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High throughput screening of mixed-mode sorbents and optimisation using pre-packed lab-scale columns for the purification of the recombinant allergen rBet v 1a☆

Virginie Brenac Brochier[a,*], Henri Chabre[b], Aurélie Lautrette[b], Vincent Ravault[a], Marie-Noëlle Couret[b], Alain Didierlaurent[b], Philippe Moingeon[b]

[a] Pall Life Sciences, 48, avenue des Genottes, 95800 Cergy St Christophe, France
[b] Stallergènes, 6, rue Alexis de Tocqueville, 92183 Antony, France

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ABSTRACT

Mixed-mode chromatography was investigated for the purification of the recombinant allergen rBet v 1a expressed in *Escherichia coli* (*E. coli*) and used as an active principle for specific immunotherapy (SIT) treatment against birch pollen allergy. The screening of micro-volumes of three mixed-mode sorbents established that rBet v 1a could be captured without any pre-treatment of the crude feedstock on HEA or PPA HyperCel sorbents equilibrated in "physiological-like" conditions. On a mini-column pre-packed with PPA HyperCel sorbent, rBet v 1a was recovered at pH 4, partially separated from a major methionine Bet v 1 contaminant and purified approximately 9-fold in a single step (85% purity).

1. Introduction

More than 95% of tree pollen allergic patients exhibit IgEs against the birch pollen major allergen Bet v 1, and up to 60% of these patients react solely to Bet v 1. Specific immunotherapy (SIT) based mainly on sublingual approaches uses natural products (i.e. extract from birch pollen) as main sources of the allergen [1]. A recombinant allergen approach has been developed for SIT using a recombinant allergen Bet v 1 (rBet v 1a isoform) expressed in *Escherichia coli* (*E. coli*) and presenting a natural conformation [2,3]. Following sub-cutaneous immunotherapy, this allergen was shown to be clinically effective in patients with allergic rhinitis [4]. The production of recombinant pharmaceutical grade Bet v 1a requires a process allowing large scale purification of a well characterized molecule. During the scale-up to reach commercial batch size, the occurrence of a N-ter methionine Bet v 1 impurity form resulting from the accumulation of uncleaved Bet v 1 was identified at the cell culture step [5]. The aim of the present study was to investigate an alternative purification process using mixed-mode chromatography to capture rBet v 1a and to eliminate the methionine Bet v 1 contaminant.

The development of the purification step was carried out using a high-throughput (HT) platform. HT platforms for screening multiple chromatography sorbents and operating conditions have recently emerged to help overcome the bottleneck of protein purification [6–9]. These platforms are based on small scale batch-chromatography experiments run in parallel in a 96-well format. Binding and elution characteristics of target and impurity proteins are screened across ranges of operating parameters including buffer formulation, pH, conductivity, displacer concentrations, etc.. . . In the present study, the HT platform was based on a robotic assisted sorbent screening combined with surface enhanced laser desorption ionisation–mass spectrometry (SELDI-MS), which has been previously proposed as a fast and sensitive analytical tool for HT analysis for process development [10–12].

A family of three mixed-mode sorbents was investigated for the purification step of rBet v 1a: HEA HyperCel™ (hexyl amine), PPA HyperCel (phenyl propyl amine) and MEP HyperCel (mercapto ethyl pyridine). Ligands of HyperCel mixed-mode sorbents are designed to provide effective binding in the absence of lyotropic or other

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* Corresponding author. Tel.: +33 134 207 829; fax: +33 134 207 878.
E-mail address: virginie_brochier@europe.pall.com (V. Brenac Brochier).

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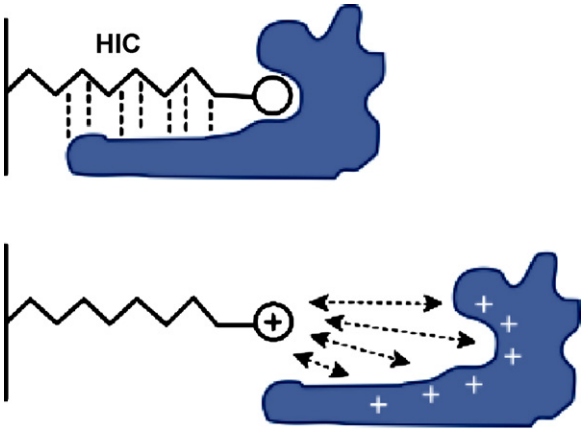

Fig. 1. Schematic of adsorption and desorption mechanisms for HyperCel mixed-mode chromatography sorbents.

salts and effective desorption based on charge repulsion as pH is reduced (Fig. 1) [13–15]. These ligands include a hydrophobic tail and an ionizable headgroup: an aromatic pyridine ring (MEP) and aliphatic (HEA) or aromatic (PPA) rings which contain amines. At physiological pH, they are uncharged and hydrophobic. Additional contributions to binding are provided by an aliphatic spacer arm. For MEP HyperCel, antibody binding is further enhanced by interaction with a thioether group. When the pH of the mobile phase is adjusted to values below the pKa of the ligand and the isoelectric point of the target molecule(s), both the ligand and the target molecule(s) take on a distinct positive charge, inducing electrostatic repulsion.

