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Biological and biophysical characterization of recombinant soluble human E-selectin purified at large scale by reversed-phase high-performance liquid chromatography

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Abstract

A first step in the development of a high-throughput screening assay for antagonists of human E-selectin is the purification and characterization of the selectin. In the present paper we describe a single-step, rapid, reversed-phase HPLC purification protocol for the recombinant, soluble form of human E-selectin (rshE-selectin) produced in Chinese hamster ovary cells. The procedure resulted in high protein yields with recoveries of greater than 98%. Characterization of the reversed-phase purified rshE-selectin showed this product to be analogous to rshE-selectin purified using conventional chromatographic techniques with respect to biological activity and molecular shape. However, the carbohydrate composition of reversed-phase purified rshE-selectin, which had been variable with conventionally purified material, was found to be constant across several isolations. The protocol described herein eliminated the high mannose component associated with previously purified rshE-selectin and provided a uniform carbohydrate composition for additional experimental studies, such as NMR. This fact, coupled with the high yield and simplicity of the present purification scheme are distinct advantages over those previously published. It is expected that other mammalian selectins, such as P-selectin and L-selectin, would also be amenable to reversed-phase HPLC purification.

1. Introduction

Leukocyte adhesion and extravasation, as a result of inflammation, is a multi-step process involving specific protein–carbohydrate and protein–protein interactions. In the initial phase of this process, leukocytes bind relatively weakly to the activated endothelium as a result of interactions between proteins and specific carbohydrate moieties. This initial binding event is mediated by the selectins, Ca^{2+}-dependent lectins ex-

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pressed in response to inflammatory cytokines. To date, three selectins have been identified: L-selectin which is constitutively expressed on all leukocytes; P-selectin which is expressed on activated platelets and endothelial cells; and E-selectin which is expressed on activated endothelial cells. The binding of the selectins to their cognate carbohydrate ligands causes the leukocytes to roll on the endothelial cells. Subsequent expression and interaction of other cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) results in much tighter binding of the leukocyte to the endothelium and promotes extravasation of the leukocytes into the surrounding tissue. For a review of the role of the selectins in inflammation see Ref. [1].

The selectins have been shown to bind the sialyl-Lewis^x antigenic determinant, [Neu-N-acetylα2,3-galactoseβ1, 4-(fucoseα1,3) N-acetyl-glucosamine-R] and the sialyl-Lewis^a antigenic determinant, [Neu-N-acetylα2,3-galactoseβ1,3-(fucoseα1,4)N-acetylglucosamine-R], where R is the remainder of the oligosaccharide covalently linked to the protein backbone. In vivo studies have demonstrated that antagonism of the first event of leukocyte-endothelial cell adhesion, i.e. the interaction of the selectins with their ligand(s), ameliorates the inflammatory response by diminishing the adhesion event per se and, therefore, the extravasation of leukocytes [2].

There are at least three possible strategies for the development of novel anti-inflammatory agents based on our current understanding of the involvement of the selectins in leukocyte adhesion/extravasation. The first approach would be the use of specific monoclonal antibodies that would neutralize the selectins, as described by Polley et al. [3]. The second approach is the large scale synthesis of sialyl-Lewis^x, or similar structures, or rational design of antagonists based on the sialyl-Lewis^x structure. While Schusta et al. [4] have made significant contributions to the field of semi-enzymatic synthesis of oligosaccharides and glycopeptides, the large-scale production of oligosaccharides as pharmaceuticals has yet to be realized. It is worth noting, however, that infusion of sialyl-Lewis^x in an IgG immune complex model of acute lung injury in the rat, is protective as assessed by changes in lung vascular permeability and hemorrhage [5,6]. These data suggest that derivatives of sialyl-Lewis^x, or glycomimetics, are *indeed* a feasible avenue for anti-inflammatory drug discovery efforts. Given this, we decided to follow a third strategy for the discovery/development of novel anti-inflammatory agents which is to implement a high throughput screening assay of natural products in the hope of identifying a compound, or compounds, that specifically antagonize the selectin–carbohydrate interaction. That is, to discover and identify a natural glycomimetic antagonist of the selectins which might be more amenable to large scale synthesis.

