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Detection of fungal carbohydrate antigens by high-performance immunoaffinity chromatography using a protein A column with covalently linked immunoglobulin G

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ABSTRACT

Fungal carbohydrate antigens were analysed by high-performance immunoaffinity chromatography (HPIAC) with immunoglobulin G (IgG) antibodies raised against extracellular polysaccharides of *Mucor racemosus*. The protein A–IgG complex was covalently bound with dimethyl pimelimidate, which enabled the use of strong acidic buffers to release the tightly bound antigens from the column. Prior to pulsed-amperometric detection, an anion-micromembrane suppressor was used to raise the pH of the effluent to above 12 without dilution. The HPIAC system provides a sophisticated method for the rapid and sensitive detection of antigenic oligomeric carbohydrates in biological samples and is proposed as an alternative to quantitative enzyme-linked immunosorbent assay techniques.

INTRODUCTION

Antibodies are widely used for affinity separation of mixtures of oligosaccharides or polysaccharides containing the target antigen [1–6]. Conventional methods of immobilizing immunoglobulin G (IgG) antibodies as ligands on solid matrices usually employ non-specific antibody–protein coupling to activated Sepharose or macroporous silica. This binding reduces the efficiency of the antibody–antigen interaction be-

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cause of the multisite attachment and orientation of the immunoglobulin molecule [7,8]. The introduction of the complex of *Staphylococcus* protein A coupled to Sepharose beads, which specifically binds non-covalently to the Fc fragment of IgG molecules, allows optimal spatial orientation of the paratopes of the IgG and therefore maximum antigen-binding efficiency [9].

The combined use of immunoaffinity chromatography and high-performance liquid chromatography (HPLC) technology has been proposed by Ohlson *et al.* [7], and is known as high-performance immunoaffinity chromatography (HPIAC), as a further refinement of the more general high-performance liquid affinity chromatography (HPLAC) [10,11]. Potentially, this approach can be used in developing biosensors for rapid chromatographic monitoring of bioprocesses such as downstream processing and fermentations [12]. Many immunochemically active oligosaccharides can be separated and detected successfully with immunoaffinity columns of antibodies coupled to tresyl-activated silica combined with HPLC [13,14]. Improvement in sensitivity was obtained by Wang *et al.* [15], who introduced the use of pulsed-amperometric detection (PAD) to this system, which allows detection of 10 ng of oligosaccharides separated with these immunoaffinity columns.

Moulds belonging to different genera of the order of Mucorales are distributed worldwide and are important in foods, causing many cases of food spoilage [16]. Medically, some species are able to cause mucormycosis in man [16]. These Mucoralean moulds excrete mixtures of polysaccharides with a common antigenic carbohydrate determinant [17,18]. Antigenic activity of fungal polysaccharides is often based on the presence of carbohydrate epitopes of three to six sugar residues linked to extracellular polysaccharides (EPSs), as demonstrated recently for EPSs from *Penicillium* and *Aspergillus* species [19,20]. To elucidate the structure of the epitopes of the EPSs produced by species belonging to the order Mucorales, many polysaccharide and oligosaccharide fractions, either isolated or synthesized, had to be tested for their immunochemical activity towards IgG antibodies raised against the EPSs of *Mucor racemosus*. To this end, series of α-linked fucose and mannose oligomers and combinations thereof were synthesized [21,22]. As a sandwich enzyme-linked immunosorbent assay (ELISA) requires the presence of at least two epitopes on one molecule, this is an inappropriate assay for testing oligosaccharides and small polysaccharides. With other ELISA techniques it is difficult to test microgram quantities of carbohydrates for their immunological properties. Therefore, other, more sensitive methods are necessary to test these fractions immunochemically.

In this study, the preparation of an HPIAC column from polyclonal IgG antibodies raised against EPSs of *Mucor racemosus* is described. The Fc fragments of the IgG antibodies were coupled to a HiTrap protein A column recently available from Pharmacia (Uppsala, Sweden). The complex of the IgG and protein A was linked covalently using dimethyl pimelimidate (DMP). This enables the use of strong acidic buffers to elute the tightly bound antigens from the HPIAC column without eluting the IgG antibodies. The oligosaccharides eluted from this column were detected with pulsed-amperometric detection.

