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Short communication

Primary isolation of *Geotrichum klebahnii* polygalacturonase by capturing with glass fiber microfilters

Arley D. Zapata Zapata[a], Claudio E. Voget[b,*]

[a] Escuela de Química, Facultad de Ciencias, Universidad Nacional de Colombia, Sede Medellín, Calle 59A Número 63 – 20, Medellín, Colombia
[b] CINDEFI (UNLP, CCT-La Plata, CONICET), Calle 47 y 115, La Plata (CP 1900), Argentina

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ABSTRACT

This study reports the use of borosilicate glass fiber microfilters (GFMs) for capturing and concentrating *Geotrichum klebahnii* polygalacturonase (GkPG) from the culture media through a simple coupled filtration–sorption process. GFM was characterized by scanning electron microscopy and intrusion mercury porosimetry. Adsorption of GkPG was almost instantaneous with maximum effectiveness at pH 3.0. Using the parameters of Langmuir model and a material balance, a filter bed consisting of a stack of GFM was designed for recovering GkPG from a certain volume of culture media. After adsorption and washing, the enzyme was efficiently eluted with a buffer solution at pH 5.0 containing 0.5 M NaCl. The filter bed was reused in several sorption cycles and enzyme recovery could be also done without prior cell removal with the benefits of using microfiltration for enzyme cell separation in a single step. Under the process conditions employed, GkPG recovery was about 80% with a concentration factor of about 4-fold.

1. Introduction

Membrane adsorbers are being increasingly used as chromatographic media with several advantages over conventional bead columns [1,2]. Specific membranes are commercially available with different functionalities, including ligands for ion exchange, metal chelate and affinity chromatography. These have been successfully used for the purification of different biomolecules and viral particles [3–5]. In a previous study [6] it was described the adsorptive properties of borosilicate glass fiber microfilters (GFMs) toward the non-acidic polygalacturonases (PGs) and other extracellular enzymes produced by *Aspergillus kawachii* in a glucose–tryptone medium. Although the GFM is a depth filter which has been mainly developed for polishing liquid streams containing colloidal and insoluble submicron particles, it was used to develop a simple filtration–sorption process for the primary isolation of *A. kawachii* PGs from the culture broth, resulting in a highly purified and concentrated PG solution. This was the first report in which a filter system was used for capturing a fungal polygalacturonase, although the separation process was not studied in detail.

Geotrichum klebahnii (= *Geotrichum penicillatum*) is a yeast like fungi that produces a single extracellular protein, i.e. an endo-polygalacturonase (GkPG), when grown in synthetic media [7]. The enzyme was characterized and its gene cloned, although it has not been used for industrial applications [8]. The enzyme was used as a very efficient biocatalyst for releasing pectin from citrus wastes and to macerate plant tissues [9]. Preliminary experiments in which the GFM was used to clarify the culture supernatants showed that, under certain conditions, the GkPG was adsorbed to the GFM and that it was readily released from the filter with a buffer solution of high ionic strength. The GkPG was therefore an interesting model protein to characterize GFM behavior as an adsorber for the primary isolation of the enzyme from the culture media.

This paper presents data on some GFM structural features, about the effect of some process conditions in GkPG adsorption, the adsorption equilibrium between the enzyme and the GFM and the design of a sorption process using a filter bed to recover and concentrate the enzyme from the culture medium. Also it was analyzed the reuse of this filter bed and the possibility of integrating the enzyme recovery and the separation from the biomass in a single filtration stage. This work provides a better understanding of the GFM performance as an adsorptive matrix for the primary isolation of polygalacturonases.

2. Experimental

2.1. Culture conditions and enzyme production

Batch (BC) and feed-batch (FBC) cultures of *G. klebahnii* were carried out at 30 °C in a 5-L bioreactor using a medium containing glucose, urea, salts and microelements [7]. Separation of biomass was achieved by centrifugation (16,000 × *g* for 10 min). The pH, conductivity, volumetric PG activity and protein content of FBC and BC supernatants were 4.8, 7.5 mS/cm, 350 U/ml, 0.51 mg/ml and 5.5, 1.2 mS/cm,

* Corresponding author.
E-mail addresses: adzapata@unal.edu.co (A.D. Zapata Zapata), voget@biotec.org.ar (C.E. Voget).

