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Anayla S. Sousa[a]; Artemízia P. Guimarães[a]; Caroline V. Gonçalves[a]; Ivanildo J. Silva Jr.[a]; Celio L. Cavalcante Jr.[a]; Diana C. S. Azevedo[a]
[a] Departamento de Engenharia Química, Grupo de Pesquisa em Separações por Adsorção (GPSA), Universidade Federal do Ceará, Campus do Pici, Fortaleza - CE, Brasil

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Purification and Characterization of Microbial Hyaluronic Acid by Solvent Precipitation and Size-Exclusion Chromatography

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Universidade Federal do Ceará, Departamento de Engenharia Química, Grupo de Pesquisa em Separações por Adsorção (GPSA), Campus do Pici, Fortaleza – CE, Brasil

Abstract: Samples of hyaluronic acid (HA) produced by submerged fermentation using a synthetic culture medium were recovered, purified, fractioned, and characterized using solvent precipitation and size-exclusion chromatography (SEC). The samples showed a wide molar mass distribution in the range 10^3–10^7 Da, most of which had an average molar mass between 10^4 and 10^5 Da after purification by sequential precipitations. Fractions of HA with molar mass above 10^5 Da were purified by SEC in a semi-preparative scale with nearly no protein contamination. Characterization and fractionation of the HA was carried out by SEC in a analytical and semi-preparative scale using Shodex OHPak SB806M HQ and Superose 6 columns, respectively.

Keywords: Hyaluronic acid, precipitation, pullulan, recovery and purification, size-exclusion chromatography

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Address correspondence to Diana C. S. Azevedo, Universidade Federal do Ceará, Departamento de Engenharia Química, Grupo de Pesquisa em Separações por Adsorção (GPSA), Campus do Pici, Bl. 709, Fortaleza – CE 60455-760, Brasil. Tel.: +55 (85) 3366-9611 (ext. 29); Fax: +55 (85) 3366 9601. E-mail: diana@gpsa.ufc.br

INTRODUCTION

Hyaluronic acid (HA) is a naturally occurring linear, unbranched, non-sulfated polysaccharide. It may be classified as a polyanionic glycosaminoglycan that has important structural and biological roles in animal tissues. HA is composed of 2,000 to 25,000 repeating disaccharide sub-units of β-(1-4)-D-glucuronic acid and β-(1-3)-*N*-acetyl-D-glucosamine (1–4). It has high lubricating properties and it may adsorb and retain water, which impacts on several cellular functions, such as migration, adhesion, and proliferation (5,6). Recent biomedical applications of HA include ophthalmic surgery, arthritis treatment, scaffolds for wound healing, tissue engineering, and as a component in implant materials (7–9).

HA is produced commercially by solvent extraction from rooster combs (10) or to a lesser extent, by microbial fermentation (11,12). HA isolation from the crude extract is a laborious process with low yield, which results in a product that is usually contaminated with allergens. On the other hand, HA obtained by microorganism cultivation using bacteria from the *Streptococcus* family has gained increasing interest due to the potential for higher yields and better control of the process, thus providing a more homogeneous product (13).

Complex biological functions of the HA are associated to its viscoelastic properties, which depend on its size or hydrodynamic volume, macromolecular conformation (14) and, mainly, on average molar mass. Consequently, cosmetic or pharmaceutical uses of HA are directly related to its molar mass distribution (15–17). However, HA samples obtained from fermentation processes do not have monodisperse molar mass; therefore, it is very important to know its molar mass distribution and how it is affected by the various purification procedures. A wide variety of methods have been described to evaluate the distribution of the molar mass of HA, such as viscosimetry (18), gel electrophoresis (19), capillary electrophoresis (20,21), and chromatographic methods (1,22–25). Some newly developed spectrometric techniques are now capable of providing very accurate molar mass distributions, but equipment is costly and not always readily available (26).

Chromatographic methods, mainly size-exclusion chromatography (SEC), have shown good performance in the separation of macromolecules based on molar mass. SEC is a chromatographic separation method based on the differences of sizes or hydrodynamic volumes of the substances. In this method, the stationary phase is constituted of inert material having a given pore size distribution. As a result, in a mixture composed of polymers of different sizes, molecules having dimensions larger than those of the pores (exclusion limit), will be

excluded from the stationary phase and will elute first. On the other hand, smaller molecules will penetrate to different extents in the pores of the stationary phase and will be eluted sequentially later. The lower the molar mass of the polymeric fraction is, the longer it will take for it to elute.