EXPERIMENTAL

Extracellular polysaccharides

EPSs from the strains *Mucor racemosus* CBS 222.81, *Mucor circinelloides* RIVM M 40, *Rhizopus oryzae* LU 581, *Rhizomucor pusillus* CBS 432.78, *Absidia corymbifera* LU 017 and *Syncephalastrum racemosum* CBS 443.59 used in this study were produced, isolated and purified by ethanol precipitation as previously described [17].

Preparation of the protein A immunoaffinity column

A HiTrap protein A column (Pharmacia–LKB, Uppsala, Sweden) containing 1 ml of Sepharose high-performance beads with approximately 3 mg of protein A (derived from *Staphylococcus aureus)* was washed with 20 ml of 0.1 *M* sodium borate buffer (pH 8.2) using a pump with

a flow of approximately 0.3 ml/min at 4°C. Lyophilized polyclonal rabbit antibodies (4.3 mg; IgG 1000/1201) raised against EPSs of *Mucor racemosus* [18] were dissolved in 1 ml of 0.1 *M* sodium borate buffer (pH 8.2) and pumped slowly through the column in 1 h, after which the column was washed with an excess (10 ml) of sodium borate buffer. The effluent was collected in fractions of 1 ml, which were all tested for protein content with the Sedmak assay [23] to determine any unbound IgG. Only 65 μg of protein could be detected in the first fraction, corresponding to 1.5% of the IgG protein applied to the column. The column was rinsed with 10 ml of 0.2 *M* triethanolamine (pH 8.2). The IgG was covalently bound to the column by rinsing the column with 20 ml (0.3 ml/min) of 50 m*M* DMP·2HCl (dimethyl ester of heptane diimidic acid; Pierce, Rockford, IL, USA, freshly made up in 0.2 *M* triethanolamine with the pH readjusted to 8.2 [9]. The column was rinsed with 10 ml of 50 m*M* ethanolamine (pH 8.2) and finally with 10 ml of 0.1 *M* sodium borate buffer of the same pH. The column was tested several times by rinsing with 20 ml of 0.1 *M* sodium citrate–sodium hydroxide buffer (pH 2.0). Only after the first washing was a minor amount (60 μg) protein released from the column. The HPIAC column was stored in 0.1 *M* sodium borate buffer (pH 8.2) supplemented with 0.02% sodium azide as an antimicrobial agent.

HPIAC system

HPIAC was carried out using a Dionex BioLC HPLC system (Sunnyvale, CA, USA) with a pulsed-amperometric detector equipped with a gold working electrode and an Ag/AgCl reference electrode. The system was run at 20°C with a flow-rate of 0.2 ml/min using 50 m*M* sodium borate buffer (pH 8.0) and 50 m*M* sodium citrate–sodium hydroxide buffer (pH 2.0) as eluents, prepared from distilled water filtered in a Nanopure II system (Sybron/Barnstead, Boston, MA, USA). In order to decrease the pulse noise from the HPLC pump, a restrictor was connected between the pump and the injection valve, providing a working pressure of 40–50 bar. After automatic injection of 20 μl of a saccharide sample (1 mg/ml) using an SP 8880 autosampler (Spectra-Physics, San Jose, CA, USA), elution was performed with 4 ml of sodium borate buffer (pH 8.2), followed by 4 ml of sodium citrate buffer (pH 2.0) and then another 4 ml of sodium borate buffer. Prior to pulsed-amperometric detection, a solution of 1.5 *M* sodium hydroxide was used to increase the pH of the effluent above 12 by means of an anion-micromembrane suppressor (AMMS II; Dionex) at a flow-rate of 4.0 ml/min using a separate pump [24].

