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Evaluation of IDA-PEVA hollow fiber membrane metal ion affinity chromatography for purification of a histidine-tagged human proinsulin

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Abstract

Inabilities to process particulate material and to allow the use of high flow rates are limitations of conventional chromatography. Membranes have been suggested as matrix for affinity separation due to advantages such as allowing high flow rates and low-pressure drops. This work evaluated the feasibility of using an iminodiacetic acid linked poly(ethylenevinyl alcohol) membrane in the immobilized metal ion affinity chromatography (IMAC) purification of a human proinsulin$(His)_6$ of an industrial insulin production process. The screening of metal ions showed Ni^{2+} as metal with higher selectivity and capacity among the Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}. The membrane showed to be equivalent to conventional chelating beads in terms of selectivity and had a lower capacity (3.68 mg/g versus 12.26 mg/g). The dynamic adsorption capacity for human proinsulin$(His)_6$ was unaffected by the mode of operation (dead-end and cross-flow filtration).

Keywords: Proinsulin-$(His)_6$; Purification; Affinity membrane; IMAC

1. Introduction

Membrane affinity chromatography has long been pointed as a solution to process related problems in the downstream of protein products. Among these problems are the high-pressure drop at high throughput and the need to operate with particle-free feed solutions, typical drawbacks of fixed-bed chromatography [1–4].

The strategy of recombinant fusion proteins to facilitated purification is a well-documented established technology [5]. Among many fusion–separation method systems, the histidine tag-immobilized metal ion affinity chromatography (His tag-IMAC) has been used to routinely purify a large variety of recombinant proteins from glutaryl acylase to insulin and at laboratory and commercial scale, using fixed and expanded beds [6–13]. IMAC is based on the fact that accessible histidine (His) residues in proteins bind with relatively high affinity to electropositive transition metals including Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} [14]. When four or six contiguous histidine residues are engineered into a tag, a stable ligand is formed that can bind to a metal chelate affinity column and withstand extensive washing for removal of non-specific bound proteins [6,15].

The coupling of the two approaches, membrane affinity chromatography and fusion proteins, to solve the mentioned problems of high flow rate and the presence of particles in feed solutions has been studied by Cattoli and Sarti [16], who used amylose linked native cellulose membrane to purify β-galactosidase and rubredoxin fused to the maltose binding protein domain [16]. Also, immobilized nickel affinity flat membrane adsorber was used as stationary phase for purification of recombinant (His_6-tagged) EcoR V, an *Escherichia coli* restriction endonucelase, from cell lysates of *E. coli* [17]. Nevertheless, data on purification of fusion proteins on immobilized metal ion affinity hollow fiber membranes are not available.

Affinity membranes with the hollow fiber configuration have the advantage of providing high surface area to volume ratio for

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separation. In this design, cross-flow operation is feasible and an effective separation for crude extract solutions or suspensions can be achieved [18]. In a recent work, an IMAC affinity hollow fiber membrane system – an iminodiacetic acid covalently linked poly(ethylenevinyl alcohol) membrane, called IDA-PEVA – was evaluated for the purification of anti-TNP IgG_1 mouse monoclonal antibody. The antibody was efficiently adsorbed and eluted with its antigenic properties conserved [19]. The present work evaluated the feasibility (in terms of capacity and selectivity) of using this IDA-PEVA membrane in the IMAC purification of a histidine-tagged human proinsulin of an industrial insulin production process. The effects of different metal ions (Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}) and operating variables (type of feed, inlet flow rate and mode of operation) on the capacity and selectivity of the system were investigated. Initial experiments were designed to determine the most appropriate chelated metal ion among Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}. The affinity membrane was then compared to a packed bed of chelating agarose for different aspect including the ease of washing and elution. The binding was also studied and the resulting adsorption isotherm analyzed with the Langmuir model for the determination of the dissociation constant and maximum binding capacity. The effects of the concentration of contaminant proteins and the filtrate flow rate on proinsulin-$(His)_6$ breakthrough curves were also studied.

2. Materials and methods

2.1. Materials

The three proinsulin preparations used in this work were produced by BIOMM (Brazil). The low purity preparation, a particulated sulfonated proinsulin-$(His)_6$ suspension containing 35% of sulfonated proinsulin-$(His)_6$ according to the supplier, named SPI in this work, was the result of processing recombinant *E. coli* homogenate. The clear supernatant obtained after centrifugation of SPI ($9000 \times g$ for 30 min) was named cSPI (38% of sulfonated proinsulin-$(His)_6$ analyzed with a HPLC). The other two preparations, renaturated proinsulin-$(His)_6$ (not sulfonated) and the relatively high purity sulfonated proinsulin-$(His)_6$, containing 76 and 84% of proinsulin-$(His)_6$, respectively, according to the supplier, were named rPI and hSPI in this work.

Sepharose 6B was purchased from Amersham Biosciences (Sweden). Epichlorohydrin and Coomassie Brilliant Blue were purchased from Merck (Germany). Tris(hydroxyethyl amino ethane), copper sulphate, zinc sulphate, nickel sulphate, cobalt sulphate, disodium ethylenediamine tetra-acetate (EDTA), β-mercaptoethanol, crystalline bovine serum albumin (BSA), iminodiacetic acid (IDA) and imidazole were purchased from Sigma (USA). The stirred ultrafiltration cell YM3 membrane (nominal molecular mass cut-off of 3 kDa), and Centricon YM3 (nominal molecular mass cut-off of 3 kDa) were purchased from Millipore (USA). The water used in all experiments was ultrapure water obtained using a Milli-Q System (Millipore, USA). All other chemicals were of analytical reagent grade.

