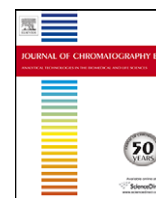




Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb



Review

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[☆]

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ARTICLE INFO

Article history:

Received 17 November 2008

Accepted 16 March 2009

Available online xxx

Keywords:

Recombinant allergen

rBet v 1a

High-throughput screening

Mixed-mode sorbents

HEA HyperCel

PPA HyperCel

MEP HyperCel

Pre-packed columns

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).

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Contents

1. Introduction.....	00
2. Materials and methods.....	00
2.1. Chemicals and equipment.....	00
2.2. Biological materials.....	00
2.3. HT mixed-mode chromatography sorbent screening.....	00
2.4. Liquid chromatography using pre-packed columns.....	00
2.5. Analysis of chromatography fractions using SELDI-MS.....	00
2.6. Analysis of chromatography fractions using SDS-PAGE.....	00
2.7. Measurement of total protein content (TPC).....	00
2.8. Evaluation of the purity of chromatography fractions by size exclusion chromatography (SEC).....	00
3. Results and discussion.....	00
3.1. Detection of rBet v 1a using SELDI-MS.....	00
3.2. HT mixed-mode chromatography sorbent screening.....	00
3.3. Optimization of the capture step on mixed-mode sorbents using pre-packed columns.....	00
4. Conclusion.....	00
References.....	00

[☆] This paper was presented at the 11th International Symposium on Biochromatography and Nanoseparations, Montpellier, France, 14–16 October 2008.

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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 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.

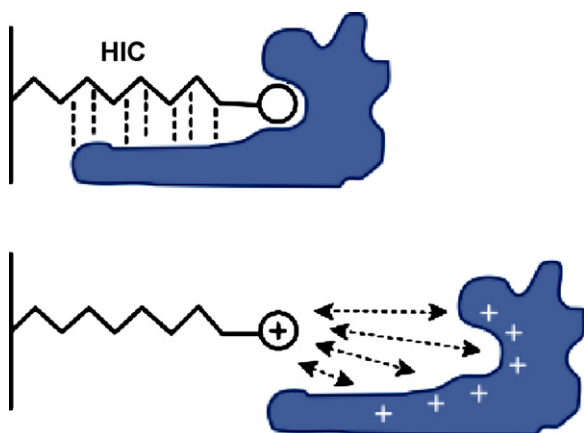


Fig. 1. Schematic of adsorption and desorption mechanisms for HyperCel mixed-mode chromatography sorbents.

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 $\mu\text{mol/mL}$ of sorbent. For MEP HyperCel, the synthetic ligand is mercapto ethyl pyridine and the ligand density is 70–125 $\mu\text{mol/mL}$ 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 μ m.

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. The slurry was agitated and 100 μ L were transferred to the filter plate for a final volume of sorbent of 50 μ L per well. An aliquot of crude feedstock (100 μ L) was added in each well and stirred vigorously for 60 min. Each well was washed twice with 200 μ L of equilibration buffer and stirred vigorously for 5 min. Then, 200 μ L of elution buffer (50 mM Na acetate, pH 5.0) were added twice in each well of the filter plate and stirred vigorously for 10 min. The process was repeated using three successive elution buffers (50 mM Na acetate, pH 4.5, pH 4.0 and pH 3.0). At each step, effluents (flowthrough, wash and elution fractions) were collected in a standard 96-well plate and stored at +4 °C.

2.4. Liquid chromatography using pre-packed columns

Each pre-packed column was equilibrated with a minimum of five column volumes (CV) of phosphate buffered saline (PBS), pH 7.4. Two CV of crude feedstock filtered at 0.2 μ m were loaded directly without pH or conductivity adjustment. The column was washed with ten CV of PBS, pH 7.4. The elution was done using 4 pH steps: ten CV of 50 mM sodium acetate, pH 5.0; 4.5; 4.0; and 3.0. The column was regenerated for 60 min with 1 M NaOH and re-equilibrated with 5 CV of 50 mM sodium acetate, pH 3.0 and in an excess of PBS, pH 7.4 (15 CV). The run was performed at 75 cm/h (0.25 mL/min) corresponding to

a 4 min residence time (RT) except for the regeneration step, at 80 cm/h.

2.5. Analysis of chromatography fractions using SELDI-MS

Crude feedstock, reference samples and chromatography fractions showing absorbance peaks at 280 nm were analysed using SELDI-MS. 5 μ L of fractions were applied directly per array spot on a normal phase array (NP20) and air-dried. Each spot was washed with $3 \times 5 \mu$ L of ultrapure water and air-dried. A saturated solution of SPA was prepared by mixing 5 mg of dried SPA in 100 μ L of acetonitrile and 100 μ L of 1% (v/v) trifluoro acetic acid (TFA). The solution was mixed for 5 min and centrifuged at 14,000 rpm for 5 min. 0.8 μ L of the saturated solution was applied twice to each spot of the array and allowed to dry. The array was introduced in the sample chamber of the PCS4000 Reader and read following an automatic protocol including the following optimized parameters: detector voltage of 25 kV, laser energy of 3500 μ J and focus mass of 17,500 Da. The PCS4000 reader was calibrated daily using protein standards of known molecular weight (equine myoglobin, 16,951.5 Da, bovine serum albumin, 66,433.0 Da and *E. coli* β -galactosidase, 116,351.0 Da).