The main applications of SEC are related to purification, analysis of purity, and estimation of molar mass of polymers (28). Determination of molar mass by using a calibration curve with standard polymers is a well-established technique (29–32). In usual SEC, the molar mass of the fractions is estimated from a calibration curve, which is normally obtained from a sufficient number of well-defined, narrow molar mass distribution standards of the polymer under study (primary standard). However, for many polymers, these standards are not available. In such case, secondary standards may be used (33), that is, polymers of known molar mass whose hydrodynamic size is similar to that of the polymer under study. Pullulan, an extra cellular polysaccharide produced by the *Aureobasidium pullulans* fungi, is a linear water soluble glucan. Its medical and industrial uses have been reported by a number of authors (34). Commercial availability of pullulan, in an extensive range of well-defined molar mass, suits this oligosaccharide as a usual calibration standard for SEC in aqueous phase (35).

Iqbal et al. (1) reported the determination of molar mass of hyaluronic acid extracted from bovine vitreous humors and a commercial preparation containing sodium hyaluronate (Healon®) by SEC using a calibration curve with pullulan polymer molar mass standard. Several studies (24,27,36) have shown that the macromolecular conformation of pullulan, in aqueous solution, is a semi-rigid worm-like structure, which is similar to HA conformation. For this reason, pullulan molecules have been used as secondary standard in the SEC method to determine the molar mass of HA samples. Recently, Yang et al. (4) satisfactorily employed only one ethanol precipitation as an initial step in the purification of HA from an unclarified *S. zooepidemics* followed by adsorption on chitosan-conjugated magnetite particles. Obviously, they have found a decrease in the protein concentration after ethanol precipitation. Despite the considerable reduction in the protein concentration a low yield of recovery was reached in this work. Up to date, the influence of pre-purification steps (like solvent precipitation and re-dissolution) on the average molar mass of microbial HA has not yet been shown, nor has the simultaneous final purification from proteic matter and size fractionation by SEC been reported.

In the present work we describe an alternative and efficient route to recover, purify, and characterize HA from *S. zooepidemicus* produced by submerged fermentation in synthetic culture media using solvent

precipitation and size-exclusion chromatography. Recovery and pre-purification was performed by centrifugation and ethanol-induced precipitation, respectively. A systematic procedure was employed to characterize molar mass distribution of HA samples by analytical SEC column. The polishing purification step by semi-preparative SEC column is also shown.

MATERIALS AND METHODS

Chemicals and Solutions

The fermented broth containing HA was kindly supplied by the Department of Biotechnological Processes of the State University of Campinas (UNICAMP, Brazil). HA was produced by submerged fermentation in synthetic medium using *S. zooepidemicus*, as reported by Ogrodowski et al. (11). A set of standard samples of pullulan (kit P-82 from Shodex, Japan) of known molar mass (5.8×10^3 to 8.53×10^5 Da) was used as molar mass references. A commercial HA standard from Sigma Aldrich (Missouri, USA) with an average molar mass of 10^5 Da was used. HPLC grade ethanol (J.T.Baker, Xalostoc, Mexico) was used in the pre-purification procedure of HA. Analytical grade sodium nitrate (Synth, São Paulo, Brazil) was used in the chromatographic analysis and solubilization procedures. All aqueous solutions were degassed in an ultrasonic bath and prepared using distilled water purified with a Milli-Q purification system (Millipore, USA).

Experimental Setup

The SEC runs were performed in a Waters Liquid Chromatography System (USA) composed of a dual pump (Waters model 1525), UV/Vis detector (Waters model 2487), and a Refractive Index (RI) detector (Waters model 2414) placed in series at the exit of the chromatographic column. The UV/Vis detector was set at 280 nm for proteic matter detection. HA does not absorb UV/Vis radiation at this wave length and was detected by its refractive index. A chromatographic analytical column Shodex OHPak SB806 M HQ (300×7.8 mm I.D.) and a semi-preparative column Superose 6 10/300GL (300×10 mm I.D.) were used for analytical determinations and final polishing purification steps, respectively. The measurements were performed at room temperature (25°C) in both scales.