There are at least two possible formats for such high throughput screening assays involving the selectins: cell-based and cell-free assay systems. We have concerned ourselves with the development of a cell-free assay system, specifically for identification of antagonists of E-selectin. A critical reagent for such an assay is the soluble form of E-selectin. We have therefore established a continuous Chinese hamster ovary (CHO) cell line expressing a recombinant, soluble form of human E-selectin, rshE-selectin, at 65–70 mg/l. The present paper describes the purification and characterization of rshE-selectin as a first step in the development of a high throughput screening assay. Purification of the rshE-selectin from conditioned media was achieved by a one-step reversed-phase chromatographic procedure with greater than 98% recovery of protein. rshE-selectin so purified is demonstrated to be biologically active in that it supports adhesion of HL60 cells and that this adhesion is specifically inhibited by anti-E-selectin monoclonal antibodies and sialyl-Lewis^x derivatives. Additionally, the rshE-selectin is demonstrated to be an elongated molecule with an axial ratio of $\approx$10:1, as has been described for recombinant, soluble P-selectin [7].

2. Experimental

2.1. Materials

HPLC-grade acetonitrile-UV was obtained from Baxter Scientific Products (Edison, NJ, USA), HPLC-grade trifluoroacetic acid (TFA) from Sigma (St. Louis, MO, USA) and HPLC-grade water from J.T. Baker (Phillipsburg, NJ, USA). The POROS II R/H HPLC analytical column and POROS 2R50 bulk resin were procured from PerSeptive Biosystems (Cambridge, MA, USA). Mini-Protean II Ready Gels 15% SDS-PAGE were purchased from Bio-Rad Laboratories (Melville, NY, USA). Electrophoresis molecular mass standards were from Pharmacia Biotech (Piscataway, NJ, USA). Synthetic oligosaccharides, 3′-sialyl-fuscosyl-lactose (3′-sfl) and 3′-fucosyl-lactose (3′-fl) were obtained from Oxford Glycosystems (Abingdon, UK). The anti-E-selectin monoclonal antibody 1.2B6 and the anti-ICAM-1 monoclonal antibody 6.5B5 were obtained from the laboratory of Dr. Dorian Haskard [8]. Frozen ampules of human umbilical vein endothelial cells were purchased from Cell Systems (Kirkland, WA, USA). 2′,7′-Bis(2-Carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) was purchased from Molecular Probes (Eugene, OR, USA). BCA protein assay reagents were obtained from Pierce Chemical Company (Rockford, IL, USA). All other chemicals were of reagent grade.

2.2. Hollow fiber fermentation of recombinant, soluble human E-selectin

The generation and characterization of the Chinese hamster ovary (CHO) cell line secreting a recombinant, soluble form of human E-selectin has been described elsewhere [9]. Cells were grown in MR1-Mod 3 media containing 100 n*M* MTX, 27–28 IU/mg protein Zn-Recomblin, 0.12 mg/l oleic acid, 0.2 mg/l linoleic acid, 0.05 mg/l linolenic acid, 0.14 mg/l lipoic acid, 0.01 mg/l methyl linoleate, 2 mg/l cholesterol, 0.05% (v/v) pleuronic F68, 11.2 m*M* ferric chloride and 1.1 *M* fructose using hollow fiber fermentation technology. An aliquot of 100 ml of media was drawn from the culture daily for a period of 4–12 weeks. The media was centrifuged, sterile filtered and stored at −70°C until use.

2.3. Preparation of hollow fiber media for reversed-phase chromatography

Hollow fiber media (HFM; 300–400 ml per run) was thawed at room temperature, then concentrated and diafiltered using a Filtron Tangential Flow Ultrafiltration System with a 30 kDa molecular mass cut-off (MWCO) screen channel membrane (Filtron Technology, Northborough, MA, USA). The media was concentrated to one-tenth of its original volume and then diluted to 3 times its original volume with 10 m*M* Tris buffer, pH 7.8. This process was repeated twice. The final volume of concentrated HFM, including the hold up volume of the Filtron Unit, was recovered and the unit washed with an equivalent volume of 1 *M* NaCl and 10 m*M* Tris buffer, pH 7.8. The salt wash was pooled with the concentrated HFM to yield a final concentration of 500 m*M* NaCl and 10 m*M* Tris buffer, pH 7.8. A final volume of 40–50 ml of concentrated HFM was obtained by ultrafiltration using an Amicon Stirred Cell equipped with a YM 30 kDa MWCO membrane (Amicon, Beverly, MA, USA). Concentrated HFM was either used immediately or stored frozen at −70°C.