The poly(ethylenevinyl alcohol) (PEVA)-hollow fiber membrane cartridges (Model Eval 4A, 1 m^2 surface area, approximately 6000 hollow fibers) were purchased from Kuraray (Japan). The hollow fiber had an internal diameter of 200 μm, a wall thickness of 20 μm and a nominal molecular mass cut-off of 600 kDa.

2.2. Methods

2.2.1. Agarose activation and IDA immobilization

Sepharose-6B activation with epichlorohydrin and coupling to IDA were carried out as described by Porath and Olin [14]. The chelating capacity of the gel for Cu^{2+} ion was determined according to the method described by Belew and Porath [20]: about 60 μmol of Cu^{2+} per gram of the dry gel. The resulting adsorber gel is referred as to IDA-Sepharose in this work.

2.2.2. Immobilization of IDA onto PEVA hollow fiber membrane

2.2.2.1. Cut membrane derivatization. A commercially available PEVA hollow fiber membrane cartridge was disassembled, the fibers were removed and finely cut to pieces of approximately 2 mm in length. The cut PEVA fibers were activated with epichlorohydrin as described by Bueno et al. [21] and coupled to IDA as described by Porath and Olin [14]. The Cu^{2+} chelating capacity of these cut fibers was about 40 μmol of Cu^{2+} per gram of dry membrane (determined by atomic absorption spectrometry using EDTA solution as a blank). The derivatized membrane is referred to as IDA-PEVA, and, in this work, this membrane with immobilized metal ion is referred to as Me^{2+}-IDA-PEVA (where Me^{2+} was Cu^{2+}, Ni^{2+}, Zn^{2+} or Co^{2+}).

2.2.2.2. Minicartridge derivatization. A small-scale cartridge of PEVA hollow fibers membrane was manufactured using fibers from a commercially available cartridge. The fibers were cut and assembled in a minicartridge with effective length of 5.7 cm. The amount of fibers in this cartridge was 0.14 g (dry mass) with 71.6 cm^2 of surface area and 0.16 cm^3 of fiber volume. The hollow fiber volume (V_b) was calculated as follows:

$$V_b = \pi(r_o^2 - r_i^2)L_e N_f \quad (1)$$

where r_o and r_i are the outer and inner radius of the hollow fibers, respectively, L_e is the length and N_f is the number of hollow fibers in the minicartridge (200). The PEVA fibers in this small-scale cartridge were modified with epichlorohydrin and IDA as described in this section.

2.2.3. Determination of protein concentration

Samples were analyzed for total protein with the Bradford method [22] (BSA used as a reference protein). Samples of the chromatographic experiments of SPI and cSPI solutions were also analyzed for sulfonated proinsulin concentration with a HPLC methodology using a Mono-Q HR 5/5 column (Amersham Biosciences, Sweden) and a Breeze HPLC System (Waters, USA). Bound proteins were eluted using a stepwise salt gradient strategy: first pure buffer A (0.050 mol/L Tris–HCl 6.0 mol/L of urea pH 8.0) to 50% buffer B (buffer A with 1.0 mol/L NaCl) in 9 min, followed by 50–80% buffer B in 3 min,

80% buffer B for 2 min, 80–81% buffer B in 1 min, 81–100% buffer B in 1 min and 100% buffer B for 2 min.

2.2.4. Sodium dodecyl sulfate–polyacrylamide gel elecrophoresis (SDS–PAGE)

The chromatographic fractions were analyzed by SDS–PAGE (15% acrylamide gels) under reducing conditions with a Protean II System (Bio-Rad, USA) according to Laemmli [23]. The gels were stained with silver nitrate according to the protocol developed by Morrissey [24]. A standard protein mixture (Amersham Biosciences, Sweden) was used as a molecular mass protein marker.

2.2.5. Column preparation

The IDA-PEVA cut fibers (1.25 g dry mass) and IDA-Sepharose gel (1.65 g dry mass) were suspended in the buffer C (0.030 mol/L Tris–HCl pH 7.5), degassed and packed into 10 cm × 1.0 cm I.D. columns (Amersham Biosciences, Sweden) to give bed volumes of approximately 5.0 mL. Copper, nickel, zinc or cobalt ions were loaded in the IDA-PEVA column, and nickel ion was loaded in the IDA-Sepharose column by pumping 0.050 mol/L sulphate solution of the given metal ion in water through the column until saturation. The matrix saturation was detected visually for all ions except for zinc that was detected in the out-stream by titration with 2.0 mol/L Na_2CO_3 [25]. Non-specifically bound metal was removed from the column by washing it sequentially with the adsorption and elution buffers used in chromatographic runs described below. The column was equilibrated with adsorption buffer (buffer C containing 0.5 mol/L NaCl and 7.5 mol/L urea) when no further metal was detected in the out-stream.

2.2.6. Fixed-bed chromatographic experiments

All chromatographic experiments were carried out using an automated Econo Liquid Chromatography System (Bio-Rad, USA) at 25 °C with continuous 280 nm absorbance measurement of the out-stream. The linear velocity of solutions through the column was 38.2 cm/h (30.0 mL/h). The metal ion saturated columns of IDA-PEVA cut fibers and IDA-Sepharose gel were washed with acetate buffer (0.025 mol/L sodium acetate with 1.0 mol/L NaCl pH 4.0) and then equilibrated with buffer C.