Sandwich ELISA for the detection of EPSs

To determine the ELISA activity of the various fractions obtained after separation with the HPIAC column, the effluent was partitioned in fractions of 0.2 ml (1 min) in a separate experiment using a Bio-Rad 2110 fraction collector after disconnecting the PAD detector. The antigenic activity of these fractions was determined using the sandwich ELISA as previously described [17], in wells of polyvinyl chloride microtitre plates (Dynatech, Chantilly, VA, USA). The IgG fraction (1000/1201) used for the preparation of the HPIAC column was also used in this ELISA. An aliquot (100 μl) containing 10 μg/ml rabbit IgG anti *M. racemosus* EPS in 0.07 *M* sodium phosphate buffer (pH 7.2) containing 0.15 *M* sodium chloride (phosphate-buffered saline, PBS) was added to each well and incubated overnight at room temperature. After rinsing with PBS containing 0.05% Tween 20 (PBS-T), 60-μl portions of samples diluted with 40 μl of 0.15 *M* PBS-T (pH 7.5) were added and incubated for 90 min at room temperature. After another rinse with PBS-T the wells were incubated with rabbit IgG (anti *M. racemosus*), conjugated to horseradish peroxidase, for 90 min. Finally, the rinsed wells were incubated at room temperature for 10 min with 100 μl of a substrate solution. The substrate solution was 3,3′,5,5′-tetramethylbenzidine in dimethyl sulphoxide (DMSO), prepared according to Bos *et al.* [25]. Just before use, 7 μl of 30% hydrogen peroxide were added to 100 ml of the substrate solution. The enzyme reaction was stopped by adding 50 μl of 2 *M* sulphuric acid to each well. The absorption of the yellow colour

was measured at 450 nm using an EAR 400 spectrophotometer (SLT, Groedig, Austria).

RESULTS

Preparation and analysis conditions of the HPIAC column

The IgG antibodies were bound covalently to the protein A column with the use of DMP with a yield of more than 95%. Only the first washing after the covalent coupling with a strong acidic buffer released some IgG protein from the HPIAC column, representing 1.4% of the initial amount. Further elution of the column with sodium citrate buffer (pH 2.0) did not release any protein.

The binding and the subsequent release of the antigenic compounds from the HPIAC column in relation to the pH and the flow-rate of the eluents were studied by ELISA detection of the effluent. Complete binding of the antigenic components of the EPSs of *M. racemosus* could be achieved with a flow-rate of 0.2 ml/min of the sodium borate buffer (pH 8.2), as no antigenic material could be detected by ELISA in the non-binding fraction (Fig. 1). Higher flow-rates also resulted in the presence of antigenic material in the effluent at the initial pH, indicating incomplete binding of the antigenic material. The influence of the enlarged size of the immobilized ligand as a result of cross-linking, which is likely to increasingly invoke steric hindrance, was not studied in detail.

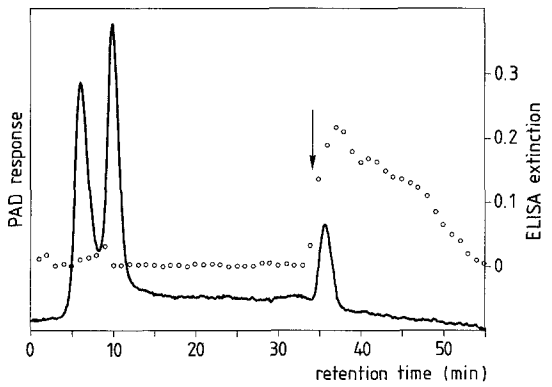

Fig. 1. High-performance immunoaffinity chromatogram of 20 μg of a preparation of extracellular polysaccharides derived from the mould *Mucor racemosus*. The binding antigenic fraction was eluted from the column with 50 m*M* sodium citrate buffer (pH 2) (arrow). Solid line, response of the pulsed-amperometric detector; ○, ELISA absorption of the different fractions at 450 nm.