2.6. Analysis of chromatography fractions using SDS-PAGE

Fractions showing absorbance peaks at 280 nm were analysed using Coomassie-stained SDS-PAGE gels [17].

2.7. Measurement of total protein content (TPC)

TPC in the fractions were analysed by spectrophotometry at 595 nm [18].

2.8. Evaluation of the purity of chromatography fractions by size exclusion chromatography (SEC)

Fractions of interest were analysed by SEC. An aliquote of 30 μ L of each fraction was loaded on the column previously equilibrated in 0.05 M sodium phosphate, 0.1 M sodium sulfate, pH 6.8 (mobile phase). The mobile phase was run at 0.7 mL/min for 30 min and thereafter the column was stored in 20% (v/v) ethanol.

All experiments were carried out at room temperature unless specified.

3. Results and discussion

A HT screening approach was used to investigate in parallel the efficiency of MEP, HEA and PPA HyperCel sorbents for capturing the recombinant allergen rBet v 1a from a crude *E. coli* feedstock.

3.1. Detection of rBet v 1a using SELDI-MS

Since fraction analysis was intended using SELDI-MS, in a preliminary feasibility study we confirmed the ability of SELDI-MS to track rBet v 1a and the N-methionine contaminant in the crude feedstock. Fig. 2A and B show that the main peak corresponding to rBet v 1a was around 17,440 Da. There was another peak at approximately 17,650 Da (+210 Da) in all the samples including the crude material. Since the nature of this peak was unknown, the detection of rBet v 1a in this study was based on the peak around 17,440 Da only.

The analysis of a partially purified sample of rBet v 1a containing 28% of N-methionine contaminant established that the contaminant peak was detected with a mass shift of 130 Da compared to rBet v 1a peak (Fig. 2A and B). It could be also detected in the crude material, therefore SELDI-MS analysis was adapted to track

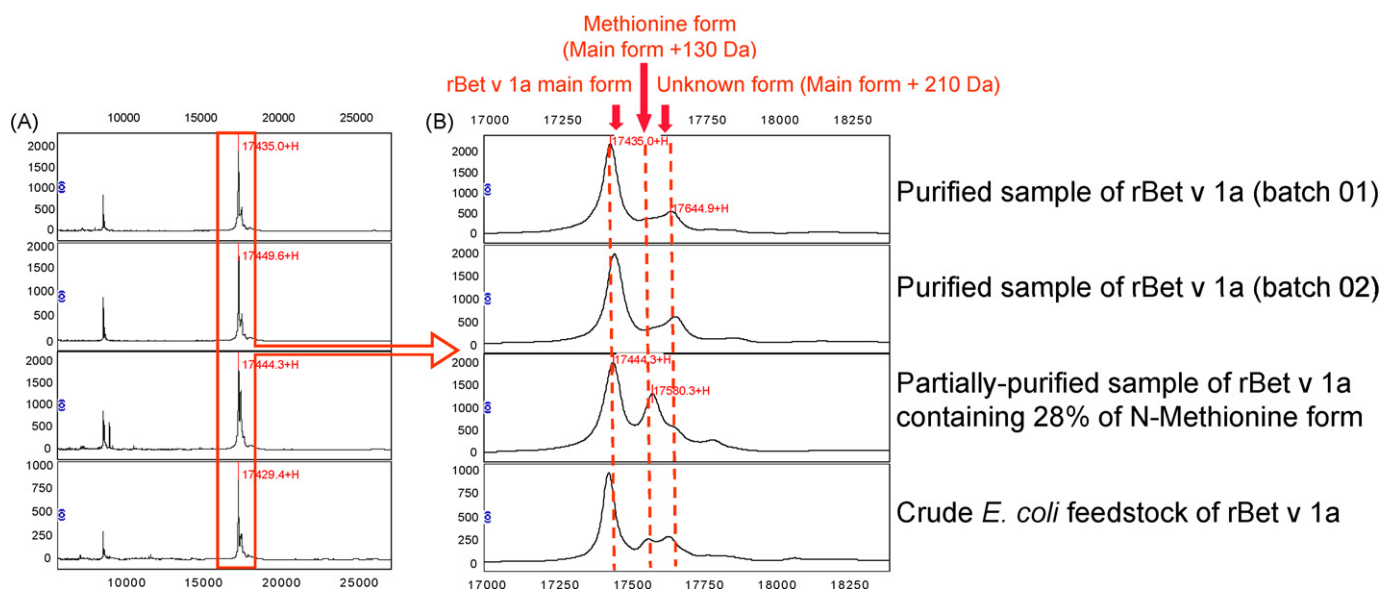


Fig. 2. SELDI-MS profiles after depositing 5 μ L of the crude *E. coli* feedstock and the reference samples (purified or partially-purified rBet v 1a) on NP20 array (A) zoom between 5 kDa and 25 kDa. (B) Zoom between 17 kDa and 18.5 kDa.

and distinguish the target protein in its natural conformation and the N-methionine contaminant during process development.