The rPI solution (about 17 mg of protein) in adsorption buffer was loaded into the column at 0.5 mL/min. For the experiments with cSPI, solutions containing about 70 mg of total protein (cSPI diluted 1:1 in the adsorption buffer) were loaded into the column. After protein injection, the column was washed using a sequence of buffer C containing 0.020 mol/L of imidazole; buffer C, and buffer C containing 6.0 mol/L urea until no protein was detected in the column out-stream [10]. Adsorbed proteins were subsequently eluted with buffer C containing 6.0 mol/L urea and 0.1 mol/L of imidazole. Regeneration of the column (removal of remaining adsorbed proteins) was achieved by washing the column with 0.050 mol/L of EDTA solution buffered with the equilibration buffer without NaCl at pH 8.0.

Fractions of 2.0 mL were collected during chromatographic experiments and their protein concentration was determined by the Bradford method [22]. The fractions were then pooled and concentrated using a stirred ultrafiltration cell with a YM3 membrane for SDS–PAGE analysis and sulfonated proinsulin quantification by HPLC.

2.2.7. Determination of adsorption isotherm

The protein adsorption experiments for isotherm determination were carried out using hSPI solutions in triplicate at 25 °C. The Ni^{2+}-IDA-PEVA finely cut fibers (19.4 mg dry mass) were weighed in 1.5 mL Eppendorf tubes. The cut fibers were equilibrated with buffer C for 15 min. Then aliquots of 1.0 mL of hSPI solution were added to Eppendorf tubes (hSPI solution diluted with adsorption buffer) to total protein concentration from 0.5 to 9.3 mg/mL. The tubes were rotated end-over-end at 6 rpm for 3 h to allow equilibrium to be established. After 3 h, the cut fibers and the liquid phase were separated by centrifugation and the unbound protein concentration in this liquid phase (C) was measured by UV spectrophotometry at 280 nm. The adsorbed protein mass, Q, was determined as the difference between the amount of protein added and that present in the liquid phase after equilibrium divided by the dry mass of the adsorbent (19.4 mg). Data were fitted to the Langmuir isotherm model [26] (Eq. (1)) using non-linear least squares and the Levenberg–Marquardt method:

$$Q = \frac{Q_m C}{K_d + C} \quad (1)$$

where Q_m is the maximum adsorbate binding capacity and K_d is the apparent dissociation constant, which represents the affinity between adsorbate and adsorbent.

2.2.8. Minicartridge experiments

Chromatography experiments with the minicartridge were carried out at 25 °C with the automated chromatography system used for the fixed-bed chromatography experiments. Two different filtration modes were used. First, experiments were carried out with an open loop dead-end mode (the lumen outlet was closed with a clamp so that all the fluid was drawn by the shell-side pump through the membrane pores) at filtrate flow rates of 0.50 and 0.30 mL/min, with residence time, t_R, of 14 and 24 s, respectively (t_R was calculated by dividing the membrane interstitial volume by the filtrate flow rate [27]. In the second filtration mode, the experiments were carried out at cross-flow filtration mode (the solution was fed through the affinity cartridge by the lumen-side pump, and part of the fluid (filtrate) was drawn through the membrane pores and out of the shell by the shell-side pump) at inlet flow rates of 0.70 and 0.43 mL/min, and the retentate was recirculated to the feed tank until its liquid volume reached 10 mL while the filtrate was discarded. In the cross-flow filtration mode, the inlet flow rate (Q_i) and filtrate flow rate (Q_F) were kept constant using two peristaltic pumps with a fixed Q_F/Q_i ratio equal to 0.70. For both filtration modes, the filtrate outlet stream passed through a detector where its absorbance at 280 nm was monitored.

Prior to the experiments, buffer C was pumped through the Ni^{2+}-IDA-PEVA minicartridge for 15–20 min in dead-end mode. A volume of 60 mL of protein feed (SPI or cSPI solutions diluted 1:1 with adsorption buffer) was pumped through

the minicartridge. After loading of the protein solution, the unadsorbed protein was washed out sequentially with buffer C containing 0.02 mol/L imidazole, buffer C, and buffer C containing 6.0 mol/L of urea.

Four washing steps at different modes were used [19]: cross-flow filtration, lumen, shell and backflushing wash. The lumen side of the fibers was washed by pumping buffer into the lumen inlet with the filtrate exit valves closed. For washing the shell side of the fibers, buffer was pumped into the shell inlet and out of the shell outlet to the waste (retentate exit valve was kept closed). The backflushing wash was carried out by closing the shell outlet and pumping buffer into the shell inlet, through the membrane, and out of the lumen outlet. For each of these steps, washing was stopped when the absorbance of effluent at 280 nm reached the baseline.

The adsorbed protein was eluted in backflushing mode with a discontinuous step gradient of imidazole: 0.1–0.3 mol/L imidazole in buffer C containing 6.0 mol/L of urea and 0.5 mol/L NaCl at the same inlet flow rate. The effluents were monitored as described previously.

After elution was completed, the cartridge was sequentially washed at frontal mode with 0.050 mol/L of EDTA pH 6.5 and with the buffer C to restore it to its initial conditions for carrying out the next experiment.

Protein concentration of the non-adsorbed and eluted fractions was determined by the Bradford method [22]. Protein molecular mass profile of the breakthrough and the non-adsorbed and eluted fractions were analyzed by SDS–PAGE under reducing conditions. Breakthrough curves were plotted as the ratio of the protein concentration in the filtrate (C_f) to that in the feed stream (C_o) as a function of the volume of protein solution throughput.

