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Purification of murine immunoglobulin E (IgE) by thiophilic interaction chromatography (TIC)

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Abstract

In addition to their known implication in allergy studies, IgE antibodies are becoming an increasingly interesting antibody class in cancer research. However, large-scale purification of IgE antibodies still poses substantial challenges, as they cannot be purified using techniques commonly used for other immunoglobulins such as protein A or protein G chromatography. Here, we have developed and optimized a gentle and simple IgE purification method based on thiophilic interaction chromatography (TIC). IgE binds to the thiophilic resin in presence of 1.2M ammonium sulfate and is eluted in low salt concentration. Monomericity of purified antibodies ranged between 54 and 73%. Preparative size-exclusion chromatography was thereafter performed to further improve the purity, which reached >95% in the final product. The overall recovery was around 30%. The purification method was tested on both hybridoma-produced and recombinantly produced IgE antibodies with reproducible results. In addition, the antigen binding activity of purified IgE antibodies was preserved, as shown by binding ELISA. Purification by TIC is cheap, gentle in terms of pH to preserve IgE folding and function, and universal as any IgE antibody can be purified irrespective of the species of origin or affinity. Potentially, it could be used for purification of other antibody isotypes as well, when gentle conditions are required.

Graphical abstract

Keywords: IgE, purification, thiophilic interaction chromatography (TIC), thiophilic adsorption chromatography

Abbreviations:

CE-SDS, Capillary electrophoresis sodium dodecyl sulfate; OVA, ovalbumin; Prep SEC, Preparative size-exclusion chromatography; UPLC-SEC, Ultra-high performance liquid chromatography-size exclusion chromatography;

1. Introduction

Antibodies are widely used in research, diagnostics and therapeutic settings and while the IgG class is the most common isotype, the interest in other antibody classes is rising. Among them, IgE antibodies have been found to play an important role in allergy and parasitic infections, but are also being investigated as potential anti-cancer therapy¹. The first clinical trial of an IgE antibody in oncology settings is ongoing (NCT02546921) due to its superior efficacy in ovarian cancer models compared to its IgG₁ counterpart². Therefore, given the increasing interest in IgE antibodies, there is also a need for a robust and reliable IgE purification method.

Purification is an important step during antibody production. Ideally, it is cost-effective, yields a pure product with high recovery and preserves biological activity of the antibody. Most commonly used methodologies such as protein G or protein A chromatography cannot be used for IgE purification, because neither the protein G nor protein A bind to IgE antibodies³. Several other techniques have been described for IgE purification, such as ammonium sulfate precipitation⁴, hydrophobic charge induction with MEP (Mercapto-Ethyl-Pyridine) HyperCel sorbent⁵ and affinity chromatography with either anti-human IgE antibody⁶, antigen or HiTrap KappaSelect resin⁷. However, all of them have certain disadvantages as they may require elution under low pH conditions (MEP HyperCel, KappaSelect) or can only be used for specific IgE antibodies (affinity-based methods). For example, KappaSelect used for the purification of the IgE which is currently in clinical trial⁷ can only capture antibodies with kappa light chain.

Thiophilic interaction chromatography (TIC) was first described in 1985⁸, and has been successfully used for IgG, IgM and IgY purification⁹. It is based on the principle that immunoglobulins interact with the ligand that contains a sulfone group proximal to a thioether group in the presence of high lyotropic salt concentration. After this salt-promoted adsorption, the immunoglobulins are eluted in low salt concentration^{8,9}. This method offers certain advantages, such as elution under neutral pH conditions which reduces aggregate formation and preserves biological activity of the antibody⁹ and, in addition, the possibility to purify any IgE antibody irrespective of species or antigen affinity. Due to the impossibility to use protein A or protein G based methods and considering the above-mentioned advantages of TIC, we decided to develop and optimise a TIC method for IgE purification.