Pre-Purification of the HA Sample

The HA sample produced by submerged fermentation was centrifuged at 3200 rpm for 20 min in order to remove cells and debris. After discarding cells, 1.5 vol. ethanol were added to 1 vol. supernatant and the solution was kept at 4°C for 1 hour to enhance HA precipitation. The supernatant was discarded and the precipitate was re-dissolved in 0.1 M $NaNO_3$ and analyzed to assess molar mass distribution, HA and protein concentration. This pre-purification procedure and respective analyses were performed up to four times.

Proteins Assays

Chromatographic analyses were performed to determine proteic contaminants in the HA samples submitted to a different number of pre-purification steps. In these analyses, an analytical SHODEX OHPak SB806M HQ column (300×7.8 mm I.D.) was used at a flow rate of 0.8 mL.min^{-1}, injection volume of 20 μL, room temperature, wavelength of 280 nm (UV-Vis), and 0.1 M $NaNO_3$ solution as mobile phase. The quantification of the proteic contaminants in the HA samples was carried out by the Bradford method.

Determination of HA Concentration

In order to determine the HA concentration after each pre-purification step, a calibration curve was plotted using standard solutions of commercial microbial HA at different concentrations (0.1; 0.5; 1.0; 1.5 e 2.0 g L^{-1}) dissolved in (0.1 M) $NaNO_3$ solution at room temperature. The chromatographic runs were performed under the same analytical conditions as described in the previous paragraph, using a RI detector.

Characterization of the HA Molar Mass by SEC in Analytical Scale

In order to estimate the molar mass of HA, a set of standard samples of pullulan was used to plot a molar mass calibration curve. Each standard polymer (5 mg) was kept in water for 12 h in order to achieve maximum swelling of the polymer particles. After that, the mobile phase was added (under agitation) to the standard solutions to a final concentration of 5 mg/mL. Each chromatographic run with pullulan samples was performed under the same conditions as those used to determine the HA

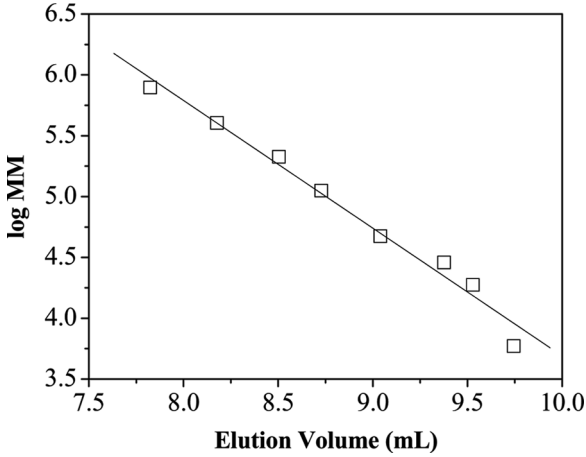

Figure 1. Molar mass calibration curve of pullulan standards on Shodex OHPak SB806 M HQ (300×7.8 mm I.D.) column. Experimental analytical conditions: $NaNO_3$ (0.1 M) as mobile phase at 0.8 mL/min, 20 μL injection loop, RI detection, and room temperature (25°C). The regression equation is log (MM) = 14.186 – 1.050×V_e (R^2 = 0.9910).

concentration. The calibration plot log MM (molar mass) versus V_e (elution volume) was constructed from pullulan chromatograms. Within the range of molar mass of standard pullulan used in this study, the calibration curve was found to be linear, as shown in Fig. 1, and an appropriate regression curve was obtained and used to estimate the molar mass distribution of HA samples.

As reported by Iqbal et al. (1), in the absence of a standard of suitable molar mass, the calibration curve may be extrapolated. It is likely that HA samples have fractions of higher molar mass than the highest molar mass pullulan standard used, thus the molar mass of HA may be slightly over or underestimated.