3.2. HT mixed-mode chromatography sorbent screening

Since MEP, HEA and PPA HyperCel sorbents can provide efficient interactions in “physiological-like” conditions (i.e. low conductivity and neutral pH) and without the addition of lyotropic salt, the binding of rBet v 1a was tested in a phosphate buffered saline at neutral pH [13]. Figs. 3A and 4A, B show that rBet v 1a was fully retained on HEA and PPA HyperCel sorbents (no signal in the flowthrough or wash fractions) but only partially on MEP HyperCel sorbent. The use of a phosphate buffer containing 1 M of sodium chloride at equilibration improved the retention of rBet v 1a on MEP HyperCel but the binding remains partial (Fig. 3B). The distinct selectivity of MEP HyperCel sorbent compared to HEA and PPA HyperCel sorbents has already been observed [16] and is explained by the fact

that the binding mechanism of MEP, a hydrophobic charge induction chromatography (HCIC) ligand, is very different from the two other mixed-mode sorbents [13,14].

Since desorption from MEP, HEA and PPA HyperCel sorbents is favoured by charge repulsion at decreasing pH, the elution of rBet v 1a was attempted in acetate buffers from pH 5 to 3 [13]. It appeared that rBet v 1a was mainly recovered in the elution fractions at pH 4.5 and 4 from MEP HyperCel; at pH 4 from HEA HyperCel and at pH 4 and 3 from PPA HyperCel (Fig. 3A and 4A, B). rBet v 1a was eluted at a higher pH on HEA than on PPA HyperCel sorbent. This trend has already been observed for model proteins (lysozyme, chymotrypsinogen A, ovalbumin, ...) and explained by the fact that the phenyl propyl group of the PPA ligand exhibited stronger hydrophobic interactions than the hexyl group of the HEA ligand [16].

Thus, a reversible capture of rBet v 1a occurred on HEA or PPA HyperCel in “physiological-like” conditions. The crude feedstock was directly loaded on the sorbents without pre-treatment (i.e.

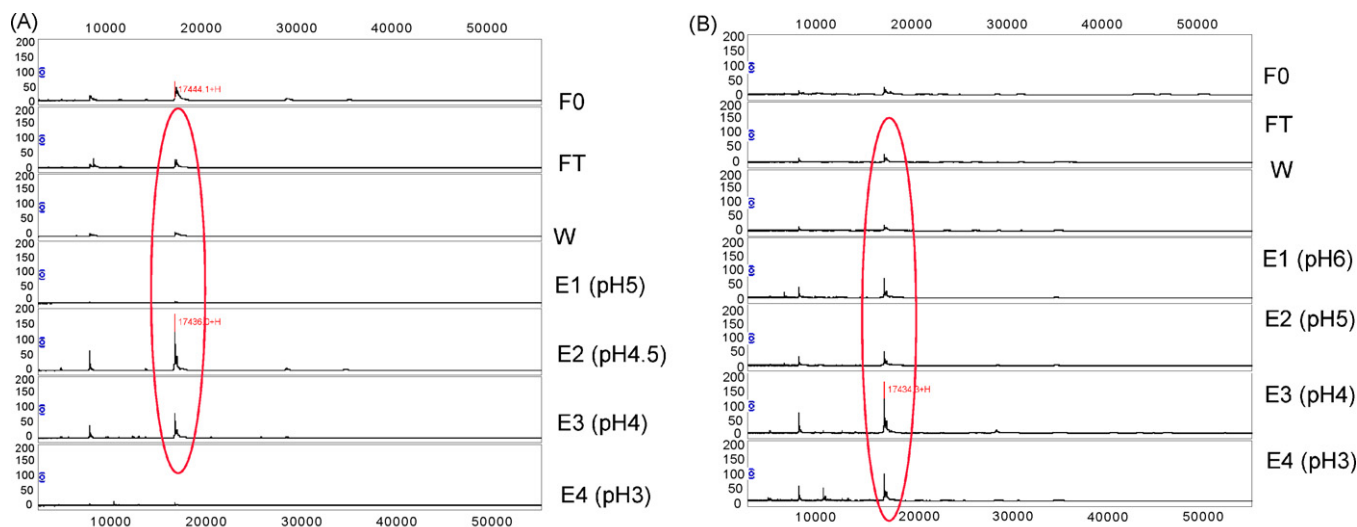


Fig. 3. SELDI-MS profiles after mini-batch screening of the crude feedstock on MEP HyperCel equilibrated in (A) PBS, pH 7.4 or (B) 20 mM Na phosphate, 1 M NaCl, pH 7. F0: starting material. FT: flowthrough. W: wash in equilibration buffer. E1: elution in (A) 50 mM Na acetate, pH 5 or (B) 20 mM Na citrate pH 6. E2: elution in 50 mM Na acetate, (A) pH 4.5 or (B) pH 5. E3: elution in 50 mM Na acetate, pH 4. E4: elution in 50 mM Na acetate, pH 3. Red circles highlight the detection of rBet v 1a peak.