Optimisation included various factors including the salt concentration that affects the strength of resin-ligand interaction, as well as the column size and sample loading flow rate. The last

two determine the residence time of the sample in the column, i.e. the duration of interaction between the antibody and the resin. The optimised purification method was then employed to purify murine IgE from hybridoma supernatant. Finally, the method was employed to purify murine IgE antibodies that were produced in a recombinant production system. To our knowledge this is the first report on thiophilic interaction chromatography for IgE purification.

2. Materials and methods

2.1 Cell culture

2C6 hybridoma cells were kindly provided by Prof. Lester Kobzik (Harvard) and were grown in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Biowest), 1% penicillin/streptomycin (Gibco), 50 μ M 2-mercaptoethanol (Gibco), 1% HT supplement (Gibco) and 1% serum-holding T24-CM (in house supernatant from T24 cells containing IL-6). FreeStyle™ 293 cells (Invitrogen) were grown in FreeStyle™ 293 Expression Medium (Invitrogen). CHO.K1 cells (ATCC Cat# CCL-61) were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco), supplemented with 10% Fetal Calf Serum (Biowest) and 1% penicillin/streptomycin (Gibco). ExpiCHO-S™ cells (Gibco) were grown in ExpiCHO™ Expression Medium (Gibco). Culture conditions were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

2.2 Antibody production by hybridoma

Anti-OVA IgE antibodies were obtained from 2C6 hybridoma. In brief, the cells were seeded to shaking erlenmeyer flask with the density of 5x10⁵ cells/ml and cultured for 7 days in hybridoma serum free culture medium (H-SFM, Gibco) with 1% penicillin/streptomycin and 1% serum-free T24-CM at 37°C, 5% CO₂ and 80 rpm. 7 days post-transfection, the cell suspension was collected and centrifuged for 10 min at 2700 g. The supernatants were filtered over a 0.22 μ m filter and stored at 4°C.

2.3 Recombinant antibody production

The OX7 hybridoma (ECACC Acc.No 84112008), which secretes anti-Thy1.1 IgG antibody, was sequenced to determine the heavy and light chain variable domains, respectively (LakePharma). Next, those sequences were used to engineer chimeric mIgE and mIgG1 heavy and light chains. The DNA constructs encoding for anti-Thy1.1 heavy and light chains were *de novo* synthesized (GeneArt). Anti-Thy1.1 IgE and IgG1 were recombinantly produced in FreeStyle293 cells and ExpiCHO-S™ cells, respectively. Briefly, cells were transfected with pcDNA3.1.(+) expression vectors encoding corresponding heavy and light chains (1:1 ratio), using 293fectin reagent (Invitrogen) or ExpiFectamine (Gibco) according to the manufacturer's recommendation, respectively. The cells were incubated for 7 days at 37°C, 8% CO₂ at 120 rpm or 90 rpm, respectively. 7 days post-transfection, the cell suspension was collected and centrifuged for 15 min at 2500 g. The supernatants were filtered over a 0.22 μ m filter and stored at 4°C.

2.4 Purification of anti-Thy1.1 IgG1

The Expi-CHO-S™ supernatant was mixed with a pre-determined amount of MabSelect SuRe LX resin (GE Lifesciences) and rotated overnight at 4°C. Following overnight capturing, the bound antibody was purified from the resin by affinity chromatography using Pierce™ Centrifuge Columns (ThermoFisher Scientific) and re-buffered to PBS using PD-10 Desalting Columns (GE healthcare) according to the manufacturer's instructions.

2.5 Sandwich ELISA for IgE quantification

96-well MaxiSorp plate (ThermoFisher Scientific) was coated with 0.15 µg/ml of goat anti-mouse IgE in PBS (Southern Biotech, 100 µL/well) overnight at 4°C. Blocking was done with 1% BSA in PBS for 1 h at 37°C. Next, supernatants containing IgE antibodies were added in serial dilutions. Initially, before serial dilutions, 2C6 hybridoma supernatant was diluted 4x, whereas FreeStyle293 supernatant containing anti-Thy1.1. IgE was initially diluted 2x with cell culture medium. Mouse IgG1 kappa was used as negative control (clone P3.6.2.8.1). Mouse IgE (Bio-Rad cat# PMP68) was used for the standard curve. The incubation was performed at room temperature for 1 h. Finally, goat anti-mouse IgE-HRP conjugate (Southern Biotech, 1:4000 in 1:1 1% BSA PBS/PBST) was added for 45 min at room temperature. Immunoreactivity was visualized with TMB Stabilized Chromogen (Invitrogen). The reaction was stopped after 15 min with 0.5 M H₂SO₄ and absorbances were read at 450 nm and 620 nm.