Release of antigenic material could only be obtained by lowering the pH of the eluent by at least 2 pH units, which was determined by using different buffers with various pH values in the range 8 to 2 (results not shown). An optimal release of the binding antigenic material in this HPIAC system was obtained by using a sodium citrate–sodium hydroxide buffer (pH 2). As shown in Fig. 1, in which the start of the elution with this buffer is indicated with an arrow, the antigenic material could be released completely from the column in 4 ml of this buffer. Lower pH values were not tested to prevent any damage to the IgG antibodies covalently linked to the HPIAC column. Successful PAD of the carbohydrate material in the effluent of the HPIAC column requires a pH of more than 12. This was obtained by post-column addition of OH^- by the use of an anion-micromembrane suppressor [24]. As the capacity of the suppressor was limited, the maximum flow-rate of the 50 m*M* sodium citrate buffer (pH 2) was 0.2 ml/min. However, exact quantification of the antigenically active material was difficult because of the unknown responses of the PAD detector towards these polysaccharides. Using these elution conditions the column exhibited negligible loss of performance after analysis of more than 200 samples during a period of one year.

Detection of fungal antigenic extracellular polysaccharides

Preparations of EPSs derived from various mould species from five different genera belonging to the order Mucorales were analysed with HPIAC with IgG antibodies raised against the EPSs of *M. racemosus*. As illustrated in Figs. 1 and 2, the preparations tested contained an antigenic fraction that was able to bind to this column. ELISA reactivity (tested in separate experiments as described; results not shown) could only be determined in the peaks with a retention time of around 36 min, representing the HPIAC-binding fractions. The non-binding material eluted in

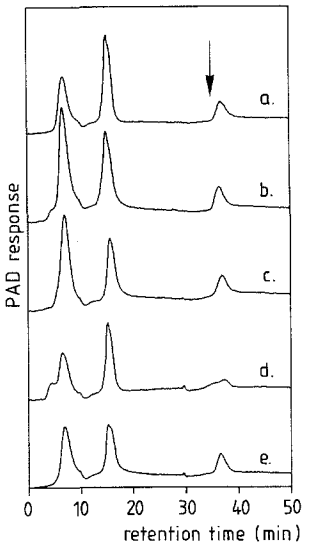

Fig. 2. High-performance immunoaffinity chromatograms of 20 μg of preparations from extracellular polysaccharides from the Mucoralean moulds *Mucor circinelloides* (a), *Rhizopus oryzae* (b), *Rhizomucor pusillus* (c), *Absidia corymbifera* (d) and *Syncephalastrum racemosum* (e). The arrow indicates the start of the elution with the sodium citrate buffer (pH 2).

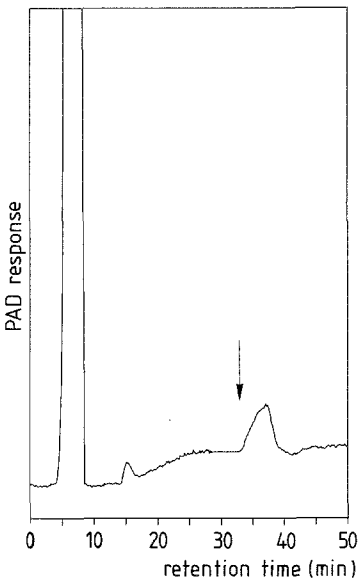

Fig. 3. High-performance immunoaffinity chromatogram of 20 μg of α(1–2) tetramer of D-mannopyranoside. The binding fraction was eluted from the column with 50 m*M* sodium citrate buffer (pH 2) (arrow).

two fractions from the HPIAC column after rinsing with sodium borate buffer (pH 8.2). This column, prepared from 4.3 mg of IgG, showed a linear relationship between peak area and the amount of injected antigenic polysaccharides between 50 and 1000 ng. Based on the peak areas after pulsed-amperometric detection, the binding fractions represent 10–15% of the total amount of EPSs.

HPIAC analysis of oligosaccharides

The characteristics of the HPIAC column used in this study were carefully determined with antigenic polysaccharides and subsequent ELISA detection as described above. Assuming a similar performance of this HPIAC column towards antigenic oligosaccharides, many synthesized carbohydrate oligomers were tested immunochemically with this column.

Oligomers of α(1–2)-, α(1–3)- and α(1–4)-linked L-fucose residues were synthesized using the iodonium ion-assisted stereospecific glycosylation procedure as recently described [21]. Also, α(1–6)- and α(1–2)-linked D-mannopyranoside oligomers, α-D-man-(1–4)-α-L-fuc-O-Me and α-L-fuc-(1–2)-α-D-man-O-Me were synthesized using the same procedure [22]. As shown in Fig. 3, the α(1–2)-tetramer of D-mannopyranoside was able to bind partly to this column. The binding capacity of the other oligomers tested in this study was negligible.