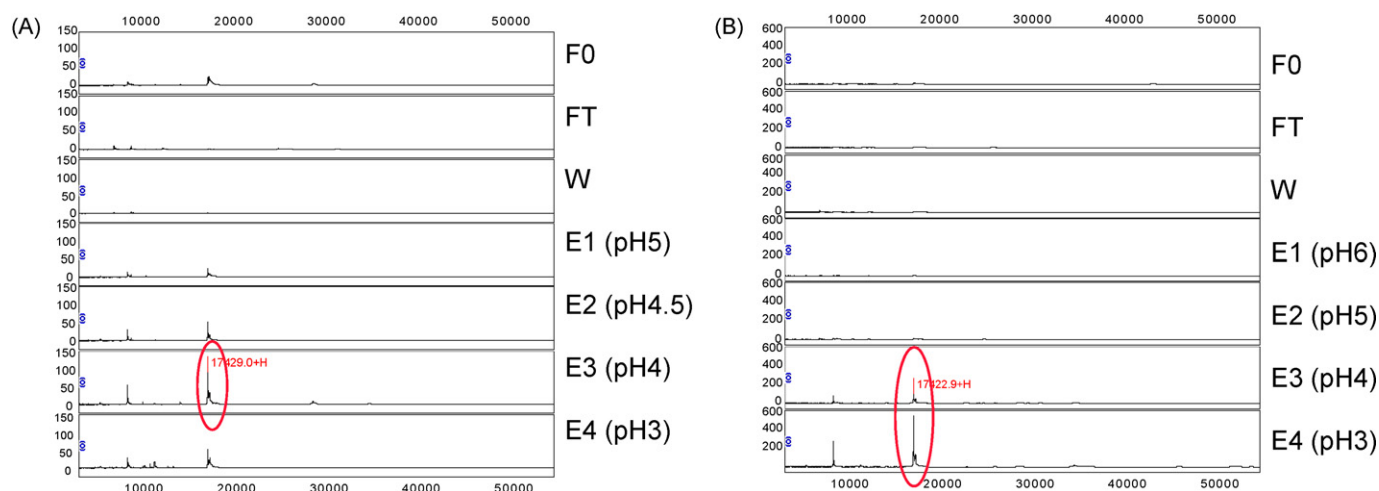


Fig. 4. SELDI-MS profiles after mini-batch screening of the crude feedstock on (A) HEA HyperCel or (B) PPA HyperCel equilibrated in PBS, pH 7.4. F0: starting material. FT: flowthrough. W: wash in equilibration buffer. E1: elution in (A) 50 mM Na acetate, pH 5 or (B) 20 mM Na citrate pH 6. E2: elution in 50 mM Na acetate, (A) pH 4.5 or (B) pH 5. E3: elution in 50 mM Na acetate, pH 4. E4: elution in 50 mM Na acetate, pH 3. Red circles highlight the detection of rBet v 1a peak.

dilution or pH adjustment often required on ion exchangers) and without addition of lyotropic salts (as usually required for conventional hydrophobic interaction chromatography (HIC) sorbents) [19]. The benefit of a direct loading on mixed-mode sorbents is to save an extra-step, leading to time and economy savings and to satisfy demands at industrial scale for processes that do not request addition of lyotropic salts which are costly, corrosive and require specific waste disposal.

3.3. Optimization of the capture step on mixed-mode sorbents using pre-packed columns

As a proof of concept of the HT mixed-mode sorbent screening, transfer of the conditions was carried out on PRC columns pre-packed with HEA and PPA HyperCel sorbents that enabled an efficient capture of rBet v 1a, but also on MEP HyperCel that did not permit the full retention of rBet v 1a in the conditions tested.