2.6 Thiophilic interaction chromatography

The required amount of salt was added gradually to the supernatant containing IgE antibodies to avoid precipitation. The samples were put on a tube roller until the salt was completely dissolved. Samples were then filtered through a 0.22 µm filter to prevent clogging of the resin. Column purification was performed using an ÄKTA pure system (GE LifeSciences). The thiophilic resin packed column (G Biosciences cat# 786-268, 6% highly cross-linked agarose as support) was equilibrated with equilibration buffer (20 mM sodium phosphate, 1.2 M sodium sulfate, pH 7.5), after which the previously prepared sample was loaded. The column was then washed with equilibration buffer until the absorbance reached baseline. The isocratic elution was performed using 20 mM sodium phosphate, pH 7.5. The collection was done starting at >50 mAU and ending with <50 mAU. The column was regenerated with 20mM sodium phosphate, 30% propan-1-ol, pH 7.5 and stored in 20% ethanol. Antibody concentration was quantified using NanoDrop spectrophotometer at 280 nm (extinction coefficient 1.6) and the samples were stored at 4°C.

2.7 Ultra-performance size-exclusion liquid chromatography (UPLC-SEC)

If necessary, the samples were concentrated prior to the analysis with Microsep Advance 10K centrifugal device (Pall Corporation). Centrifugation was performed at 3000 g for 10-20 min. Monomericity of purified antibodies was determined using an ACQUITY UPLC Protein BEH

SEC column (Waters, Cat# 186005225) with Acquity H-class Bio system. Separations were carried out in 50 mM Sodium Phosphate, 0.2 M Sodium Chloride pH 7.

2.8 Preparative size-exclusion chromatography (prep SEC)

The samples were concentrated by using Microsep Advance 10K centrifugal device (Pall Corporation) to a final volume of 6-7ml. Prep-SEC based fractioning was done using a HiLoad 26/600 Superdex 200pg column (GE28-9893-36, V=319ml, d=26mm, h=60cm) using an ÄKTA pure system. Equilibration was done overnight with PBS for 2 column volumes. The sample was loaded onto the column with a velocity of 25 cm/h (2.212 mL/min). The antibody was eluted with PBS and collected in 2ml fractions. Concentrations were quantified by spectrophotometry using NanoDrop and fractions were analyzed on UPLC-SEC as described above.

2.9 SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2µg of each antibody was diluted in Laemmli Sample Buffer (Bio-Rad) for analysis under non-reducing conditions or Laemmli Sample Buffer containing 10% β-mercaptoethanol for analysis under reducing conditions. Samples were heated either at 95°C for 5 min (reduced) or at 90°C for 2 min (non-reduced). Samples and EZ-Run™ Prestained Rec Protein Ladder (Fisher BioReagents) were subjected to either 10% polyacrylamide gel at 120 V (reduced samples) or 8% polyacrylamide gel at 100 V (non-reduced samples). Gels were stained using GelCode™ Blue Safe Protein Stain (Thermo Scientific), fixed for 30 min in buffer containing 40 % ethanol and 10% acetic acid, destained with distilled water and scanned using ChemiDoc™ Touch Imaging System (Bio-Rad).