DISCUSSION

An HPIAC column was prepared from a protein A column with IgG antibodies raised against EPSs of *M. racemosus*. As the binding between the paratopes of the antibodies and the epitopes of the antigenic polymers was found to be very strong, covalent binding of the IgG–protein A complex of the column was necessary to be able to use strong acidic buffers to release the antigens from the column after binding. Stabilization of the complex of protein A and the Fc fragment of the IgG through covalent binding with DMP was possible with an efficiency of more than 95%.

The resulting HPIAC column with optimal spatial orientation of the IgG paratopes could be used to separate the antigenic polysaccharides completely. Repeated experiments revealed reproducible peak areas of the binding fraction. However, there is no evidence that these circumstances provide a maximized stoichiometry of the interaction between antibody and antigen. This HPIAC column can easily be incorporated into any HPLC system, providing considerable improvements compared with HPIAC columns described previously [11,15].

Electrochemical detection of the effluent using a PAD detector requires alkalization of the effluent prior to detection. Previously, this was performed by the addition of a solution of sodium hydroxide to the effluent [15], which resulted in a two-fold greater dilution of the effluent. To avoid this problem, an anion-micromembrane suppressor was introduced, enabling alkalization of the effluent without dilution [24]. The sensitivity and reproducibility of the HPIAC system was increased considerably as a result of this.

The use of Sepharose beads as a matrix of the HPIAC column also resulted in partial separation of the non-binding fractions (Figs. 1 and 2). As the molecular mass of these polysaccharides is known to be less than 100 kDa [17], the doublet near the void cannot originate from size-exclusion effects of this Sepharose-based column. It is known that the EPSs of Mucorales consist of a mixture of neutral polysaccharides and negatively charged polysaccharides containing glucuronic acid [17]. Therefore, this phenomenon can probably be explained by assuming some weak interaction with the glucuronic acid-containing polysaccharides present in these preparations.

The analysis of several EPS preparations of various moulds belonging to Mucorales revealed a similar antigenic fraction. This fraction, representing 10–15% of the initial polysaccharides, could be separated reproducibly from the non-antigenic material. This strongly suggests that the epitopes of Mucorales are not randomly distributed among the excreted polysaccharides, but are located only on specific polysaccharides that are a minor fraction of the total amount excreted. Previously, it was established that these antigenic polysaccharides of Mucorales have a unimodal molecular weight distribution with an average molecular mass of 30 kDa [17].

Immunochemical analysis of oligomers was possible on microgram quantities, which is approximately ten times less than the amounts needed for testing these oligomers with conventional ELISA techniques [16,19]. The range of sensitivity of this column can be improved by increasing the amount of IgG coupled to the column. As the α-L-fucose oligomers, the dimer of α(1–6)-linked mannopyranose, oligomers and α-D-man-(1–4)-α-L-fuc-O-Me and α-L-fuc-(1–2)-α-D-man-O-Me did not bind to this column, it can be assumed that they play no role in the antigenicity of the EPSs of Mucorales.

Miyazaki *et al.* [26] suggested that α(1–6) mannose residues are the antigenic determinants of Mucoralean polysaccharides, but our results do not confirm this suggestion. Some binding of the α(1–2)-linked D-mannopyranoside tetramers was found with this HPIAC system, indicating a role for these mannose residues in the epitopes of EPSs of Mucorales. However, the binding was incomplete and it is therefore unlikely that the Mucoralean epitopes are composed of nothing else than α(1–2)-linked D-mannopyranoside sequences as suggested by Yamada *et al.* [27].

In conclusion, the HPIAC column described in this study can be considered as an important improvement of the HPIAC carbohydrate oligomeric antigens, and can be used as an alternative to ELISA techniques, allowing ten-fold lower concentrations of antigens to be detected. Potentially, such immunochromatographic biosensor systems can be used to monitor the level of almost any carbohydrate against which a suitable antibody can be raised [12,28].

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