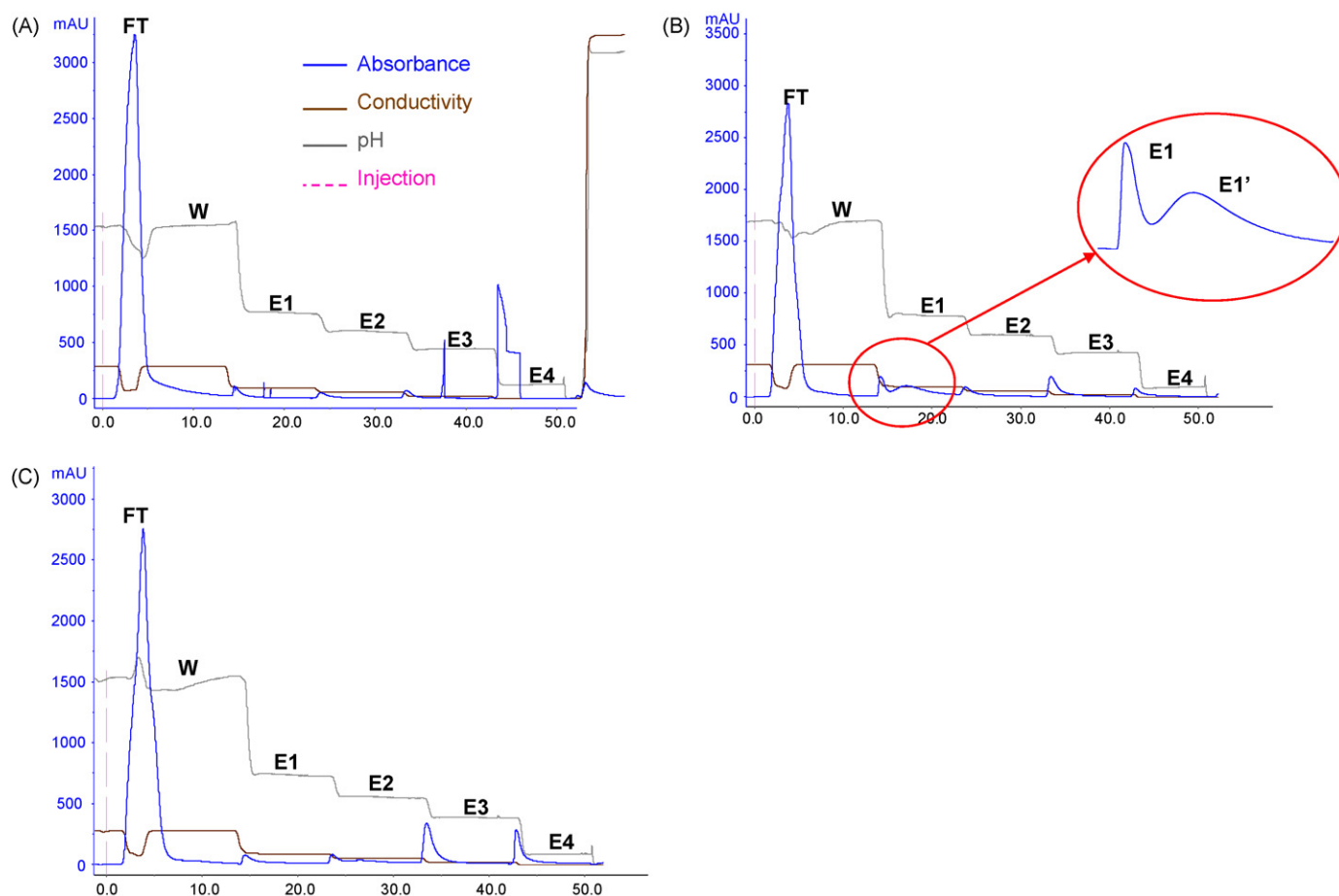


Fig. 5. Chromatograms after loading two CV of crude feedstock on (A) MEP HyperCel (B) HEA HyperCel (C) PPA HyperCel equilibrated in PBS, pH 7.4. FT: flowthrough. W: wash in 10 CV of PBS, pH 7.4. Elution in 10 CV of E1: 50 mM Na acetate, pH 5. E2: elution in 50 mM Na acetate, pH 4.5. E3: elution in 50 mM Na acetate, pH 4. E4: elution in 50 mM Na acetate, pH 3.

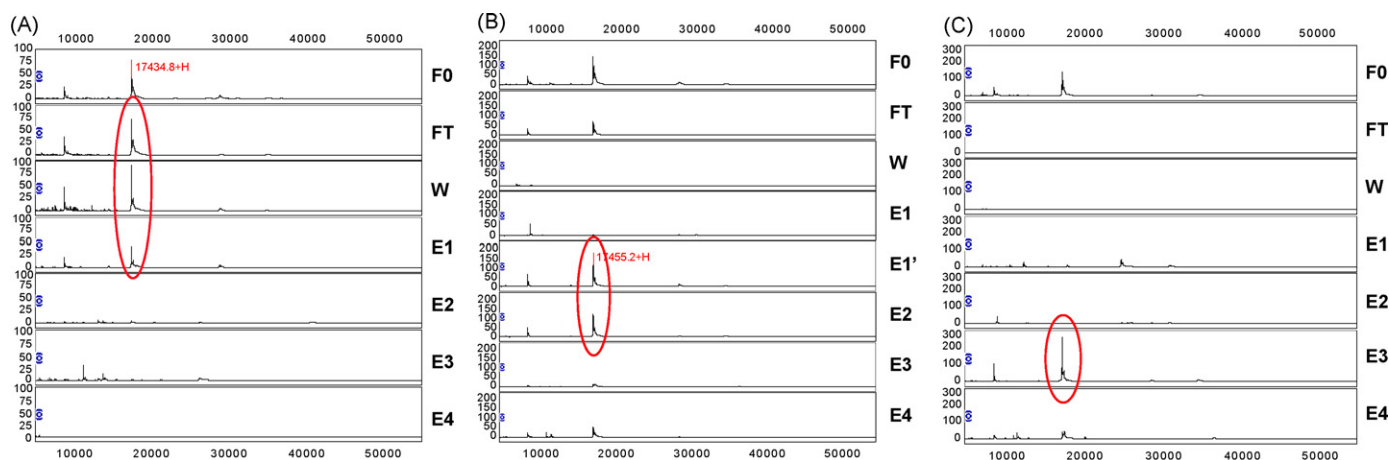


Fig. 6. SELDI-MS profiles after chromatography of two CV of crude feedstock on (A) MEP HyperCel, (B) HEA HyperCel or (C) PPA HyperCel equilibrated in PBS, pH 7.4. F0: starting material. FT: flowthrough. W: wash in equilibration buffer. Elution in E1: 50 mM Na acetate, pH 5. E2: 50 mM Na acetate, pH 4.5. E3: 50 mM Na acetate, pH 4. E4: 50 mM Na acetate, pH 3. Red circles highlight the detection of rBet v 1a peak.