2.4. Column selection and preparation

Preliminary studies on the purification of rshE-selectin by reversed-phase chromatography were performed using a PerSeptive Biosystems POROS II R/H column (50 mm × 4.6 mm, I.D.). For preparative purposes, 50 g of POROS 2R50 resin were rehydrated with 0.1% TFA in HPLC-grade water and flow packed at 689 kPa into a Waters AP-2 60 cm × 2.0 cm I.D. column. The column was then equilibrated with 10% acetonitrile–0.1% TFA (v/v). A Waters 650E

HPLC system with a Waters 486 Tunable Absorbance Detector was used throughout.

2.5. Reversed-phase HPLC

Maximal protein loading of the column was determined using bovine serum albumin fraction V (BSA V). It was concluded from these studies that, for optimal resolution, the total protein load for the preparative column should not exceed 250 mg, equivalent to approximately 50 ml of concentrated HFM. Concentrated HFM was injected onto the column at a flow-rate of 40 ml/min. The column was washed with 10% acetonitrile–0.1% TFA (v/v) in water until baseline was obtained ($A_{280nm} < 0.075$). The column was then eluted with a linear gradient of acetonitrile (10–36%) containing 0.1% TFA (v/v) at a flow-rate of 40 ml/min. rshE-selectin eluted from the column at approximately 34% acetonitrile. The column was then washed with 60% acetonitrile–0.1% TFA (v/v) to elute the remaining bound protein, primarily BSA. The column was then re-equilibrated with 10% acetontrile–0.1% TFA (v/v) and was ready for a second cycle. An entire purification cycle took about 45 min. Fractions containing rshE-selectin were pooled and dialyzed overnight against multiple changes of PBS, pH 7.8. Alternatively, pooled fractions containing rshE-selectin were dialyzed extensively against ultra pure water and then lyophilized to dryness (Labconco Freeze Dryer 4.5). Protein concentrations of dialyzed rshE-selectin were determined by BCA protein assay. Purity of rshE-selectin was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) performed as described previously [10], using Mini-Protean II Ready Gels (15% acrylamide). Gels were stained with Coomassie Blue R-250.

2.6. E-Selectin activity assay

In order to assess biological activity of rshE-selectin we employed a coated plate assay modified from a previously described method [11]. Microtiter plates (96-well Immunolon-2, Dynatech, Chantilly, VA, USA) were coated with rshE-selectin, diluted in phosphate-buffered saline (PBS) (10 m*M* sodium phosphate, 150 m*M* NaCl, pH 7.4), for 16 h at 4°C. Wells were then blocked by addition of 200 μl 2% BSA (w/v) in PBS and incubated for 1 h at room temperature. HL60 cells, grown in RPMI 1640 media containing 10% fetal bovine serum, were washed once in PBS and resuspended to $5 \cdot 10^6$ cells/ml. BCECF/AM (2',7'-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein acetoxymethyl ester) was added to a final concentration of 25 μM from a 10 m*M* stock solution made in dimethyl sulfoxide. Cells were incubated for 30 min at 37°C, washed with PBS and resuspended to $2 \cdot 10^6$ cells/ml in complete Hank's buffered saline solution (0.3 m*M* sodium phosphate, dibasic, 0.4 m*M* pottasium phosphate monobasic, 1.25 m*M* calcium chloride, 0.8 m*M* magnesium sulfate, 5.4 m*M* potassium chloride, 4.2 m*M* sodium bicarbonate, 136 m*M* sodium chloride, 10 m*M* HEPES, pH 7.1) containing 1% (w/v) BSA ($HBSS^+$). Prior to the addition of labelled HL60 cells, plates were washed once with 200 μl $HBSS^+$. Subsequently, 100 μl of dye labeled HL60 cell suspension was added to each well. Cells were allowed to adhere for 30 min at room temperature. Wells were washed twice with 200 μl $HBSS^+$ by vacuum aspiration through a 20-gauge needle and gentle addition of $HBSS^+$ with an 8-channel multipipettor. Adherent cells were lysed for 10 min in 100 μl 0.1 *M* NaOH, 0.1% SDS and plates read in a Fluoroskan II Plate Reader (Labsystems, Helsinki, Finland) at excitation wavelength 485 nm and emission wavelength 538 nm.