2.10 Capillary electrophoresis sodium dodecyl sulfate (CE-SDS)

The purity of IgE antibodies was tested by Capillary electrophoresis sodium dodecyl sulfate (CE-SDS). CE-SDS analysis was carried out on a CE system PA800 Plus machine (Beckman Coulter). Samples were diluted to 1mg/mL with 10 kDa internal standard and iodoacetamide in SDS-MW sample buffer and heated to 70°C for 10 min. 95 µL were transferred into sample vials and loaded into the machine. Separations were performed in a bare-fused silica 50µm I.D capillary at 22°C. Effective separation length was 20cm, run time 40 min and antibody fragments detected at a wavelength of 220nm. The capillary was flushed with 0.1 M HCl, then 1 M NaOH, then water and run buffer before sample loading at 5 kV for 20 sec. Noise was recorded for 3 min from the run buffer. Data analysis was carried out using 32Karat software (version 9.2).

2.11 OVA binding ELISA

96-well MaxiSorp plate (ThermoFisher Scientific) was coated with 5 µg/ml of rabbit polyclonal anti-OVA in PBS (EMD Millipore, 100 µL/well) overnight at 4°C. Blocking was done with 1% BSA in PBS for 1 h at 37°C. Next, PBS (blank) or 0.08-10 µg/ml of chicken

ovalbumin (Sigma A5503) were added. The incubation was performed at room temperature for 1 h. Next, purified anti-OVA IgE was added and the plate was incubated for 1 h at room temperature. Finally, goat anti-mouse IgE-HRP conjugate (Southern Biotech, 1:4000 in 1:1 1% BSA PBS/PBST) was added for 45 min at room temperature. Immunoreactivity was visualized with TMB Stabilized Chromogen (Invitrogen). The reaction was stopped after 15 min with 0.5M H₂SO₄ and absorbances were read at 450 nm and 620 nm.

2.12 Thy1.1 transient transfection and cell ELISA

24 µg of pcDNA3.1.(+)-Thy1.1 plasmid was transfected into CHO.K1 cells (10 mm Petri dish, 80% confluent) using the lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendation. The following day, cells were plated into a 96-well plate (50000 cells/well). Two days after transfection binding ELISA was performed. The cell supernatant was discarded and either anti-Thy1.1 IgE or IgG1 were added in serial dilutions. The incubation was performed at room temperature for 1h. Next, goat anti-mouse IgE-HRP conjugate (Southern Biotech, 1:4000) or goat anti-mouse IgG Fc-HRP (Jackson Immuno Research 1:5000) in 1:1 1% BSA PBS/PBST were added for 45 min at room temperature. Immunoreactivity was visualized with TMB Stabilized Chromogen (Invitrogen). Reactions were stopped after 15 min with 0.5M H₂SO₄ and absorbances were read at 450 nm and 620 nm.

3. Results

3.1 Optimisation of salt concentration and residence time for murine IgE purification by TIC

In order to optimise factors affecting the binding of IgE antibodies to thiophilic resin, 2C6 hybridoma supernatant at a concentration of about 128 mg/L of anti-OVA mIgE antibody was used as starting material (Fig.S1A). Maximal antibody recovery rate was determined at an ammonium sulfate concentration of 1.2 M and a residence time of at least 10 minutes; leading to a maximal yield of about 61% (Table 1).

Table 1. Purification optimisation and IgE recovery

Supernatant volume	Column length	Column diameter	(NH ₄) ₂ SO ₄ conc.	Column loading flow rate	Residence time	Recovered Ab	Estimated yield
50ml	4.2cm	0.5cm	0.8M	158cm/h	1.6min	0.76mg	12%
25ml	15.8cm	0.5cm	1.2M	170cm/h	5.6min	1.44mg	45%
25ml	15.8cm	0.5cm	2M	170cm/h	5.6min	1.04mg	32.4%
25ml	15.8cm	0.5cm	1.2M	91.7cm/h	10.34min	1.96mg	61%

3.2 Monomericity of purified IgE

The monomericity of anti-OVA IgE antibody purified under four different conditions (Table 1) was analysed using UPLC-SEC (Fig. 1A-D). Consistent peak patterns were observed across

samples with the main peak retention time around 3.4 minutes and a monomericity ranging between 54 and 61%, while the aggregates levels were under 5.2% in most samples.