3. Results and discussion

3.1. Evaluation of the metal ions for the adsorption of proinsulin

Prior to the study of adsorption of proinsulin from SPI and cSPI solutions, the effect of the chelated metal ion on adsorption capacity of proinsulin was investigated at fixed-bed chromatography with finely cut IDA-PEVA fibers. In order to identify the best metal ion among Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}, preliminary adsorption experiments were carried out using imidazole as a competitive agent and rPI solutions at 6.0 mg/mL of protein in adsorption buffer. A washing buffer containing imidazole at concentration of 0.020 mol/L was utilized to eliminate adsorption of weakly bound proteins. After washing the non-adsorbed or weakly adsorbed proteins out of the column, the imidazole concentration in the washing buffer was increased to 0.1 mol/L to elute the adsorbed proinsulin, and EDTA was used to remove the chelated metal ions and strongly bound proteins (Table 1). The protein content of the rPI preparation (mostly proinsulin) was almost completely adsorbed in Cu^{2+}-IDA-PEVA and Ni^{2+}-IDA-PEVA. Only 3.3 and 3.4% of the loaded protein was displaced from Cu^{2+}-IDA-PEVA and Ni^{2+}-IDA-PEVA, respectively, at the washing steps, while for Zn^{2+}-IDA-PEVA and Co^{2+}-IDA-PEVA, 39.3 and 96.4% of protein was displaced, respectively.

The empirical ordering for the strength of adsorption of proteins with accessible histidines onto immobilized metals ($Cu^{2+} > Ni^{2+} > Zn^{2+} \geq Co^{2+}$) established by Sulkowski [28] was observed in these experiments; increased mass of protein were eluted out of the column as the metal ion was changed from Co^{2+} to Zn^{2+}, Ni^{2+} and Cu^{2+}. Thus, among the tested metal ions, Cu^{2+} and Ni^{2+} were selected for the subsequent experiments.

3.2. Cu^{2+}-IDA-PEVA and Ni^{2+}-IDA-PEVA fixed-bed chromatographies of cSPI

To evaluate the binding efficiency of proinsulin to Cu^{2+}-IDA-PEVA and Ni^{2+}-IDA-PEVA, the same amount of cSPI solution was chromatographed in fixed-bed columns of these Me^{2+}-IDA-PEVA cut fibers (Table 2, Fig. 1). The total protein adsorption capacities of these two adsorbents for the case of this feed solution (maximum of 5.2 mg for the case of Ni^{2+}-IDA-PEVA) were lower when compared with the case of rPI as the feed solution (minimum of 13.65 for the case of Ni^{2+}-IDA-PEVA). A higher adsorption capacity was observed for Ni^{2+}-IDA-PEVA than for the adsorbent with Cu^{2+} (4.60 mg of proinsulin for the former and 1.13 mg for the latter). Moreover, the Ni^{2+}-IDA-PEVA had a higher selectivity for proinsulin than for contaminant proteins: fewer contaminants where present at the eluted fractions

Table 1
Mass balances of fixed-bed chromatographies of rPI[a] for the evaluation of the different metal ions

	Protein recovery							
	Cu^{2+}		Ni^{2+}		Zn^{2+}		Co^{2+}	
	(mg)[b]	(%)[c]	(mg)[b]	(%)[c]	(mg)[b]	(%)[c]	(mg)[b]	(%)[c]
Feed	18.00	100.0	15.40	100.0	15.00	100.0	16.60	100.0
Washing	0.60	3.3	0.53	3.4	5.90	39.3	16.00	96.4
Elution	17.23	95.7	13.65	88.6	7.15	47.7	3.14	18.9
Regeneration[d]	0.18	1.0	0.31	2.0	0.15	1.0	0.07	0.4
Total	18.01	100.0	14.50	94.1	13.20	88.0	19.21	115.7

[a] Chromatographies with finely cut Me^{2+}-IDA-PEVA fibers.
[b] Mass calculated from protein concentration determined by Bradford [22].
[c] Percentage relative to injected protein mass.
[d] EDTA concentration: 50 mmol/L.

Table 2
Mass balance of fixed-bed chromatographies of cSPI for comparison of Cu^{2+}-IDA-PEVA and of Ni^{2+}-IDA-PEVA

Method	Adsorbent	Feed (mg)	Washing (mg)	Elution (mg)	Regeneration (mg)	Recovery (mg)
Bradford (total protein)	Cu^{2+}-IDA-PEVA	70.24	53.36	2.53	0.13	56.02
	Ni^{2+}-IDA-PEVA	68.11	61.00	5.20	0.22	66.42
HPLC (sulfonated proinsulin)	Cu^{2+}-IDA-PEVA	15.85	13.61	1.13	–	14.74
	Ni^{2+}-IDA-PEVA	16.12	8.20	4.60	–	12.8

Bed volumes of 5.0 mL of finely cut Me^{2+}-IDA-PEVA fibers.

(Fig. 1c). This suggests that the binding between the Ni^{2+}-IDA-PEVA and proinsulin is more specific and that this adsorber has a higher capacity when compared to the Cu^{2+}-IDA-PEVA fibers.