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85 U/ml, 0.21 mg/ml respectively. The coefficient of variation of these parameters between different independent cultures was within 10–20%. The FBC supernatant was concentrated ~10× by vacuum evaporation, stored at −20 °C and when required, thawed in a water bath at 37 °C. No loss of enzyme activity was observed after the concentration/thawing process. For adsorption experiments, the concentrated medium was diluted with deionized water. Dilutions were referred to the original FBC supernatant. The BC supernatants or whole broth containing 5 g/L of dry biomass were used within the 24 h after finishing the culture and they were not diluted. The pH of the samples to be filtered was adjusted to the desired value with HCl or NaOH.

2.2. GFM characterization

The borosilicate glass fiber binder filters with 0.5 μm nominal pore size and thickness 450 μm were purchased from GE-Osmonics Inc. (Minnetonka, USA). GFMs of different diameters (cross-section areas): 47 mm (17.4 cm^2), 90 mm (63.6 cm^2) and 110 mm (95.0 cm^2) were used as received without any treatment and were wetted with deionized water before applying the feed. The surface density was calculated from the weight of dry filters and their corresponding cross-section area. The water holding capacity was determined by the weight of water retained in a single filter after filtrating deionized water under the flow conditions used for protein adsorption. Average values obtained from 10 different samples of different GFM sizes were 7.26×10^{-3} g/cm^2 and ~0.05 ml water/cm^2 or 6.9 ml water/g, respectively. The standard deviation of these values was within 2–5%. The wet bed volume was estimated to be 0.1 ml/cm^2 or 14 ml/g on a wet basis.

Appearance of GFM was studied by scanning electron microscope SEM (Jeol, 5910 L V, Japan). Although the GFM may not be considered a porous material, a rough estimation of the void volume, pore size distribution and specific surface area was done by intrusion mercury porosimetry. (Hg-Porosimeter 2000, Carlo Erba Instruments, Milan/Italy) assuming that pores were cylindrical, a surface tension value for Hg of 0.48 N/m and a contact angle of 140° [10].

2.3. Experimental set-up

Filtration was carried out using a common bench-scale dead-end vacuum filtration unit equipped with a circular ceramic filter holder of varying diameter size according to the GFM used, a silicon stopper and a vacuum side flask connected to a polypropylene vacuum pump aspirator. The GFMs were stacked inside the circular holder without using a special housing. To initiate a filtration run, an exact volume of culture supernatant or whole broth, called from now on the feed solution (FS), was carefully poured onto the surface of the wetted filter, after which vacuum was applied. Filtration was carried out at 22–25 °C at a flow rate of 20–50 ml/min. Depending on the filter size, these values corresponded to linear flow rates in the range of 12.6–172 cm/h. The amount of enzyme adsorbed was determined from the difference between the FS activity and that remaining in the permeate.

2.4. Determination of adsorption isotherms

For constructing the adsorption isotherms, the filter was gradually saturated with enzyme by filtering several serial volume fractions of FS. Each FS volume was refiltered once to assure adsorption equilibrium. The equilibrium data was analyzed according to the monolayer Langmuir model:

$$q = \frac{q_m C}{K_d + C} \quad (1)$$

where C is the equilibrium liquid phase activity (U/ml), q is the equilibrium activity bound to the GFM (U/cm^2), q_m is the monolayer adsorption capacity or maximum adsorption capacity of the GFM and K_d an equilibrium constant reflecting the affinity of the protein for the adsorbent.

2.5. Sorption experiments

Adsorption/desorption was carried out as a typical batch process. The Langmuir model parameters and a material balance were used to design a stack of GFMs for recovering the GkPG from a certain volume of culture media. The material balance was as follows:

$$C_{FS}V_{FS} = CV_{FS} + qA \quad (2)$$

where A is the total cross-section area (cm^2), V_{FS} and C_{FS}, the volume and enzyme activities of FS, respectively.