Purification of the HA Samples using SEC in Semi-Preparative Scale

SEC experiments in a semi-preparative scale were performed as a final polishing step of HA, after the centrifugation and solvent precipitation steps. Semi-preparative chromatographic runs were performed using Superose 6 10/300GL column (300×10 mm I.D.), at a flow rate of 0.8 mL.min^{-1}, injection volume of 250 μL, room temperature and 0.1 M $NaNO_3$ solution as mobile phase. Samples of HA submitted to increasing

numbers of pre-purification steps were injected and the effluent concentration was monitored on-line by both an RI and UV detectors. Effluent samples were also collected at every two minutes and analyzed by SEC-HPLC, under the analytical conditions described previously for the determination of HA concentration.

RESULTS AND DISCUSSION

Protein Assays and Effect of Pre-Purification Procedures

The HA produced by submerged fermentation in synthetic medium was characterized in terms of its protein contamination. Proteic contaminants were detected by chromatographic analysis (UV/Vis detection) evidenced by the peaks co-eluted from 11 to 13 min, as shown in Fig. 2(b). Note that the increase in the number of pre-purification steps seems to decrease the protein concentration in the HA samples, as may be inferred from the decrease in the peak areas in Fig. 2(b).

Fig. 2(a) shows the RI-signal chromatograms obtained for the HA samples submitted to up to four precipitation steps. Peaks are co-eluted from 7.5 to 16 minutes, which seems to indicate that there is a wide distribution of molar mass in the samples. There is a large overlap of peaks between 7 to 14 minutes and a narrow chromatographic peak at 15.5 minutes. Additional chromatographic injections (not shown) indicate that the peak at 15.5 min is due to entrapped ethanol used in the pre-purification steps. The same analysis was carried out using the HA commercial standard and a single chromatographic flat peak was detected at the time range between 8 and 9 min (chromatogram not shown). This peak is also present in all samples under study, although most of the peaks in Fig. 2(a) lie in the range of 10 to 14 minutes. This confirms the observation that the studied samples do contain HA in a wide distribution of molar mass, particularly below 10^6 Da. A decrease of the chromatographic area with the increase of the precipitation steps is observed, which suggests that the HA concentration is reduced at each pre-purification procedure. This is especially true for the peaks between 10 to 14 minutes, which account for fractions with lower molar mass than that of the commercial standard (10^5 Da). Lower molar mass HA fractions tend to remain in fluid phase during the solvent precipitation steps, possibly due to a lower concentration in hydrophobic patches that prevents their precipitation.

The protein contents were quantified by the Bradford method (37). Mean and standard deviations for proteins concentration measured for all HA samples and HA concentrations calculated from a standard

