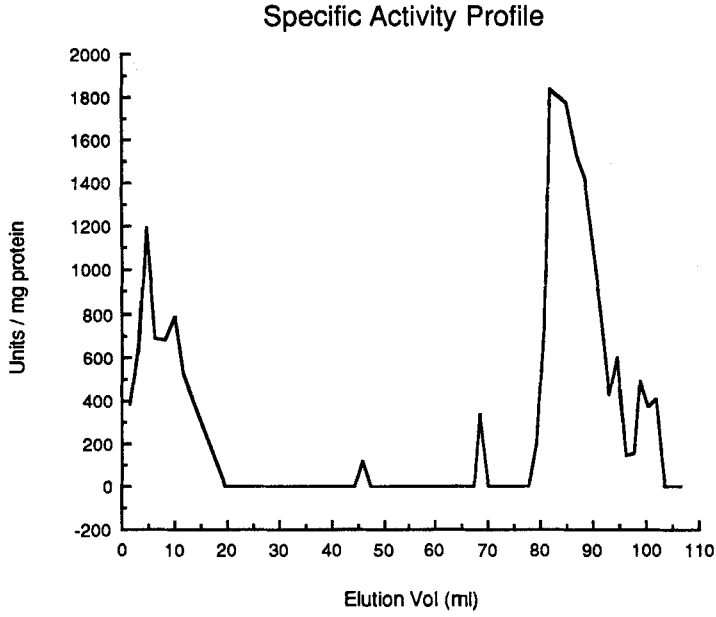


Figure 4 (continued)

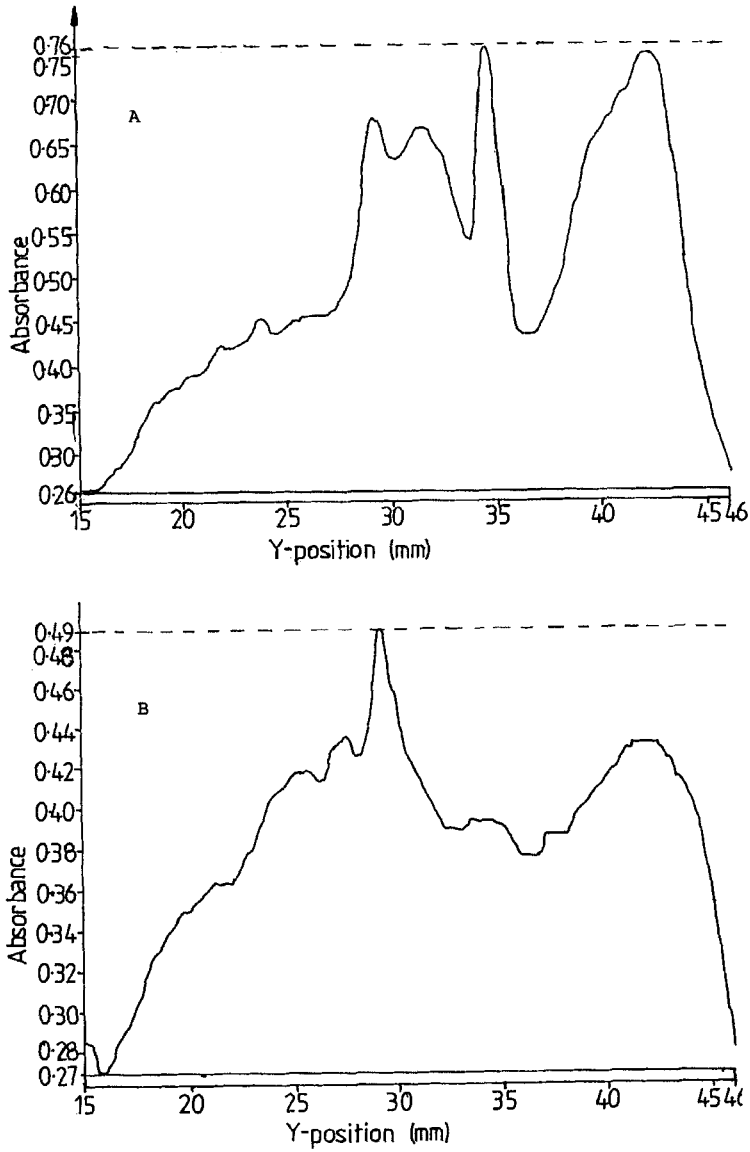
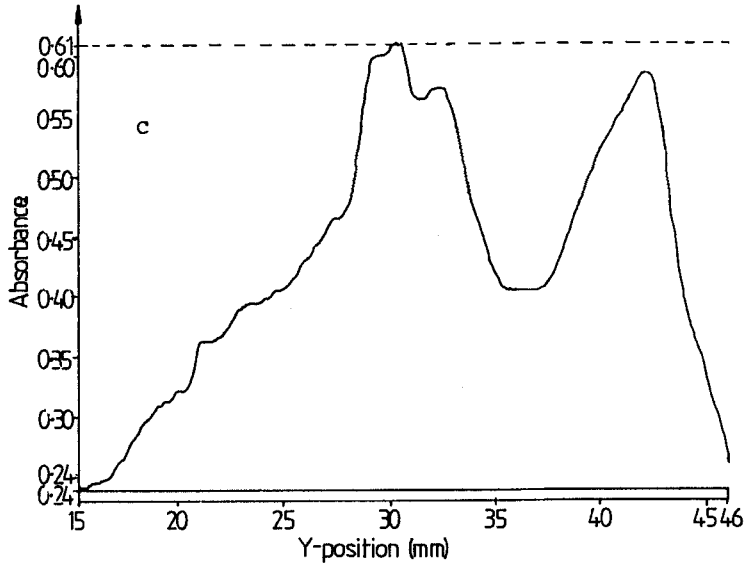