Three PRC columns pre-packed with the three mixed-mode sorbents were run in parallel following the conditions applied during the HT screening. Each chromatography run lasted approximately 3 h including regeneration and re-equilibration of the column. Chromatograms are shown in Fig. 5A–C. Analytical data for the three runs were obtained in a couple of hours using SELDI-MS and in a day using SDS-PAGE, SEC and Bradford assay. It confirmed that rBet v 1a was hardly retained on MEP HyperCel sorbent: it was partly found in the flowthrough and wash fractions and partly recovered at pH 5 (Figs. 6 and 7; Figs. 6A and 7A; Table 1).

It also confirmed that rBet v 1a was retained on HEA HyperCel sorbent in “physiological-like” conditions and without pre-treatment of the crude feedstock (Figs. 6B and 7B; Table 1). rBet v 1a was recovered in 50 mM Na acetate pH 5 and 4.5 (Figs. 6B and 7B). This observation was not exactly in accordance with the data obtained from the HT screening where rBet v 1a was recovered at pH 4. The elution profile observed using the 96-well microplate and the pre-packed column may differ, due to a modification of the interactions between the batch mode and the dynamic flow mode. The chromatogram obtained using the PRC column revealed that two absorbance peaks were observed during the first elution step at pH 5 (Fig. 5B) and only the second absorbance peak (E1') corresponded to rBet v 1a (Figs. 6B and 7B). rBet v 1a was estimated to be 85.2% pure using SEC in the fraction at pH 5 (E1') and 58.7% at pH 4.5 (E2), compared to 9.3% in the crude feedstock (F0) (Table 1). It corresponds to a 9-fold increase of the purification of rBet v 1a in the pH 5 fraction and a 6-fold increase in the pH 4.5 fraction.

Finally, it confirmed that rBet v 1a was fully retained on PPA HyperCel sorbent without pre-treatment of the crude feedstock and recovered in 50 mM sodium acetate pH 4 and 3 (Figs. 6C and 7C; Table 1), as previously determined during the HT screening. rBet v 1a was estimated to be 85.7% pure using SEC in the pH 4 fraction (E3) compared to 9.3% in the crude feedstock (F0) (Table 1). It corresponds therefore to a 9-fold increase of the purity level of rBet v 1a.

Further, rBet v 1a was recovered in a single elution step on PPA HyperCel, while it was recovered in two elution steps on HEA HyperCel. Quantities of rBet v 1a recovered were identical, however the overall purity of rBet v 1a was higher on PPA than on HEA HyperCel sorbent (Table 1).

A focus on the SELDI-MS profiles was carried out to determine whether the main form of rBet v 1a was separated from the N-methionine contaminant. On HEA HyperCel, the contaminant was recovered at pH 3 and 4 (Fig. 8A) compared to pH 4.5 and 5 for rBet v 1a. On PPA HyperCel, the contaminant was recovered at pH 3 (Fig. 8B) compared to pH 4 for rBet v 1a. Therefore, on both sorbents, rBet v 1a was separated from the N-methionine contaminant. Such separation, even partial, of two proteins differing only from one amino acid, provides evidence for the powerful selectivity of mixed-mode sorbents. Indeed, proteins with close biochemical characteristics are often difficult to separate based on a single property and the use of several orthogonal steps may be required. A study on the retention of a model protein (lysozyme) on a HIC sorbent showed that retention was only affected by amino acid substitutions occurring on the surface of the protein opposite to the catalytic cleft and that this deviation was also affected by the