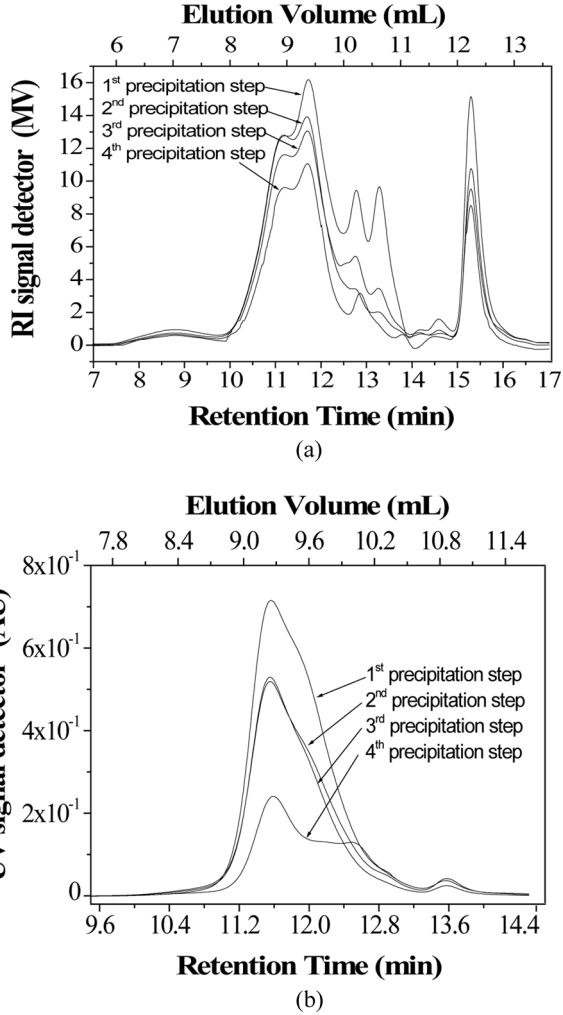

Figure 2. Chromatograms of HA samples injected in Shodex OHPak SB806 M HQ (300×7.8 mm I.D.) column, for an increasing number of pre-purification steps (ethanol precipitation and re-suspension in 0.1 M $NaNO_3$). Experimental analytical conditions: $NaNO_3$ (0.1 M) as mobile phase at 0.8 mL/min, 20 μL injection loop, and room temperature. The plots show the RI detection signal (a) and the UV/Vis signal (b) at a wave length of 280 nm.

calibration curve are given in Table 1. These results confirm the qualitative observations stated for Fig. 2: there is a decrease of both proteic contaminants and HA concentration with the increase in number of

Table 1. Protein and HA concentration after each ethanol precipitation and resuspension (in 0.1 M $NaNO_3$) step

Number of pre-purification steps	Protein concentration ($\mu g \cdot mL^{-1}$)	HA Concentration ($\mu g \cdot mL^{-1}$)
1	590.9 ± 54.3	933.7
2	270.2 ± 10.3	805.9
3	97.2 ± 2.0	796.3
4	77.7 ± 11.4	783.7

precipitation steps using ethanol. Although a reduction of almost 87% in protein concentration after four precipitation steps has been achieved, the efficiency of each purification procedure reduces with the increasing number of pre-purification steps. For instance, the concentration of the proteic contaminants reduces only 20% in the third precipitation step as compared to the first one (45%). The overall recovery yield of HA is also expected to decrease with the increasing number of purification steps, but if one takes into account the reduction of 87% in the protein concentration, HA losses may be considered small as compared to the achieved degree of purification. HA concentration was reduced from 933.7 down to 783.7 mg/L after the fourth precipitation/dissolution step. This means that the recovery yield of the precipitation steps—defined as the concentration after each step divided by the concentration after the first precipitation step—was 100%, 86%, 85%, and 84% after 1, 2, 3, and 4 purification steps. As the protein content is reduced, the recovery yield seems to level off, which is clearly seen after the second precipitation.

Although the precipitation steps presents a reasonable decrease of proteic contaminants with small losses of HA, detectable protein contamination still remains, which is unacceptable given HA medical and cosmetic applications. The commercial standard sample itself is exempt from proteins, as verified from the Bradford method. Therefore, additional chromatographic operations may be required not only to obtain narrower molar mass fractions, but also to help achieve higher purity.

Characterization of HA by SEC in Analytical Scale

In the chromatograms of HA sample with one precipitation step (shown in Fig. 3a) a large chromatographic peak may be observed in the interval of 7 to 14 minutes, which is indicative of a wide molar mass distribution. The elution volumes recorded for each pullulan standard in the Shodex