3.3 Scaled up purification of IgE antibodies produced by hybridoma or in a recombinant production system

Once the purification conditions were optimised, larger scale IgE purification was established. As starting material, 770 ml of the same 2C6 hybridoma supernatant was used. A bigger column with diameter of 1 cm was packed to a height of 19.1 cm and residence time of 17.65 minutes. A recovery rate of 59.3% (Table 2) and a monomericity of 73% (Fig. 2A) were achieved. In order to obtain even higher purity, preparative size-exclusion chromatography was performed (prep SEC). To this end, samples were eluted in 2 ml fractions and six fractions at the borders of the main peak (three in front and three in back) were analysed by UPLC-SEC (Fig. 2B). After pooling the monomeric fractions (fractions 23-31), a monomericity of 98.8% was achieved (Fig. 2C). 30.4 mg of highly pure IgE antibody was obtained as final product, constituting an overall recovery rate of 31% (Table 2).

In addition, TIC purification method was tested for recombinantly produced IgE antibodies. To this end, anti-Thy1.1 IgE antibodies recombinantly produced in FreeStyle293 cells with an estimated concentration of about 222 mg/L in the cell supernatant (Fig. S1B) were loaded to the column (d=1cm, h=20.8cm). Primary eluted sample showed a similar peak pattern as samples obtained from hybridoma, yielding a recovery rate of 65% and a monomericity of 55% (Fig. 2D). After prepSEC, the monomericity was improved to 97.7% (Fig. 2E), yielding an overall recovery rate of 27% (Table 2).

Table 2. Recovery rates of purified IgE antibodies produced by hybridoma or recombinantly

	Step	Sample description	Volume [ml]	A280 (protein conc.)	IgE conc. [mg/ml]	Total IgE [mg]	IgE conc. ELISA [mg/l]	Total IgE [mg]	Yield [%]
hybridoma	Supernatant	Anti-OVA IgE	770				128	98,56	100
	Thiophilic resin	Purified IgE (elution)	17	5,51	3,44	58,5			59,3
	Prep SEC	Final product	16	3,04	1,9	30,4			30,8
FreeStyle293	Supernatant	Anti-Thy1.1 IgE	300				222	66,6	100
	Thiophilic resin	Purified IgE (elution)	22	3,13	1,95	42,9			64,4
	Prep SEC	Final product	19	1,5	0,94	17,86			26,8

3.4 Purified IgE antibodies reach high purity and show expected molecular weight and conserved antigen binding activity

Molecular weight of purified antibodies was assessed by SDS-PAGE analysis. Anti-Thy1.1 IgG1 antibody was included for comparison purposes. Under reducing conditions, bands

corresponding to heavy (about 95kDa for IgE and 55kDa for IgG₁) and light chains (28-30kDa) were detected (Figure 3A). The molecular sizes are within expected range for IgE antibodies¹⁰. Under non-reducing conditions, both IgE antibodies showed the same molecular size, whereas IgG₁ antibody was slightly smaller (Figure 3B). No free heavy or light chain were detected, indicating that they are properly assembled into complete antibodies. The purity of final IgE products was tested on CE-SDS. No fragmentation was observed and the purity of both IgE antibodies was above 99% (Fig. 3C,D). Antigen binding activity of the anti-OVA and anti-Thy1.1 IgE antibodies was preserved as shown by binding ELISA with chicken ovalbumin and Thy1.1 expressing CHO cells, respectively (Fig. 3E,F). No difference in binding activity was observed between anti-Thy1.1 IgE and IgG₁, indicating that Ig format and purification method do not affect the binding to the antigen.

4. Discussion

With an increasing medical interest in IgE antibodies, there is also an increasing need for a cheap, simple and efficient purification method. Here, we report development and optimisation of IgE purification by thiophilic interaction chromatography.