Desorption of the bound PG activity was carried out by passing through the filter a known volume of elution buffer (EB), after washing the filter with 10 mM citrate buffer, pH 3.0, (washing buffer, WB, conductivity 0.71 mS/cm). Enzyme recovery after a sorption (adsorption–washing–elution) cycle was calculated as follows:

$$\text{recovery}(\%) = \frac{V_E \times C_E}{V_{FS} \times C_{FS}} \times 100 \quad (3)$$

where V_E, C_E are the volume and enzyme activity of EB, respectively. The concentration factor was defined as C_E/C_{FS}.

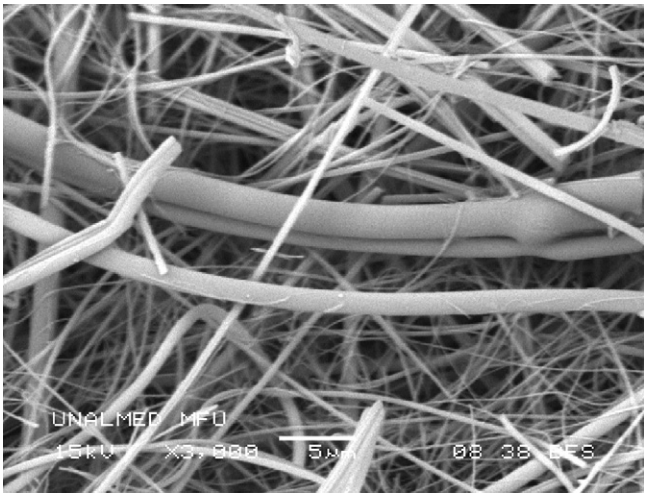

Fig. 1. Scanning electron micrograph of a GFM.

2.6. Analytical procedures

Polygalacturonase activity was assayed at 37 °C as previously described by measuring the increase in reduced groups by Somogyi–Nelson method [11]. The experimental error was ±5%. Protein was determined by Lowry method using bovine serum albumin as standard [11]. The ionic strength of buffer or protein solutions were assessed by measuring the solution conductivity with a conductivity meter (Hanna HI 3291) and was expressed in mS/cm (0.1 M KCl, 12.88 mS/cm at 25 °C).

3. Results and discussion

3.1. Characterization of the GFM

The scanning electron micrograph of a GFM (Fig. 1) shows an open fibrous matrix with a great heterogeneity in the fiber sizes (0.2–2.0 μm). Pore size distribution determined by mercury porosimetry is shown in Fig. 2. The total interfiber void space or pore volume was 4.2 cm^3/g dry material. The macropores with diameters in the range of 0.4–8.0 μm and mesopores (8–60 nm) take about 90 and 10% of the total pore volume, respectively. Macroporosity is within the range of affinity membranes and makes concentration gradient in the radial direction negligible [5]. The specific surface area of the microfibers for macropores was ~54 m^2/g dry basis. Although data on specific surface area of chromatographic membranes are scarce this value is in the same order of some ionic-exchange and microfiltration membranes [12,13].

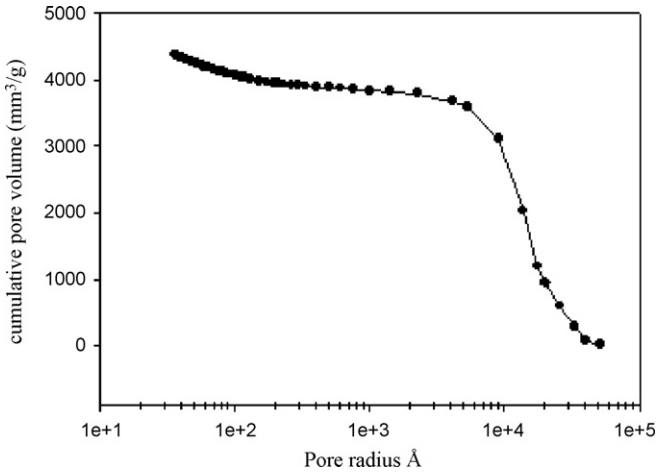

Fig. 2. Pore size distribution in GFM as determined by intrusion mercury porosimetry.