2.7. Amino acid and carbohydrate analysis of rshE-selectin

rshE-selectin was hydrolyzed in vacuo under 6N HCl for 20 h at 110°C. The resulting hydrolyzate was analyzed by ion-exchange amino acid analysis using post-column ninhydrin detection on a Beckman 6300 analyzer equipped with a System Gold data acquisition system (Beckman Instruments, Fullerton, CA, USA). For monosaccharide composition analysis, rshE-selectin was hydrolyzed in 20% TFA in water (v/v) at

100°C for 6 h then analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) as described previously [12].

2.8. N-terminal sequence analysis

N-terminal sequence analysis was performed on an Applied Biosystems 470A gas-phase protein sequencer (Applied Biosystems, Foster City, CA, USA) equipped with a Beckman 126/166 system (Beckman Instruments, Fullerton, CA, USA) for on-line PTH analysis. Data was acquired using System Gold chromatography software (Beckman Instruments). Samples were spotted directly onto Polybrene-coated GF/C filters (Applied Biosystems) and standard ABI sequencing cycles were used.

2.9. Analytical ultracentrifugation

Equilibrium sedimentation and equilibrium velocity experiments were performed with a Beckman XLA analytical ultracentrifuge (Beckman Instruments) using an An-60 Ti rotor. Double sector cells with charcoal filled epon centerpieces and sapphire windows were used. Sedimentation experiments were performed at 25°C in PBS, pH 7.8 with 1 μM rshE-selectin. The partial specific volume, $\bar{v}$, was calculated using the partial specific volumes of the component amino acids [13] and an estimate of the carbohydrate distribution, yielding a value of 0.688 ml g^{-1}. The solvent density ρ, was estimated at 1.006 g ml^{-1}. Sedimentation equilibrium data were analyzed using nonlinear least squares methods [14] under the control of a modified version of IGOR (Wavemetrics, Lake Oswego, OR, USA) running on a Macintosh Quadra 800. Data sets were collected after reaching equilibrium, usually about 18 h, at a rotor speed of 15 000 rpm. At equilibrium, the concentration distribution of a single, homogeneous species is given by:

$$c_r = c_0 \exp[M(1 - \bar{v}\rho)\omega^2(r^2 - r_m^2)/2RT] \quad (1)$$

In Eq. (1), c_r and c_0 are the concentrations of the protein at a radial position r and at a reference position, usually the meniscus, respectively. M is the protein molecular mass, $\bar{v}$ is the partial specific volume of the protein, ρ is the solvent density, ω is the angular velocity, r is the distance in cm from the center of rotation, r_m is the radial position of the reference position in cm, R is the universal gas constant and T is the absolute temperature. Sedimentation velocity data were analyzed using the program VELGAMMA (Beckman Instruments). Twelve data sets were collected, 200 s apart, starting from the time the rotor reached 60 000 rpm.

2.10. Mass spectrometry

Matrix-assisted laser desorption mass spectrometry (MALD-MS) data for rshE-selectin were obtained on a Vestec LaserTec Research laser desorption time-of-flight mass spectrometer (Vestec, Houston, TX, USA). Samples were prepared for analysis by mixing rshE-selectin, in PBS, pH 7.8, with sinapinic acid [saturated solution in 33% CH_3CN–0.1% TFA (v/v)] for a final concentration of 1 pmol/μl. Phosphorylase b from rabbit muscle (Sigma) was included as an internal calibrant ($MH^+ = 97219$ Da). Desorption/ionization was accomplished using a 337 nm nitrogen laser. Spectra were averaged over ca. 50 laser scans. Calibrations were carried out using a customized version of IGOR on a Macintosh personal computer.

3. Results and discussion

3.1. Purification of rshE-selectin

The major contaminant in the hollow fiber media (HFM) is BSA which is incorporated into the CHO cell growth media. The concentration of rshE-selectin in the HFM was determined to be 65–70 μg/ml with a total protein concentration of 450 μg/ml. Concentration/diafiltration of the HFM aids in the removal of proteins below 30 kDa, as well as salts, but is primarily used to decrease the volume to be loaded onto the reversed-phase column. No loss of rshE-