UPLC-SEC data showed that the obtained antibodies were 54-73% monomeric, with the presence of several small molecular weight peaks. This indicates that the thiophilic resin might not be as specific for immunoglobulins and that other proteins are being captured as well. Therefore, preparative SEC was required as additional purification step in order to improve monomericity and purity to >95%, which was set as the lower limit. If even higher monomericity is required, this could be achieved by an additional ion exchange step or, alternatively, by selecting exclusively monomeric fractions after preparative SEC.

For IgE purification on industrial scale with large mass loading, preparative SEC may pose certain limitations as very large columns would be required. Therefore, more scalable methods such as adsorption method may be more suitable. In addition, the need for an additional purification step such as preparative SEC might be avoided by further optimisation of the elution step of TIC. Here we performed isocratic elution, but longer gradient elution with decreasing ammonium sulfate concentration might better separate the impurities and provide an improved result and will, therefore, be the subject of further studies.

The overall IgE recovery from initial supernatant was around 30%. However, it must be taken into consideration that ELISA was used for IgE quantification in the supernatant. This method has limited accuracy and can only provide an estimation of actual antibody concentration. The concentration of antibodies after purification was measured spectrophotometrically with high accuracy based on the absorbance at 280nm. Further method optimisation in terms of column cross-section and loading settings may increase the recovery rate.

Furthermore, the purification method was tested on recombinantly produced IgE antibody. Recombinant antibody production offers a wide range of antibody designing possibilities and

is becoming increasingly important. Our method showed consistent and reproducible results for both hybridoma produced and for recombinantly produced IgE antibodies. In addition, the conserved functionality of purified antibodies was confirmed in binding ELISA.

In conclusion, we have successfully purified murine IgE antibodies by thiophilic interaction chromatography. This method has a broad diversity and can be applied to any IgE irrespective of its specificity, light chain isotype or production system used. Although not tested with human IgE, given the nature of the interaction between the thiophilic resin and immunoglobulins, we expect this method to be applicable to human IgE as well. Potentially, thiophilic interaction chromatography could be given advantage over protein G or protein A based purification methods for other antibody isotypes as well, when gentle conditions in terms of pH are required.

Declaration of Interest

None.

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Figure 1. UPLC-SEC profiles of IgE samples purified by thiophilic chromatography under different conditions. (A) 0.8 M $(\text{NH}_4)_2\text{SO}_4$, retention time 1.6 min; (B) 1.2 M $(\text{NH}_4)_2\text{SO}_4$, retention time 5.6 min; (C) 2 M $(\text{NH}_4)_2\text{SO}_4$, retention time 5.6 min; (D) 1.2 M $(\text{NH}_4)_2\text{SO}_4$, retention time 10.3 min. Percentages of aggregates and monomeric antibodies are shown.

Figure 2. Large scale purification of IgE antibodies produced by hybridoma and recombinant production system. UPLC-SEC profiles of (A) anti-OVA IgE antibody produced by 2C6 hybridoma and purified with thiophilic chromatography; (B) fractions of interest after prep SEC of purified anti-OVA IgE; (C) final anti-OVA IgE sample after the fractions 23-31 obtained by prep SEC were pooled together; (D) recombinantly produced anti-Thy1.1 IgE antibody and purified with thiophilic chromatography (before prep SEC); (E) recombinantly produced anti-Thy1.1 IgE antibody after prep SEC; Percentages of monomeric fraction are shown on chromatograms.

Figure 3. Characterization of purified IgE antibodies. SDS-PAGE analysis of purified antibodies under (A) reducing and (B) non-reducing conditions; CE-SDS analysis of (C) anti-OVA IgE; (D) anti-Thy1.1 IgE; (E) binding ELISA of anti-OVA IgE; (F) binding ELISA of anti-Thy1.1 IgE and IgG1

Supplementary figure 1. ELISA quantification of IgE antibodies in the cell supernatant. (A) anti-OVA IgE in the supernatant of 2C6 hybridoma; (B) recombinant anti-Thy1.1 IgE in the supernatant of FreeStyle 293 cells; Commercial mIgE was used for the standard curve, whereas mIgG1 was used as negative control, both starting at 5 μ g/mL.