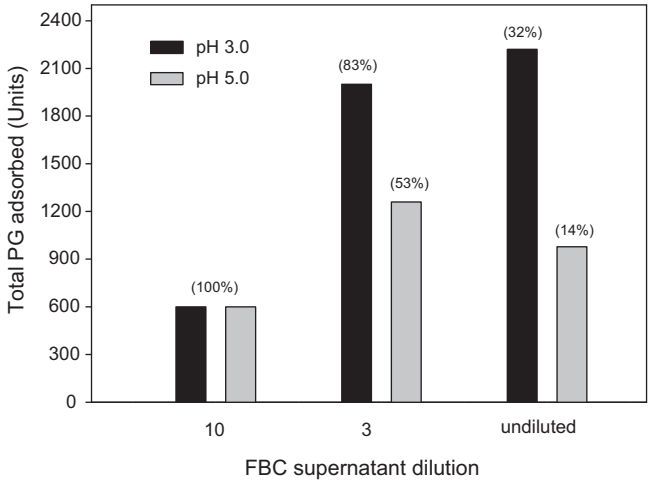

Fig. 3. Adsorption of *G. klebahnii* PG on GFM. FS: 20 ml of FBC supernatant undiluted or diluted 1:3 and 1:10 with deionized water at pH 3.0 or 5.0. The undiluted supernatant conductivity, PG activity and protein concentration was 7.5 mS/cm, 350 U/ml and 0.51 mg/ml, respectively. Adsorber: a single 47 mm (17.35 cm^2) GFM. A new GFM was used for each pH/dilution experiment. Each FS sample was filtered twice through the same GFM (by recycling the filtrate) at 20–25 ml/min at 25 °C. The numbers in parentheses represent the % of enzyme adsorbed.

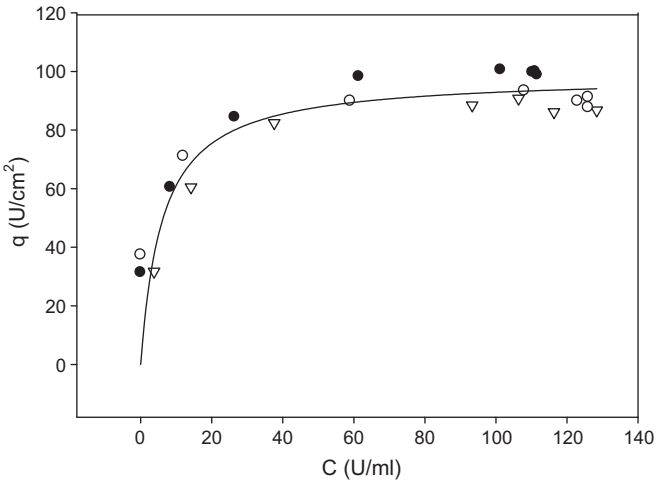

Fig. 4. Adsorption isotherms of the system *G. klebahnii* PG-GFM
FS: FBC supernatant diluted 1:3 with deionized water, pH 3.0, Conductivity, PG activity and protein concentration was 2.5 mS/cm, 120 U/ml and 0.17 mg/ml, respectively. Equilibrium data was obtained by filtering in serial several volume fractions of FS up to the saturation of the filter capacity. Adsorbers: filter diameter (area, A), volume fraction filtered (V): ●, 47 mm (17.4 cm^2), 5 ml; ○, two 47 mm (34.8 cm^2), 10 ml; ▽, 90 mm (63,6 cm^2), 20 ml. In all cases the relationship *V*/*A* was 0.29–0.31 ml/cm^2. Filtration conditions were those described in Fig. 3.

3.2. Adsorption experiments with FBC

Given that the main advantage of using membrane adsorbers is to minimize diffusional constraints [3], a filtration experiment was carried out to validate this hypothesis for our system. Typically, 20–100 ml of FBC supernatant at pH 3.0 was passed through a 47 mm GFM and the permeate obtained refiltered twice through the same filter. Activity values measured in the permeates showed that more than 95% of the PG activity was adsorbed after only one passage of the enzyme solution through the filter. It was therefore concluded that under the flow conditions employed, adsorption reached equilibrium almost instantaneously.