Figure 5 Gel electrophoresis data

a) Crude AHSA
b) Unretained protein fraction
c) EC elution (-0.06V vs Ag/AgCl)
d) Glycine elution after EC elution
e) Glycine elution (no previous EC elution)

(continued)



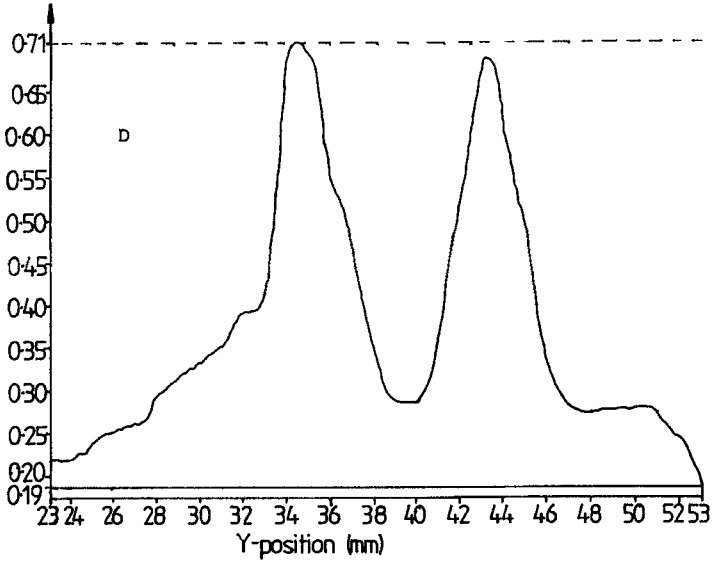


Figure 5 (continued)

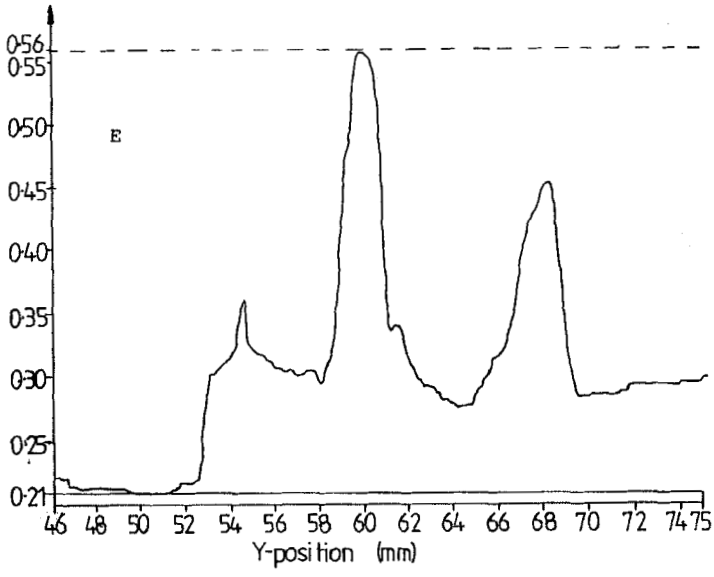


Figure 5 (continued)

Application of an anodic potential (Table 5) had little effect on the total amount of protein retained, however, selectivity was improved. This fact was borne out by the specific activities attainable in the glycine wash. In addition, the recovery of material was much higher (95-97%) for purification with application of an anodic potential. At these potentials polypyrrole is more porous and less hydrophobic.

Initial experiments that involved loading 1mL of 5 mg/mL AHSA solution did not show any EC elution upon the application of potential. Consequently a more concentrated solution (1mL of 20 mg/mL AHSA) was loaded on to the column. In this case a greater amount of total protein was retained. Results are summarised in Table 6 and also in Figure 4. It was found that with applied potentials of less than 0.00V, small amounts of antibody could be eluted with a phosphate buffer. It was found that by returning the

polymer to less negative potentials (0.00V) vs Ag/Ag Cl and then returning to the cathodic region, small amounts of antibody could be eluted. It is well known that the application of negative potentials will release materials from conducting polymers (12,13). However, once the polymer becomes insulating further release becomes difficult. In this work an additional problem arose due to the fact that at more negative potentials the polymer backbone itself becomes more hydrophobic thus preventing efficient electrochemical elution of proteins. Elution at more negative potentials, down to -0.40V, without returning to positive potentials did not improve the efficiency of EC elution. Following EC elution it was found that glycine buffer could be used to remove the remaining trapped antibody.

The data in Table 6 indicated that application of potentials even to only -0.10V caused an irreversible loss of substantial amounts of AHSA (total yield dropped to 70%).

During the EC elution experiment (down to -0.10V) fractions were collected and their components separated using SDS-PAGE. The Ultrascan traces of various separated fractions are shown in Figure 5. The traces show that with both EC and glycine elution only the antibody and not the antigen was removed from the conducting polymer based column. It also confirmed that these elution methods give fractions more pure than the starting material.

CONCLUSIONS

It has been shown that the preparation of affinity chromatographic stationary phases based on conducting polymers is feasible.

The use of applied potential to affect both the trapping and elution stages has been demonstrated and future work will be concerned with the refinement of EC controlled affinity chromatography.

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