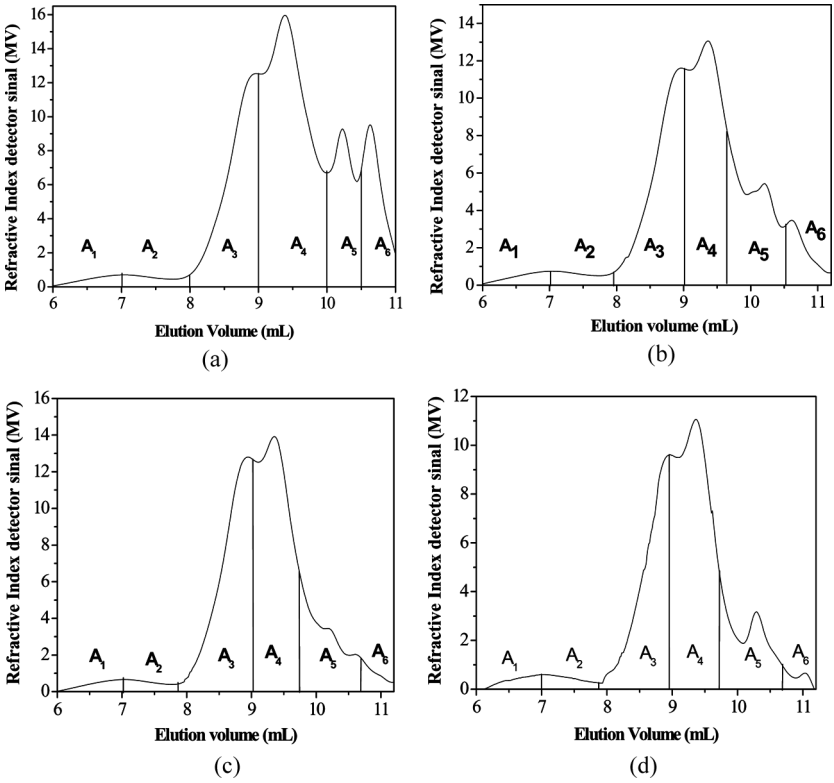

Figure 3. Sectioned chromatograms of HA samples injected in Shodex OHPak SB806 M HQ (300×7.8 mm I.D.) column, for an increasing number of pre-purification steps (ethanol precipitation and re-suspension in 0.1 M $NaNO_3$): one step (a), two steps (b), three steps (c) and four steps (d). Fractions were chosen so as to be centered in the characteristic retention times of the pullulan molar mass standards. Analytical conditions were: $NaNO_3$ (0.1 M) as mobile phase at 0.8 mL/min, 20 μL injection loop, RI detection, and room temperature.

column were used to obtain the semilogaritmic calibration curve, as presented in Fig. 1. According to Cunico et al. (38), if the separation of polymer samples are performed under constant flow conditions, molecules of a given molar mass will elute at a characteristic volume and therefore at a predictable time after sample injection. In this case, the retention volume (or elution volume) of the sample peaks can be directly compared with the retention volume of standard molecules of known molar mass to estimate the MM of the samples under study. In Figs. 3(a), (b), (c), and (d), the chromatograms of HA samples after the first, second, third, and

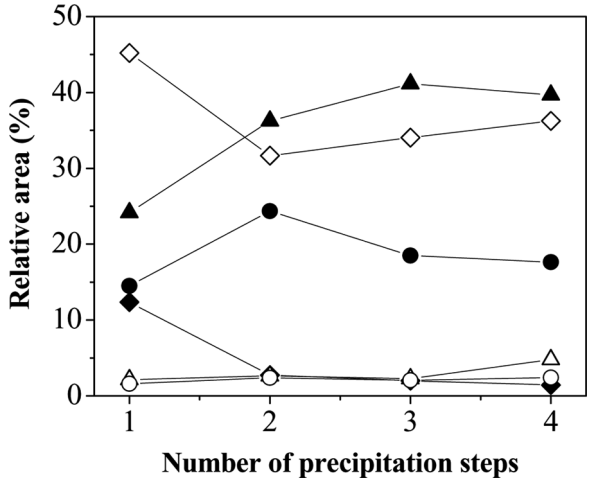

Figure 4. Percent amount of molar mass fractions present in the HA samples under study as a function of the number of ethanol precipitation steps: $<10^3$ Da (◆), 10^3 Da (●), 10^4 Da (◇), 10^5 Da (▲), 10^6 Da (△) and $>10^6$ Da (○).

fourth pre-purification steps are respectively shown. The total area of the chromatograms was divided into six particular elution sections, corresponding to the distinct molar masses of the pullulan standards. Estimation of the average molar mass of each HA sample was performed by relating the elution volume of each chromatogram section to the respective molar mass estimated from the calibration curve (Fig. 1).

The molar mass distribution in the four samples ranged from 10^3 to 10^6 Da, although the relative amount of each molar mass fraction varied, according to the number of precipitation steps that the sample had been submitted to. Figure 4 shows the percent amount of each distinct molar mass fraction present in the samples as a function of the number of pre-purification steps. According to the results, we can observe that the average molar mass of the samples ranges from 10^4 to 10^5 Da. This fact is in agreement with conclusions drawn by Ogrodowski et al. (11) from viscosimetric measurements. The data on Fig. 4 also shows that the amount of lower molar mass fractions (below 10^4 Da) decreases in the samples submitted to a larger number of solvent precipitation steps. It seems that these fractions of lower molar mass tend to remain in solution despite the addition of ethanol. Hence, the solvent precipitation steps not only reduce efficiently most of the protein impurities, but also favor the precipitation of higher molar mass HA fractions.