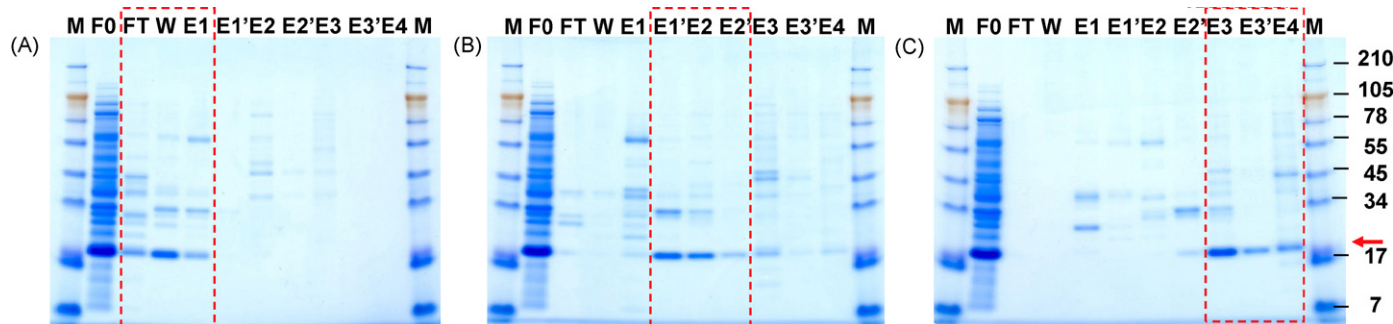
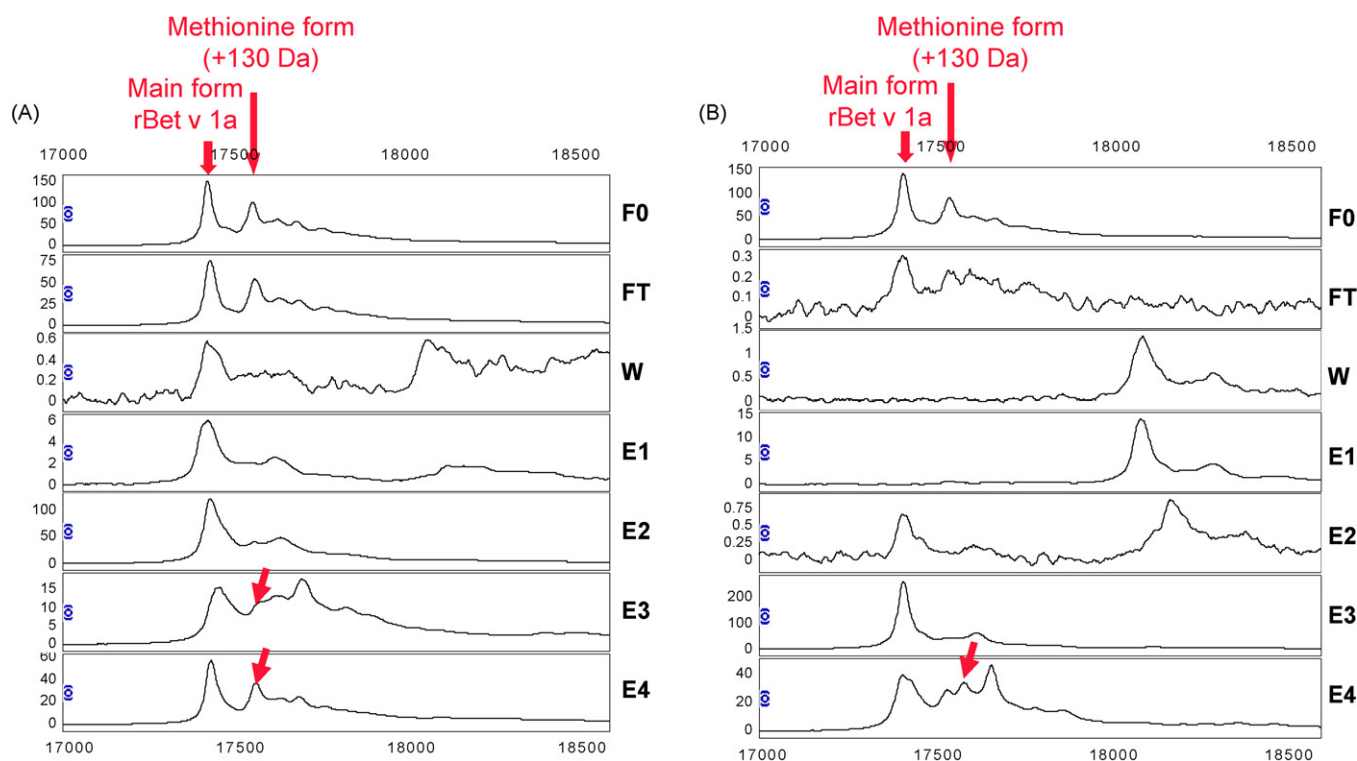


Fig. 7. Coomassie-stained SDS-PAGE gels after chromatography of two CV of crude feedstock on (A) MEP HyperCel, (B) HEA HyperCel or (C) PPA HyperCel equilibrated in PBS, pH 7.4. F0: starting material. FT: flowthrough. W: wash in equilibration buffer. Elution in E1 and E1': 50 mM Na acetate, pH 5. E2 and E2': 50 mM Na acetate, pH 4.5. E3 and E3': 50 mM Na acetate, pH 4. E4: 50 mM Na acetate, pH 3. M: molecular weight markers. The red arrow indicates the band corresponding to rBet v 1a.

Table 1Total protein content, total protein recovery and purity of rBet v 1a after the chromatography run of the crude *E. coli* feedstock on MEP, HEA or PPA HyperCel PRC columns.

Fraction	Total protein content (mg) ^a	Total protein recovery (%) ^b	Purity of rBet v 1a (%) ^c
MEP HyperCel			
F0 [*]	7.36	–	9.3
FT ^{**}	1.58	21.5	1.4
Wash	2.45	33.2	65.8
Elution 1	0.62	8.4	45.4
Elution 2	1.01	13.8	6.0
Elution 3	0.34	4.6	9.6
Elution 4	0.14	1.9	0.0
Total	6.23	83.4	–
HEA HyperCel			
F0 [*]	7.32	–	9.3
FT ^{**}	0.30	4.1	3.5
Wash	0.43	5.9	0.0
Elution 1	0.49	6.7	0.0
Elution 1'	2.07	28.3	85.2
Elution 2	1.05	14.4	58.7
Elution 3	1.03	14.1	0.0
Elution 4	0.36	4.9	0.0
Total	5.73	78.3	–
PPA HyperCel			
F0 [*]	7.16	–	9.3
FT ^{**}	0.08	1.0	0.0
Wash	0.10	1.3	0.0
Elution 1	0.46	6.5	0.0
Elution 2	0.98	13.7	0.0
Elution 3	2.66	37.2	85.7
Elution 4	1.03	14.4	8.3
Total	4.02	74.1	–