According to Sulkowski, [28] and Porath [29], for IMAC with metal ions immobilized on IDA, the selectivity of the adsorption increases, whereas the amount of bound protein decreases in the following order: Cu^{2+}-IDA, Ni^{2+}-IDA, Zn^{2+}-IDA and Co^{2+}-IDA. It is surprising to observe for the cSPI proinsulin solution that Cu^{2+} ions immobilized on IDA-PEVA have a lower amount of bound sulfonated proinsulin than the amount for Ni^{2+}. This result seems to contradict the one verified for rPI solutions, but we have to note that the feed solution was different in each case. The lower adsorption capacity observed for Cu^{2+}-IDA-PEVA may also be due to a damage caused by metal-catalyzed oxidation reactions (MCO) of the sulfonated proinsulin. The occurrence of MCO reactions in IMAC systems for immobilized Cu^{2+}-IDA in the presence of reducing agents such as ascorbate and thiol agents (GSH, cysteine, etc.) has been reported by Krishnamurthy et al. [30] and Bush and Lumpkin [31]. The addition of these reducing agents is relevant to bioprocess as thiol reducing agents such as dithiothreitol, glutatione, sulfite, and β-mercaptoethanol in the millimolar range are often used for refolding and solubilization of inclusion bodies [30]. The results obtained by Krishnamurthy et al. [30] imply that Cu^{2+}-IDA chromatography may result in a damaged protein product when using crude extract containing reductants and/or oxidants.

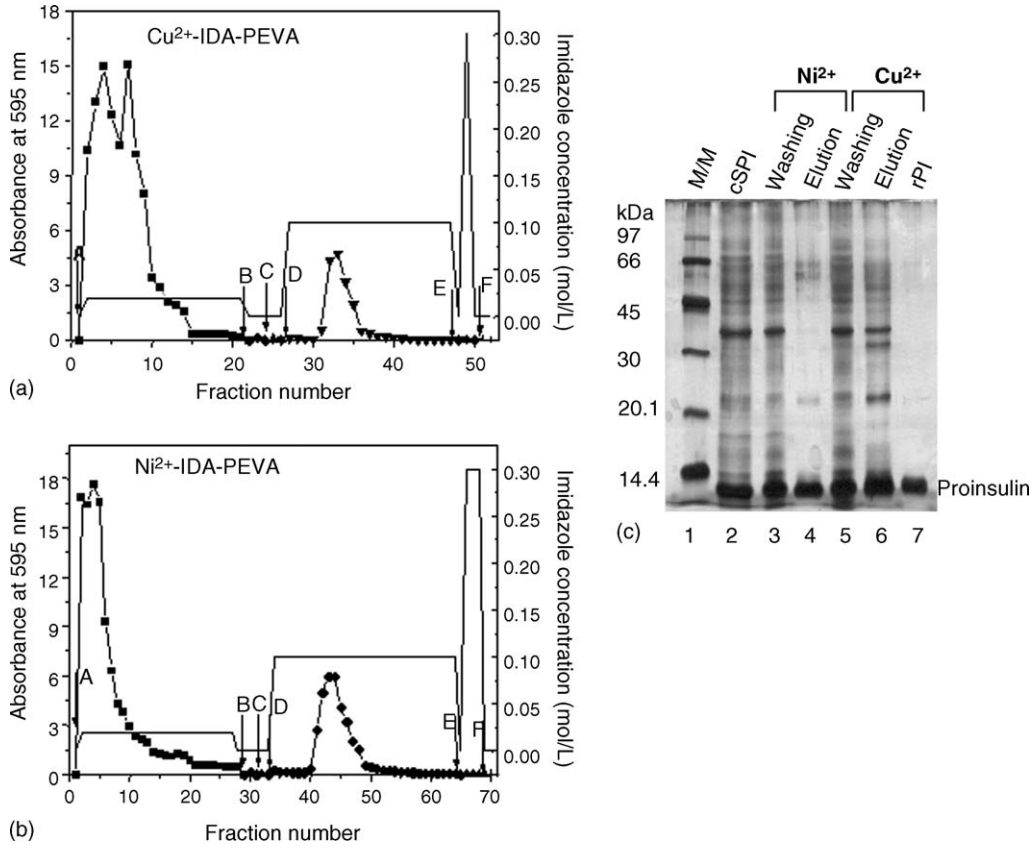

Fig. 1. Chromatography of proinsulin from cSPI solutions onto: (a) Cu^{2+}-IDA-PEVA and (b) Ni^{2+}-IDA-PEVA on fixed-bed columns of finely cut fibers; bed volume, 5.0 mL. Injection: 20 mL at 3.5 mg/mL of total protein. Flow rate: 0.5 mL/min. Washing: (A) buffer C containing 0.02 mol/L of imidazole; (B) buffer C; and (C) buffer C containing 6 mol/L of urea. Elution: (D) buffer C containing 6 mol/L of urea, 0.05 mol/L NaCl, and 0.1 mol/L of imidazole; and (E) buffer C containing 6 mol/L of urea, 0.05 mol/L of NaCl, and 0.3 mol/L of imidazole. Regeneration: (F) EDTA 0.05 mol/L pH 8.0. (c) SDS–PAGE analysis under reducing conditions (15% acrylamide). (Lane 1) MM (molecular mass makers). (Lane 2) cSPI solution. (Lane 3) Washing fractions from Ni^{2+}-IDA-PEVA chromatography. (Lane 4) Elution fractions of Ni^{2+}-IDA-PEVA chromatography. (Lane 5) Washing fractions from Cu^{2+}-IDA-PEVA chromatography. (Lane 6) Elution fractions of Cu^{2+}-IDA-PEVA chromatography. (Lane 7) rPI.