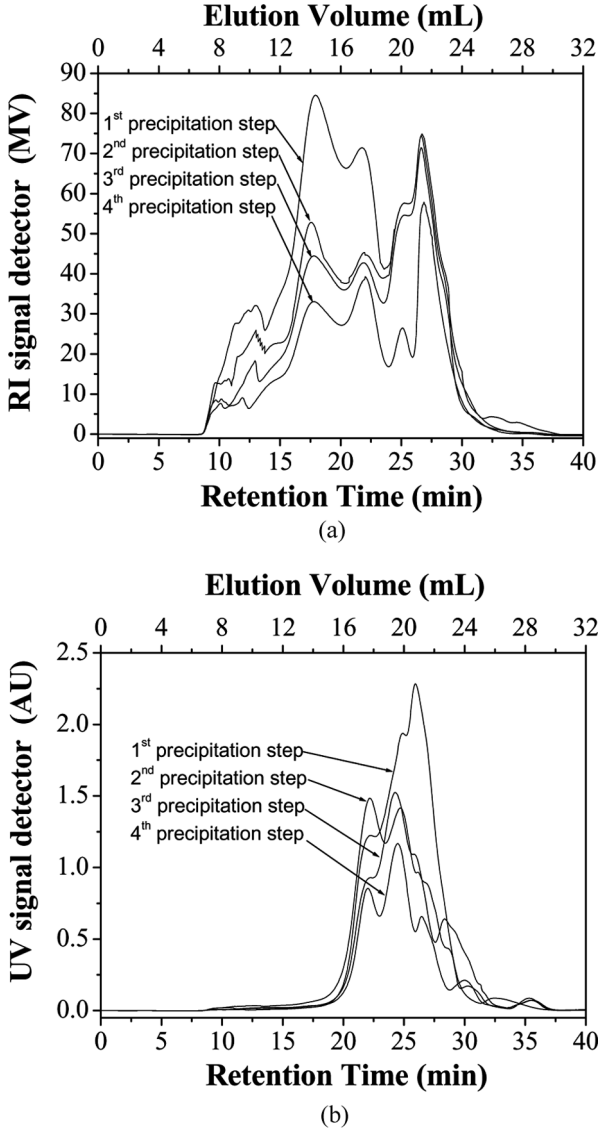

Figure 5. Chromatograms of HA samples injected in Superose 6 column for an increasing number of pre-purification steps (ethanol precipitation and re-suspension in 0.1 M $NaNO_3$). Experimental conditions: $NaNO_3$ (0.1 M) as mobile phase at 0.8 mL/min, 250 μL injection loop, and room temperature. The plots show the RI detection signal (a) and the UV/Vis signal (b) at a wave length of 280 nm.