^{*}F0: crude feedstock.^{**}FT: Flowthrough.^aTotal protein content (TPC) assessed using a Bradford assay.^bTotal protein recovery (%) = TPC of the fraction/TPC in F0 × 100.^cPurity of rBet v 1a (%) assessed using size exclusion chromatography. The red fractions contain the target protein rBet v 1a.**Fig. 8.** Zoom of SELDI-MS profiles after chromatography of two CV of crude feedstock on (A) HEA HyperCel or (B) PPA HyperCel equilibrated in PBS, pH 7.4. F0: starting material. FT: flowthrough. W: wash in equilibration buffer. Elution in E1: 50 mM Na acetate, pH 5. E2: 50 mM Na acetate, pH 4.5. E3: 50 mM Na acetate, pH 4. E4: 50 mM Na acetate, pH 3.

charge of the amino acid residue [20]. The mixed-mode sorbents investigated here proved to offer new selectivities inherent to the multi-modal mechanism involved at binding and desorption. This has been also observed recently on new multimodal pH-HIC sorbents using green fluorescent protein mutants modified with single or double amino acid substitutions [21].

The use of PRC columns facilitated the comparison of the separation performance of the three mixed-mode sorbents, since it saved packing operations and provided data from identical column bed geometry. Even at this column scale (1 mL), the resolution was sufficient to highlight a 2-step desorption of rBet v1a (at pH 5 and 4.5) on HEA HyperCel and also to demonstrate the separation at elution of rBet v 1a from a contaminant with a close conformation, on both HEA and PPA HyperCel sorbents. The ability of the two sorbents to partly eliminate the methionine form is critical since the occurrence of this contaminant results from the accumulation of non enzymatically cleaved rBet v 1a during fermentation, specifically observed in *E. coli* host cell system at high protein concentration [5].

The use of PRC columns of identical volume also facilitates the comparison of sorbent dynamic binding capacity (DBC). One perspective of the work described here is to evaluate the DBC of rBet v1a on HEA and PPA HyperCel sorbents. For protein models like bovine serum albumin, a typical DBC of 40 to 60 mg/mL of sorbent is obtained at 10% breakthrough on HEA and PPA HyperCel in PBS, pH 7.4. It is high compared to the DBC obtained in the same conditions (138 mM NaCl, pH 7.4) for conventional HIC sorbents since optimal DBC is obtained in relatively high lyotropic salt concentrations for these sorbents. A relatively high DBC of rBet v1a on PPA HyperCel sorbent in “physiological-like” conditions could result in important economic and environmental benefits at production scale.

4. Conclusion

The selection and development of the capture step of a recombinant allergen rBet v 1a expressed in *E. coli* using mixed-mode sorbents was carried out in one day on a high throughput platform including batch chromatography in a 96-well microplate combined with SELDI-MS analysis. It revealed that HEA and PPA HyperCel sorbents allowed the capture of rBet v 1a in “physiological-like” conditions while MEP HyperCel sorbent offered only partial binding. No pre-treatment of the crude extract was needed for the capture of rBet v 1a on HEA and PPA HyperCel sorbents, saving the cost and time of an extra step often necessary prior to ion exchange chromatography (i.e. dilution or diafiltration) or conventional HIC (addition of lyotropic salts).

The mini-batch screening is usually intended to guide the selection of the lead sorbents and the best running conditions, narrowing the number of chromatography runs to transfer on lab-scale chromatography columns. Here, the conditions used during the small-scale screening were transferred on the three sorbents tested, as a proof of concept of the approach. The use of pre-packed PRC columns facilitated the comparison of the performance of the three mixed-mode sorbents: in three days, it confirmed that HEA and PPA HyperCel enabled the reversible capture of rBet v 1a (unlike MEP HyperCel), in the conditions tested. The elution profiles observed on the PPA HyperCel column was identical to the profiles obtained during the mini-batch screening. A distinction between the batch and the dynamic mode was observed on HEA HyperCel sorbent. Indeed during the elution step at pH 5 on the PRC column, two separate

absorbance peaks could be observed (the former corresponding to impurities and the latter one, to rBet v 1a). This observation evidences the high resolution of separation on the PRC column despite the small scale (1 mL) and highlights the need for optimizing the conditions of a chromatography step on lab-scale columns prior to a larger scale-up.

The transfer on PRC columns suggested that the recovery of rBet v 1a in a single elution fraction on PPA HyperCel sorbent would be facilitated compared to the recovery in two elution steps on HEA HyperCel sorbent. Moreover, the purity of rBet v 1a was evaluated to be around 85% in the PPA HyperCel fraction compared to 85% and 58% in the two HEA HyperCel fractions. Based on a purity of 9% in the crude feedstock, the increase in purity levels of rBet v 1a was estimated to be approximately 9-fold during the capture step on PPA HyperCel sorbent.

Moreover, SELDI-MS analysis of the elution fractions provided evidence for the partial elimination at elution of a major contaminant, both on HEA and PPA HyperCel sorbents. The ability of the two sorbents to separate two proteins that differed only at one amino acid relies likely on the multi-modal mechanism of mixed-mode sorbents. This is critical since the production of the recombinant pharmaceutical grade Bet v 1 should rely on a process leading to a highly purified molecule while eliminating contaminants potentially accumulated during the fermentation step.

The approach chosen here for the development and optimization of a purification step proved in four days that HEA and PPA HyperCel mixed-mode sorbents were of interest for the capture and the 9-fold purification of the recombinant allergen rBet v 1a in a single chromatography step and without the pre-treatment of the crude *E. coli* feedstock.

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