Previous studies carried out with *A. kawachii* PGs showed that enzyme adsorption was maximum at pH 3.0 under low ionic strength conditions being negligible at pH 5.0 [6]. Therefore, GkPG capturing was initially studied at pH 3.0 and 5.0 with FBC supernatant either undiluted or diluted (1.3 and 1:10) with deionized water. Adsorption was carried out using a single 47 mm GFM and a V_{FS} of 20 ml. The results are shown in Fig. 3. The amount of adsorbed enzyme at both pHs depended on FS concentration. At the highest dilution (1:10) all the loading activity (~600 U) was adsorbed at both pH values. As FS became more concentrated, the total amount of adsorbed enzyme was higher at pH 3.0, being increased up to a maximum value of ~2000 U/filter. As shown below, this value is close to the saturation filter capacity under these operating conditions. This situation also determined that the proportion of adsorbed enzyme at pH 3.0 was higher in the intermediate dilution. On the other hand, at pH 5.0 not only was the amount of adsorbed enzyme 40% lower but also it was greatly reduced in the undiluted supernatant as compared to the 1:3 dilution.

The GKPG isoelectric point is about 7.7 [8], thus the enzyme displays a net positive charge under acidic conditions, therefore a simple ion exchange mechanism cannot explain the adsorption results at pH 3.0 and 5.0. The differences cannot be attributed either to the ionic strength since the conductivities of protein solutions of same dilution were of the same order. It is generally acknowledged that protein adsorption is always subject to a multitude of different interactions. The surface of borosilicate glass exhibited a variety of functional groups to interact with proteins so that it is difficult to attribute the adsorption to a particular mechanism [14,15]. Probably, some compounds of the culture medium may interfere with enzyme adsorption at pH 5.0 [16]. Further research is needed to understand the molecular mechanism of PG adsorption onto the borosilicate glass fibers. Hereafter adsorption experiments were done with the FBC supernatant diluted 1:3 with deionized water at pH 3.0.

3.3. Adsorption isotherms

Fig. 4 shows three adsorption isotherms in which the relationship between the fraction volume filtered and the cross-section filter area was 0.29 ml/cm^2. With values below this relationship, the isotherm points were rather scattered probably because of poor flow distribution [1]. Statistical analysis (F test) showed that the three isotherms were of the Langmuir type ($R^2 > 0.990$), and were equivalent ($p < 0.05$). The combined adsorption isotherm (solid lane) showed the non-linear fitting of the Langmuir equation to obtain values for q_m and K_d of 98.6 ± 7.5 U/cm^2 (0.14 mg protein/cm^2 or 1.48 mg protein/ml wet bed) and 6.1 ± 3.4 U/mL (0.0087 mg protein/ml).

Although experimental data of PGs adsorption on chromatographic membranes are not available; some comparison is possible with published information concerning conventional or special prepared porous membranes and proteins different from PGs. The affinity constant suggested a favorable affinity of the GkPG for the GFM but the adsorption capacity was lower than the published data >1 mg protein/cm^2 and 20 mg protein/ml wet bed [12,17–21].

3.4. Design of a sorption process

The above results were applied to capture and concentrate GkPG from higher volumes of culture media. Three independent experiments were carried out in which 400 ml of FS (FBC supernatant 1:3 dilution, pH 3.0) was processed for theoretically capturing 85% of the enzyme activity using a filter bed constructed with 90 mm GFM. The total area was estimated in 530 cm^2 (Eq. (2)) which corresponds to a stack of ~8 GFM and the liquid volume of this filter bed was about 25 ml. A complete separation sequence should include a washing and elution step. Previous studies using a single GFM showed that effective desorption (elution) of the PG activity was

Table 1
Recovery of *G. klebahnii* PG from batch cultures using GFM.