These three sorbents are intended to purify proteins at high yield and recovery under low to moderate salt concentrations and to offer orthogonal selectivities to other standard chromatography techniques. Based on multi-modal interactions, mixed-mode sorbents may not lead to an easy prediction of protein binding and desorption conditions, therefore, HT screening appeared especially indicated here.

When applicable to the mixed-mode sorbents investigated, the chromatography step was optimized by transfer to pre-packed mini-columns to provide fast and reproducible results for the comparison of the main chromatographic variables [16].

The combination of HT mini-batch screening platform and pre-packed mini-columns for the development and optimization of a chromatography step is discussed here, as well as the advantages of mixed-mode chromatography for the purification of the recombinant allergen rBet v 1a from a crude *E. coli* feedstock.

2. Materials and methods

2.1. Chemicals and equipment

Batches F905 of HEA HyperCel™, F904 of PPA HyperCel and H266 of MEP HyperCel chromatography sorbents were supplied by Pall life Sciences (Pall BioSepra Cergy, France). HyperCel media are constituted of beads of high porosity cross-linked cellulose with an average particle size of 90 μm and a particle distribution of 70–110 μm. Synthetic ligands are hexyl amine for HEA HyperCel and phenyl propyl amine for PPA HyperCel and the ligand density is 50–95 μmol/mL of sorbent. For MEP HyperCel, the synthetic ligand is mercapto ethyl pyridine and the ligand density is 70–125 μmol/mL of sorbent. AcroWell™ 96-well filter plates with a 0.45 μm hydrophilic polypropylene (GHP) pore-membrane and 350 μL wells were provided by Pall Life Sciences (Saint Germain-en-Laye, France). Automation of microplate preparation was done using a liquid handling Biomek® 2000 Robot (Beckman Coulter, Villepinte, France) including a DPC Micromix® 5 Titer Plate Shaker.

Pre-packed resolute (PRC) columns of 5 mm internal diameter (I.D.) and 50 mm height (h) supplied by Pall Life Sciences contained 1 mL of HEA HyperCel (F901), PPA HyperCel (F902) or MEP HyperCel (G206) sorbent.

Buffers were prepared using chemicals of analytical grade from Sigma–Aldrich (Saint Quentin Fallavier, France) and ultrapure water. Buffers and sample were filtered using Supor® 200 0.2 μm 47 mm membrane filters and Acrodisc® 25 mm PF syringe filters, respectively. Liquid chromatography was performed on an ÄKTA Explorer 100 system (GE HealthCare, Uppsala, Sweden).

SELDI-MS analyses were performed using a ProteinChip® PCS4000 Reader, NP20 ProteinChip Arrays and sinapinic acid (SPA) from BioRad (Marnes La Coquette, France). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed using 10% pre-cast Novex® tricine gels (1 mm thick) in a XCell SureLock™ mini-cell apparatus and gel staining, using Simply Blue™ safe stain from Invitrogen (Cergy Pontoise, France).

The total protein concentration (TPC) was determined using a Coomassie® plus protein assay reagent kit from interchim (Montluçon, France). Size Exclusion Chromatography (SEC) analyses were performed using a TSK-gel® 3000SW column from Tosoh Bioscience (Stuttgart, Germany) and a high performance liquid chromatography (HPLC) system from Waters (Saint Quentin en Yvelines, France).

2.2. Biological materials

A crude feedstock of *E. coli* lysate at 3 mg/mL of TPC and expressing rBet v 1a was produced and 50 mL obtained for analyses. The fermentation process was based on an *E. coli* strain that expressed rBet v 1a in the cell cytoplasm after induction by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture medium. From a working cell bank (WCB) vial, initial cultures were made using shake flasks and medium fermentor. The main fermentation was a continuous fed-batch process in a 1500 L fermentor (37 °C) where a concentrated glucose solution was continuously added. When the main culture reached an absorbance of 75 ± 5 units at 600 nm (A600), the production of rBet v 1a was induced by addition of IPTG to a final concentration of 0.1 mM for 8–12 h. When A600 nm reached 110 ± 10 units, bacteria were harvested by centrifugation. Cells were then resuspended in 20 mM Tris–HCl, pH 8.0. The homogeneous suspension was passed twice through a high pressure homogenizer to ensure that the entire product was released from the cells. The homogenate was clarified using a 750 kDa hollow fiber filter to remove components, to prevent clogging the chromatographic column in the subsequent purification step. Lastly, the product was concentrated using standard tangential flow filtration (TFF), diafiltrated against 20 mM Tris–HCl, pH 8.0 and filtered at 0.2 μm.

Reference samples included two batches of rBet v 1a at 3 mg/mL TPC purified using the existing process (not communicated) and a sample containing approximately 28% of the N methionine rBet v 1a impurity were provided by Stallergènes.

2.3. HT mixed-mode chromatography sorbent screening

2 mL of each sorbent were re-suspended in approximately 10 mL of equilibration buffer (phosphate buffered saline, pH 7.4 or 20 mM Na phosphate, 1 M NaCl, pH 7.0) and agitated to get a homogeneous suspension. The slurry was centrifuged for 5 min at 2000 rpm to decant the sorbent. The supernatant was discarded and the process was repeated three times. Part of the supernatant was removed to get a final slurry concentration of 50% (v/v) in equilibration buffer.