selectin was observed during the concentration/diafiltration process since the concentration of rshE-selectin in the concentrated HFM was determined to be 650–700 μg/ml (total protein 4 mg/ml). Analytical reversed-phase HPLC experiments were performed using a POROS II R/H 50 mm × 4.6 mm I.D. column to determine conditions for loading, washing and elution of rshE-selectin. Direct scale-up of the analytical column to a preparative column was achieved using POROS II 2R50 reversed-phase resin. Experiments demonstrated the maximal loading capacity of this resin to be approximately 0.7 mg of BSA/ml of resin (not shown). Therefore, a column of dimensions 56 cm × 2.0 cm I.P. as used here, was calculated to have a capacity of approximately 250 mg total protein. Given this, 45–50 ml concentrated HFM (total protein 180–200 mg; equivalent to approximately 450–500 ml original HFM) was loaded onto the column per cycle. A representative purification cycle is presented in Fig. 1. After sample loading, the column was washed with the equilibration solvent until a stable base line was achieved. A linear gradient from 10–36% acetonitrile–0.1% TFA (v/v) was then used to elute the rshE-selectin from the column which reproducibly eluted at approximately 34% acetonitrile (Fig. 1). After stripping with 60% acetonitrile–0.1% TFA (v/v) to remove the BSA and other minor contaminants, the column was re-equilibrated with 10% acetonitrile–0.1% TFA (v/v). Average purification cycle time, from loading through re-equilibration, was 45 min. No carry-over of contaminating proteins was seen from cycle to cycle. Yields of rshE-selectin per purification cycle averaged from 660–680 μg/ml concentrated HFM loaded onto the column, or approximately 30 mg rshE-selectin. The estimated recovery of rshE-selectin using this procedure was greater than 98% of the applied rshE-selectin. The short cycle time therefore allows for the

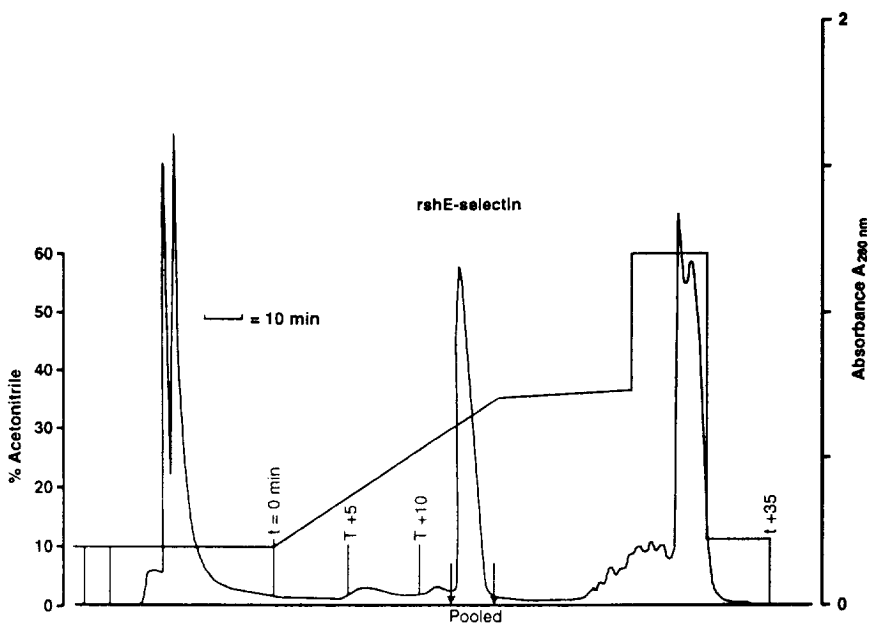

Fig. 1. Preparative reversed-phase chromatography of rshE-selectin. Concentrated HFM (45–50 ml; prepared as described under Experimental) was loaded onto a POROS II 2R50 reversed-phase column (56 cm × 2.0 cm I.D.) equilibrated with 10% acetonitrile–0.1% TFA (v/v). The column was then washed with the same solvent until a stable baseline was obtained. The column was developed with a linear gradient of 10–36% acetonitrile–0.1% TFA (v/v) and then washed with 60% acetonitrile–0.1% TFA (v/v). Finally, the column was re-equilibrated with 10% acetonitrile–0.1% TFA (v/v). rshE-selectin eluted at approximately 34% acetonitrile. The arrows indicate the fractions pooled and further processed.

purification of large amounts (tens of milligrams) of rshE-selectin very quickly. Acetonitrile and TFA were removed from the eluted protein by dialysis against either PBS or ultra-pure water. Samples of rshE-selectin dialyzed against water were lyophilized and it was demonstrated that the lyophilized rshE-selectin could be reconstituted at concentrations in excess of 10 mg/ml with no detectable loss in biological activity (data not shown).