Run	FS	Adsorption (%)	Elution (%)	Recovery (%)	CF
1	S (1000)	94	88	83	4.1
2	S (1000)	91	91	83	4.1
3	S (1000)	93	70	65	2.7
4	WB (760)	82	95	78	3.0
5	WB (1070)	85	90	76.5	4.1

Adsorber: Stack of 21 GFM of 110 mm, total area 2000 cm^2. S, supernatant; WB, whole broth containing 5 g/l dry biomass; CF: concentration factor. The supernatant conductivity, PG activity and protein concentration were 1.2 mS/cm, 85 U/mL and 0.12 mg/ml respectively. In brackets V_{FS}. Washing: 200 ml of 10 mM citrate buffer at pH 3.0. Elution: 200 ml of 10 mM citrate at pH 5.0, NaCl 0.5 M.

obtained with different buffer solutions at pH 5.0 whose conductivities were at least 20 mS/cm (not shown). Therefore, in each experiment, the desorption step was performed with an EB consisting of 10 mM citrate buffer, pH 5.0 containing 0.2, 0.5 or 0.75 M NaCl whose conductivities were 26, 38 and 58 mS/cm, respectively. Elution was done by filtering 75 ml of EB (~3 times the liquid volume of the filter bed). Before elution, the filter bed was washed with 100 ml of WB. Adsorption was done by passing FS twice through the filter bed while the washing and desorption steps were achieved by passing the buffer solutions once through the filter bed. Adsorption was slightly lower than the calculated theoretical value, but with a very good batch-to-batch reproducibility (80 ± 2.3%). The effectiveness of the elution process depended on EB conductivity with an optimum at 0.5 M NaCl (95% elution). Under this condition enzyme recovery (Eq. (3)) was 76–80% and the concentration factor about 4.0-fold.

3.5. Reusing the filter bed. Sorption process with the whole broth

Batch cultures without dilution were used to evaluate the reusability of the filter bed. The sorption process was repeated three times, in this case with BC supernatant and twice with BC crude broth, i.e. without prior cell removal. After an adsorption and desorption step, regeneration was accomplished by washing the GFM with 0.5 M NaCl and deionized water. The FS volume was around 1000 ml and the filter bed was constructed with GFM of 110 mm (95 cm^2) size. Although enzyme environment in BC was not the same as the FBC, parameters like conductivity, protein content and PG activity of BC supernatant was in the same order as the 1:3 diluted FBC supernatant. The parameters of the Langmuir equation were then used to design the filter bed, in this case for capturing 95% on the enzyme activity. The total area was estimated in 2000 cm^2 corresponding to ~21 GFM. To complete the sorption cycle, the bed filter was washed with 200 ml of WB and the adsorbed activity eluted with 200 ml of 10 mM citrate buffer, pH 5.0 containing 0.5 M NaCl (Table 1).

The GFM can be reused in multiple adsorption/desorption cycles maintaining the binding capacity performance. Adsorption with the supernatant was within the predicted value by the model but decreased in 10% when the medium was filtered with the biomass. Due to the nature of the GFM and cell morphology, the biomass was trapped on the surface and probably within the filter matrix of the uppermost filter. Then, it is likely that the biomass presence does not allow a good feeding distribution through the filter bed, thus affecting the adsorption. In fact, a third sorption cycle with the whole broth was conducted, but the filter plugging by cells made impracticable the filtration process. Although these drawbacks could be theoretically overcome by replacing the uppermost GFM after each sorption cycle, this was not done.

Elution of the adsorbed activity was near 90% resulting in an enzyme recovery of 80%. In the third sorption cycle there was a decrease in the eluted activity for unknown reasons. The concentration factor in these experiments was about 4.0-fold, as observed with FBC supernatants.

4. Conclusions

The process outlined above demonstrates that the borosilicate glass fiber microfilter, though not designed as membrane adsorber, can be used to capture the *G. klebanii* polygalacturonase directly from the culture media through a simple filtration process. However, it is required a relatively high number of filters (large area) to achieve this goal because the adsorption capacity of the microfilter is limited. The adsorbed activity could be easily eluted with a buffer solution at pH 5.0 containing 0.5 M NaCl. The structural characteristic of the microfilter allows retaining the biomass during filtering and therefore it is possible to recover and concentrate the enzyme directly from a crude broth without prior cell removal and without affecting enzyme recovery yields. From the point of view of purification, the sorption process, in this case, may be considered a polishing step since the enzyme is the only extracellular protein secreted by the fungus into the culture media. The use of GFM can be extended to recover recombinant polygalacturonases that are secreted to the culture medium in high yields. It would also be interesting to study the adsorption of other enzymes to the GFM in order to develop a similar recovery process.

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