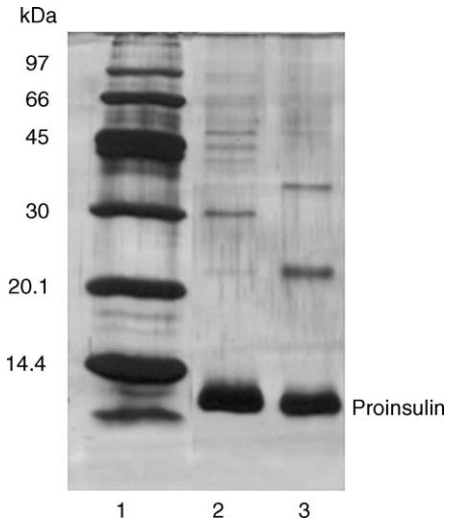

Fig. 2. SDS–PAGE analysis under reducing conditions (15% acrylamide): (lane 1) molecular mass makers; (lane 2) elution fractions of Ni^{2+}-IDA-Sepharose chromatography; and (lane 3) elution fractions of Ni^{2+}-IDA-PEVA chromatography.

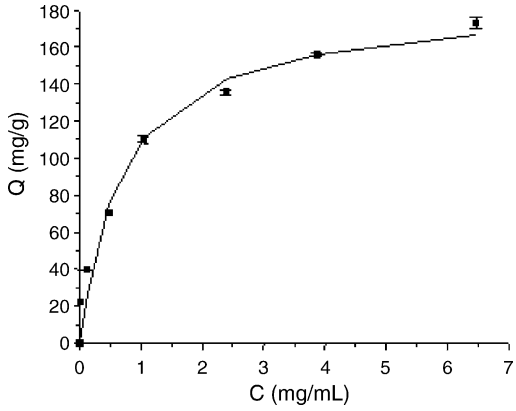

Fig. 3. Experimental adsorption isotherms (symbol) for hSPI on Ni^{2+}-IDA-PEVA with buffer C (0.03 mol/L Tris–HCl pH 7.5 at 25 °C). The solid lines correspond to fitting (non-linear regression) of experimental values on the basis of Langmuir model.

In the case of proteins, MCO reactions occur at metal binding sites on the protein, and the oxidative product attacks the side chains of amino acids at that site [32,33]. Amino acids that are most susceptible to MCO reactions are histidine, lysine, cysteine, proline, arginine and methionine [34,35]. Since the proinsulin studied here has a His-tag and the cSPI solution contains a reduction agent (sodium sulfite), MCO reactions can be suggested as the cause of low sulfonated proinsulin adsorption in Cu^{2+}-IDA-PEVA.

Thus, since Ni^{2+}-IDA-PEVA was the ligand that provided higher selectivity and capacity for the adsorpotion of proinsulin, it was chosen to be used in the experiments that followed.

3.3. Comparison of proinsulin chromatographies on fixed beds of affinity cut fibers and affinity gel

After selecting a metal ion (Ni^{2+}) for binding the proinsulin, experiments were performed to compare the performance of the affinity membrane (Ni^{2+}-IDA-PEVA, bed volume of 5 mL, 1.25 dry mass) and affinity gel (Ni^{2+}-IDA-Sepharose, bed volume of 5 mL, 1.65 dry mass) for adsorption of sulfonated proinsulin from cSPI solution.

The selectivity of Ni^{2+}-IDA-PEVA for proinsulin was similar to that of Ni^{2+}-IDA-Sepharose (Fig. 2). However, the adsorption capacity was much higher for Sepharose-based adsorbent (12.26 mg of protein/g adsorbent) than for membrane-based adsorber (3.68 mg of protein/g adsorbent) (Table 3). Although it showed a relative low capacity, the Ni^{2+}-IDA-PEVA required a shorter time for the purification: the washing step was completed within 80 min whereas for Ni^{2+}-IDA-Sepharose it took 240 min. A strong brown colour band (accumulated protein) was formed at the inlet bottom of the Ni^{2+}-IDA-Sepharose column increasing the washing step time. During washing steps, the brown colour band started smearing up the column of Ni^{2+}-IDA-Sepharose and completely disappeared after washing with buffer C containing 6.0 mol/L of urea.

3.4. Thermodynamics of proinsulin adsorption

In order to evaluate the thermodynamic parameters binding capacity (Q_m) and dissociation constant (K_d), adsorption isotherms for Ni^{2+}-IDA-PEVA were determined using hSPI. The experimental data were obtained from batch adsorption experiments at 25 °C with adsorption buffer (Fig. 3). The isotherm was analyzed using the Langmuir model (Table 4). The Langmuir isotherm equation describes satisfactorily the adsorption data (correlaction coefficient = 0.99). The K_d values measured for the Langmuir model were of the order of 10^{-5} M, indicating a medium affinity, which is typical for this kind of pseudobiospecific affinity ligand [36].

Table 3
Sulfonated proinsulin mass balances of fixed-bed chromatographies of cSPI solution for comparison of Ni^{2+}-IDA-PEVA cut fibers and Ni^{2+}-IDA-Sepharose beads[a]

Adsorbent	Feed (mg)	Washing (mg)	Elution (mg)	Proinsulin adsorbed (percentage of protein fed)	Adsorbed proinsulin (mg/g dry adsorbent)
Ni^{2+}-IDA-PEVA[b]	15.97	8.20	4.60	28.8	3.68
Ni^{2+}-IDA-Sepharose[c]	26.37	4.75	20.23	76.7	12.26

[a] Analysis with HPLC.
[b] Bed volume: 5.0 mL, 1.25 g of dry mass (injection of 12.78 mg proinsulin/g cut fiber).
[c] Bed volume: 5.0 mL, 1.65 g of dry mass (injection of 15.98 mg proinsulin/g gel).

Table 4
Thermodynamic parameter for the adsorption of hSPI to Ni^{2+}-IDA-PEVA

Parameters	Values
Q_m (mg/g)	184.28 ± 10.78
K_d (mol/L)	$(7.73 \pm 1.72) \times 10^{-5}$
Coefficient of correlation	0.989
Variance	84.34
Standard deviation	9.18

Variance $= \sum (Q_{calculated} - Q_{experimental})^2/(N-1)$; Standard deviation = (variance)$^{1/2}$.