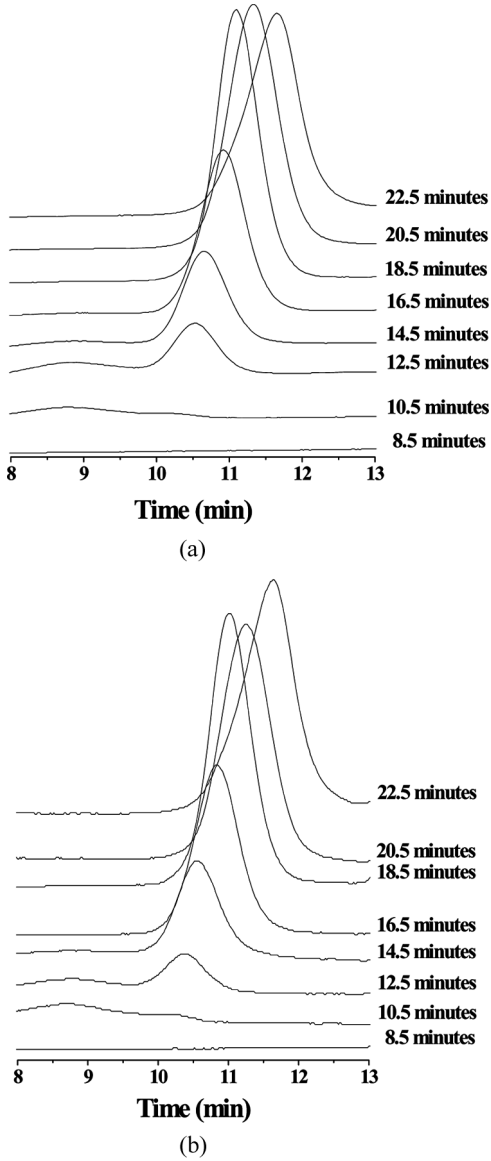

Figure 6. Chromatograms of effluent fractions collected during the experimental runs reported in Figure 8 for injected HA samples that had been submitted to ethanol precipitation and re-suspension in 0.1 M $NaNO_3$ once (a) and four times (b). Experimental analytical conditions: $NaNO_3$ (0.1 M) as mobile phase at 0.8 mL/min, 20 μL injection loop, RI detection, and room temperature.

Purification of HA in Semi-Preparative Scale

SEC experiments in semi-preparative scale were performed using the Superose 6 10/300GL (300×10 mm I.D.) column in order to achieve further purification of the HA samples and molar mass based separation on a larger scale. The chromatograms presented in Fig. 5(a) show the elution of HA samples submitted to an increasing number of solvent precipitation steps. There is a large co-elution peak in the range between 10 and 30 minutes, which is obviously due to the large molar mass distribution of the HA samples. A decrease in HA concentration with the increase in the number of pre-purification steps is also observed. Likewise, Fig. 5(b) shows a decrease of proteic contaminants following each pre-purification step. Most of these proteins elute between 20 and 30 min, having similar retention times as lower molar mass HA fractions. Injection of pullulan markers show that Superose 6 column has a fractionation range between 9 and 26.5 minutes under these experimental conditions. Hence, higher molar mass HA samples ($>10^5$ Da) leave the SEC column between 9 and 20 minutes, free of proteic contaminants.

In order to verify this assumption, fractions of HA eluted from the Superose 6 column were collected every 2 min between the elution times of 8.5 and 22.5 minutes, which were analyzed according to the analytical conditions described before. In Figs. 6(a) and (b), the chromatograms of the collected fractions are shown for the injected HA samples submitted to one and four solvent precipitation steps, respectively. Well-defined molar mass fractions detected in increasingly larger retention times were observed, starting at 10.5 minutes elution time from the Superose column. Nearly no proteic contaminants were observed (data not shown) for samples collected up to 20 minutes elution time. Thus, the assumption that protein-free fractions of high molar mass HA (above 10^5 Da) could be obtained by SEC using the Superose 6 column at elution times between 9 and 20 minutes was confirmed. These data (characteristic elution volumes of proteins and distinct molar-mass HA fractions) may be useful in the scale up of a separation process using SEC in continuous or semi-continuous mode, like for example a simulated moving bed. Fundamental results obtained in a single column in batch mode are essential to model and design these continuous processes, which have widespread use in biotech and pharmaceutical industry.

CONCLUSIONS

In this study, procedures to recover, purify, and characterize microbial HA by solvent precipitation and SEC chromatography were reported.

After four ethanol precipitation steps, a significant reduction of the proteic contaminants (around 87%) was achieved. A decrease in HA concentration was also verified but at much lower levels than the reduction in proteic contaminants. On the other hand, these pre-purification steps contributed to the decrease of HA fractions of low molar mass. The remaining traces of protein could be further removed by preparative-scale SEC.

Microbial HA samples were characterized in analytical scale and showed a large molar mass distribution ($<10^3$ to 10^7 Da). However, the average molar mass of the HA samples lied within the range of 10^4 to 10^5 Da, which favors its use in cosmetic and some pharmaceutical applications. Pre-purified HA (by solvent precipitation procedures) could be fractioned by SEC in semi preparative scale, using a Superose 6 column under a flow rate of 0.8 mL/min and injection volume of 250 µl. High molar mass HA fractions ($>10^5$ Da) were obtained—free of proteins—during the elution time interval of 9 to 20 min. These results show a favorable potential to scale-up the polishing purification steps of microbial HA by size-exclusion chromatography, preferably in continuous mode (e.g. SMB or external steady-state recycling) so as to increase stationary phase productivity and reduce solvent consumption.

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