3.2. Characterization of purified rshE-selectin

Analysis of the purified rshE-selectin by SDS-PAGE is shown in Fig. 2. Since rshE-selectin is a highly glycosylated protein, some heterogeneity is seen on SDS-PAGE. However, purified rshE-selectin resulted in a single peak on analytical reversed-phase HPLC (Fig. 2, Panel B). MALD-MS analysis (Fig. 3) gave a molecular mass of 80 500 ± 3500 Da for the purified protein. rshE-selectin, which has 11 potential sites for N-glycosylation, is clearly highly glycosylated as can be seen from the observed peak broadening (Fig. 3). Amino acid composition analysis (Table 1) demonstrated the expected composition and N-terminal sequence analysis agreed with that predicted for rshE-selectin (data not shown). Carbohydrate analysis of conventionally purified [9] versus the RP-HPLC purified material shows a marked decrease in the ratio of mannose to rshE-selectin (Table 2). The reduction in contaminating mannose as well as the uniform carbohydrate composition of the RP-HPLC purified rshE-selectin proved beneficial to NMR studies (data not shown). Data from sedimentation equilibrium analysis of rshE-selectin is shown in Fig. 4. The data from 6 replicate experiments were best fit to a single sedimenting species. Use of Eq. (1), yielded an average apparent molecular mass of 77 700 ± 3300 Da. This value is in good agreement with that determined by MALD-MS (Fig. 3). Sedimentation velocity experiments gave a sedimentation coefficient for rshE-selectin of 4.25 s. Using a value of 0.35 g H_2O/g protein for the hydration, and assuming a prolate shape, rshE-selectin has an axial ratio of ≈10:1 and dimensions of approximately 25 Å by 250 Å. The axial ratio and overall dimensions for rshE-selectin purified by RP-HPLC are in good agreement with those previously determined for conventionally purified rshE-selectin [9]. In addition, the rod shape determined for rshE-selectin, as described herein, is consistent with the molecular dimensions previously described for P-selectin [7], another member of the mammalian Ca^{2+}-dependent selectin family. Overall, therefore, the biophysical characterization of rshE-selectin purified by RP-HPLC demonstrates that this molecule is equivalent to that previously described purified by a combination of low-pressure chromatographic techniques. The advantages of the present procedure, apart from decreasing the contaminating mannose seen previously, are those of speed and product yield.

3.3. Biological activity

While RP-HPLC purified rshE-selectin was shown to be biophysically indistinguishable from that purified by conventional means, it remained to be determined whether or not it was biologically active. For this purpose adhesion of HL60 cells to rshE-selectin coated microtiter plates was used. As seen in Fig. 5A, greater than 90% of the input HL60 cells adhere to rshE-selectin coated wells in a dose dependent manner. Adhesion to uncoated wells is typically 5 ± 3% of input cells. Maximal adhesion was found using 50 ng rshE-selectin per well. Adhesion of HL60 cells to rshE-selectin coated wells was demonstrated to be calcium dependent; if calcium was not included in the assay buffer, or if the assay was performed in the presence of 5 m*M* EDTA, adhesion was entirely abrogated (data not shown). In order to further prove that this adhesion was mediated through rshE-selectin, rshE-selectin coated wells were pre-treated with an analog of the sialyl Lewis^x antigenic determinant, namely, 3′-sialyl-fucosyl-lactose (3′-sfl). 3′-sfl has a glucose replacement for the N-acetylglucosamine present in sialyl-Lewis^x at the reducing termini and has been shown to be an inhibitor of E-selectin dependent adhesion