3.5. Minicartridge experiments: effects of feed composition and flow conditions on the adsorption of proinsulin

Breakthrough curves were determined to study the effect of the nature of the proinsulin solution (cSPI and SPI) on Ni^{2+}-IDA-PEVA cartridge filtration (cross-flow mode of operation) at filtrate flow rate of 0.5 mL/min (Figs. 4 and 5). The curves for total protein (quantified by the Bradford method) were S-shaped: at first, no protein was detected in the flow out of the cartridge until the breakthrough point was achieved and protein was detected at increasing concentrations. At the end, saturation was achieved when a plateau was formed. The breakthrough point was approximately the same for both types of feed (in the range of 6–9 mL). For the case of cSPI, the SDS–PAGE analysis of the fractions corroborate this finding: a protein band with molecular mass of approximately 9 kDa (approximately the molecular mass of proinsulin) was weakly detected in SDS–PAGE gel, reflecting extensive adsorption of the proinsulin by the immobilized Ni^{2+} until the breakthrough point was achieved and an intense band was present at fractions collected later on (although C/C_o is close to zero before breakthrough, one must consider the high protein concentration in the feed and that the sensitivity of the SDS–PAGE silver staining is much higher than the Bradford method, detecting proinsulin that is not adsorbed in the pores due to the dynamic state of the system and that passed through the pores on the initial portion of the fiber which gets saturated before its terminal part). However, this phenomenon was not observed for SPI feed solution, where a protein band corresponding to a molecular mass of the proinsulin was detected in all fractions. The total mass of protein adsorbed was calculated for both cases from the amount of protein eluted in backflushing mode: 20.77 and 15.40 mg of total protein/g dry membrane, respectively, for cSPI and SPI feed solutions. The results were similar to those obtained with the Ni^{2+}-IDA-PEVA cut fibers (data not shown).

Minicartridge chromatographic experiments of different proinsulin solutions were carried out in dead-end and cross-flow filtration modes with different inlet flow rates and feed streams (Table 5). The protein mass eluted increased with decreasing filtrate flow rate for both filtration modes. No significant difference was observed in the protein adsorption capacity from cSPI solution when the cross-flow and dead-end filtration mode were tested. Decreasing the flow rate from 0.3 to 0.1 mL/min did not change the results (data not shown). When SPI solution was used as feed, it was impossible the application of dead-end filtration mode, probably because of the presence of particulate material.

Feeding hSPI, the dynamic capacity (17.00 mg/g) was much lower than the equilibrium capacity (approximately 184 mg/g, Table 4). An explanation for this may be based on the fact that the fiber pore sizes are distributed over a range of values. The per-

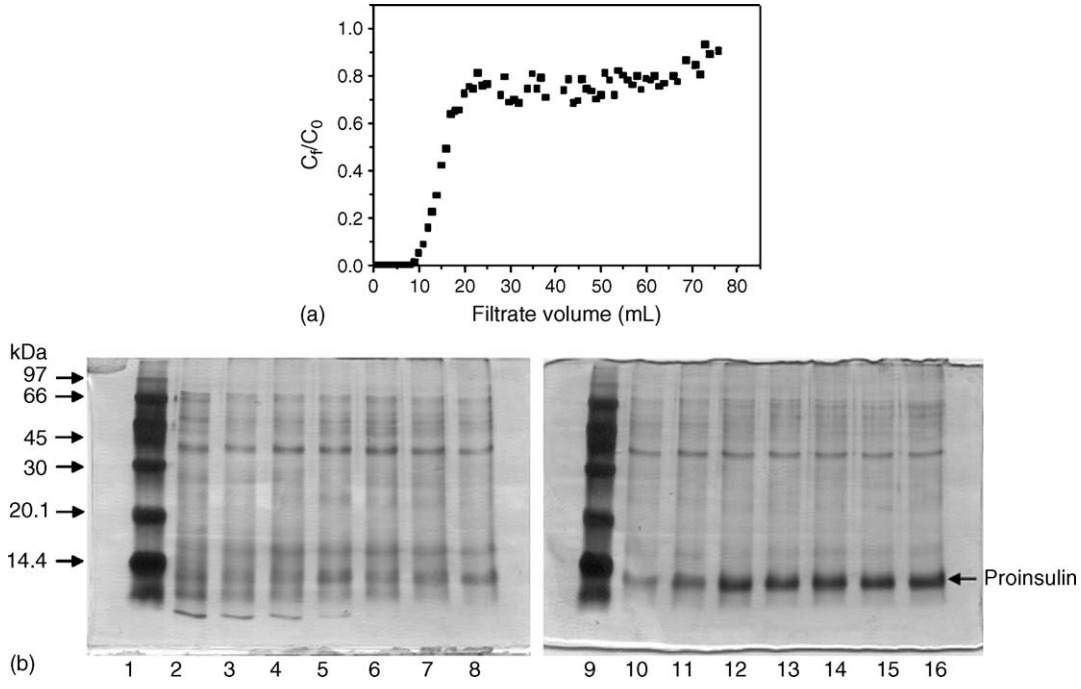

Fig. 4. Cross-flow filtration of cSPI solution on the minicartridge: (a) breakthrough curve (based on the total protein concentration) on Ni^{2+}-IDA-PEVA minicartridge for cSPI as feed stream at initial protein concentration (C_o) of 3.3 mg/mL and filtrate flow rate of 0.5 mL/min and (b) SDS–PAGE analysis under reducing conditions of the samples from the breakthrough experiments. Numbered lanes represent aliquots of the corresponding fractions, except for lanes 1 and 9 which contained protein molecular mass markers.

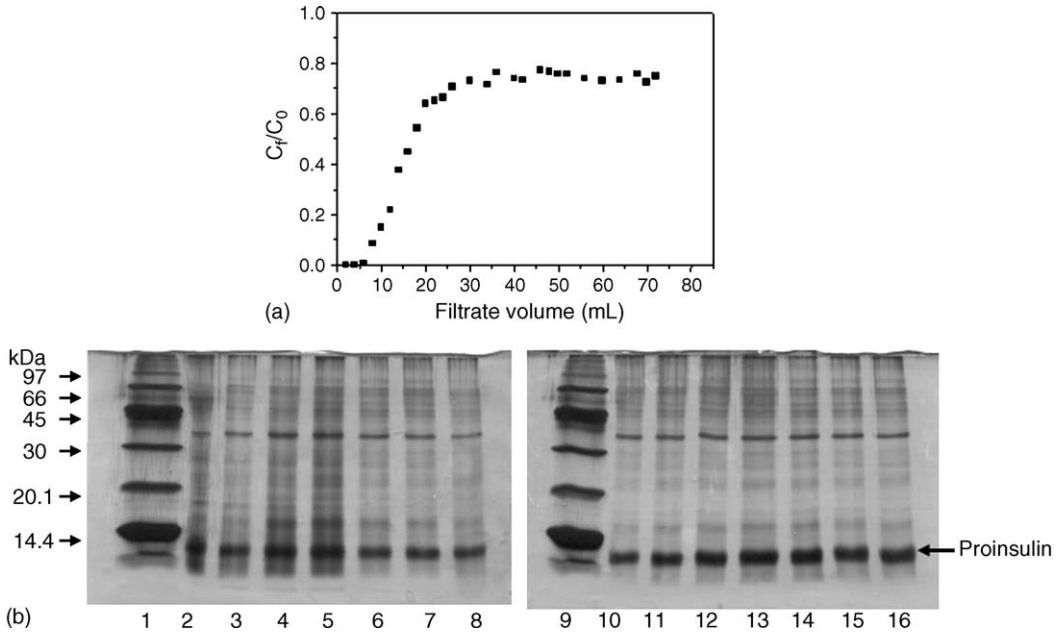

Fig. 5. Cross-flow filtration of SPI solution on the minicartridge: (a) breakthrough curve (based on the total protein concentration) on Ni^{2+}-IDA-PEVA minicartridge for SPI as feed stream at initial protein concentration (C_o) of 3.5 mg/mL and filtrate flow rate of 0.5 mL/min and (b) SDS–PAGE analysis under reducing conditions of the samples from the breakthrough experiments. Numbered lanes represent aliquots of the corresponding fractions, except for lanes 1 and 9 which contained protein molecular mass markers.

Table 5
Effect of operating conditions and feed composition on protein adsorption on Ni^{2+}-IDA-PEVA cartridge

Filtration mode	Feed stream	Inlet flow rate (mL/min)	Filtrate flow rate (mL/min)	Initial protein concentration (mg/mL)	Injection (mg)	Elution (mg)	Dynamic adsorption capacity (mg/g)
Dead-end	cSPI	0.50	0.50	3.10	186.00	2.26	20.00
		0.30	0.30	3.60	187.20	5.10	42.00
	hSPI	0.70	0.50	2.70	162.00	2.17	17.00
Cross-flow	cSPI	0.70	0.50	3.30	198.00	2.10	20.77
		0.43	0.30	3.40	187.00	4.75	40.00
	SPI	0.70	0.50	3.50	247.30	1.80	15.40
		0.43	0.30	3.70	222.30	6.90	56.46

Analytical method: 595 nm (Bradford method).

fusion flow at constant pressure will pass preferentially through the larger pores, as the flow is inversely proportional to pore size. Therefore, the Ni^{2+}-IDA sites on the wall of the large pores will be saturated first, and further perfusion of protein solution does not lead to any capture of proinsulin. If the small pores, which have not saturated with proinsulin, are not perfused, the capacity of the fibers must be lower in the dynamic mode than in the equilibrium mode where preferential perfusion does not apply [37].

Protein profiles given by SDS–PAGE analysis of eluted fractions from the minicartridge were similar to the ones verified for eluted samples from fixed bed of cut fibers (data not shown). No reduction of the transmembrane flow was experienced when feeding SPI solution. Removal of proteins in the washing step was easily carried out by inversion of flow (backflushing mode).

4. Conclusion

The combinantion of affinity membrane chromatography and an affinity fusion strategy is known as a simple and efficient protein recovery and purification process. We have demonstrated that the Ni^{2+}-IDA-PEVA hollow fiber membrane system is a potential alternative for the purification of recombinant proinsulin-$(His)_6$. The occurrence of MCO reactions in the Cu^{2+}-IDA-PEVA columns are probable, since Cu^{2+} immobilized in IDA-PEVA had a lower amount of bound sulfonated proinsulin than the Ni^{2+} column. The performance of the Ni^{2+}-IDA-PEVA membrane compared to the conventional agarose bead system – Ni^{2+}-IDA-Sepharose – showed a similar selectivity and a lower capacity for the membrane configuration. Due to the high selectivity of the separation and the high efficiency of the washing procedure observed in this work, associated with the high productivity expected for membrane systems, we considered the Me^{2+}-IDA-PEVA membrane a potential system for the purification of other His-tag proteins.

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