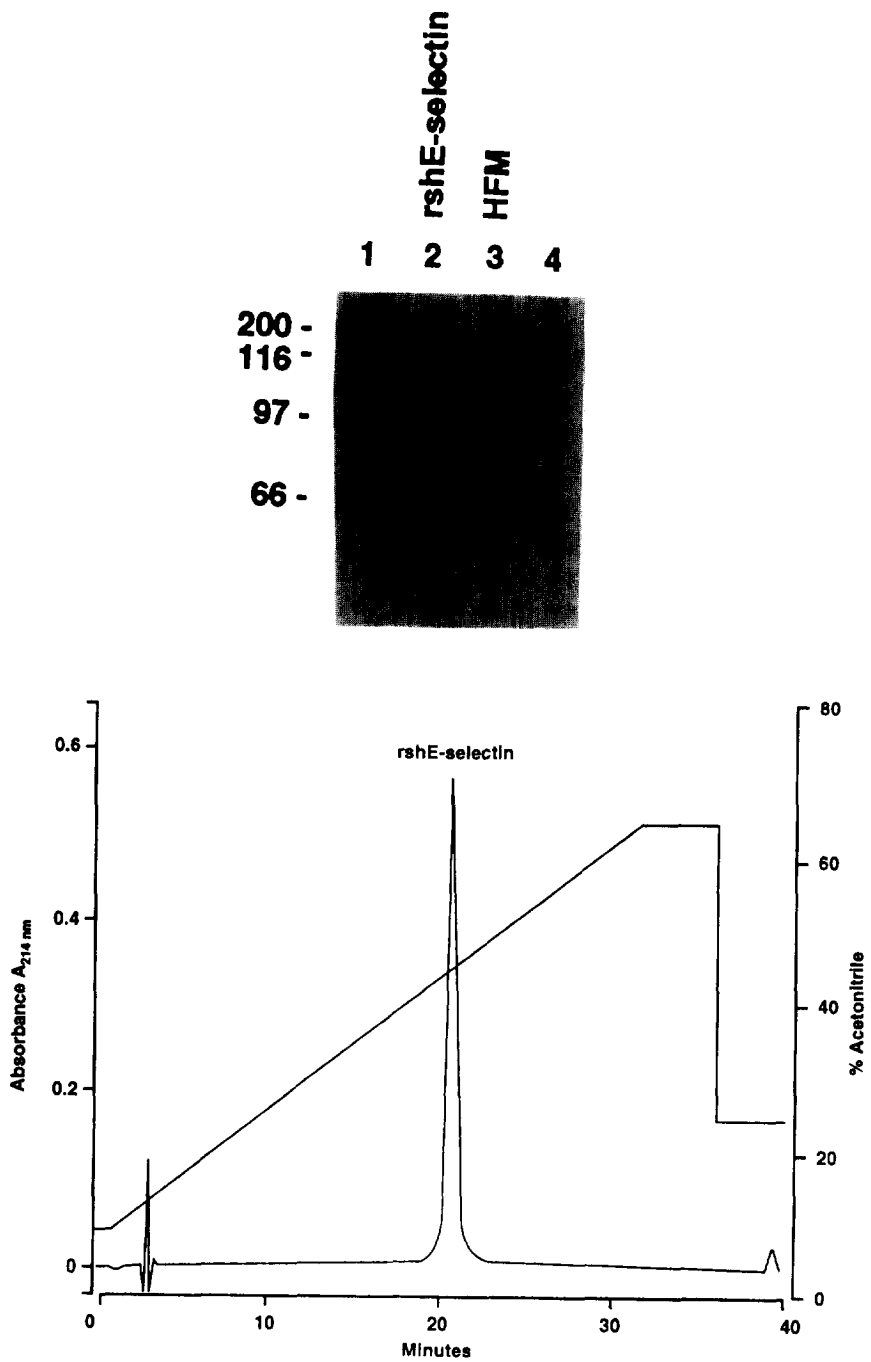

Fig. 2. SDS-PAGE analysis of RP-HPLC purified rshE-selectin. Panel A: SDS-PAGE was performed as described [10] using Mini-Protean II Ready Gels (Bio-Rad, 15% acrylamide). Staining was with Coomassie Blue R-250. Lanes 1&4: Molecular mass standards: 200, 116, 97 and 66 kDa; Lane 2: RP-HPLC purified rshE-selectin (20 μg protein loaded); Lane 3: Concentrated HFM (20 μg total protein loaded). Panel B: Analytical RP-HPLC of rshE-selectin. Five micrograms purified rshE-selectin were injected onto a Vydac C4 column (15 cm × 6.6 mm I.D.) and an acetonitrile–TFA gradient (10–65% acetonitrile over 30 min) developed at a flow-rate of 1.0 ml/min. Absorbance was monitored at 214 nm.

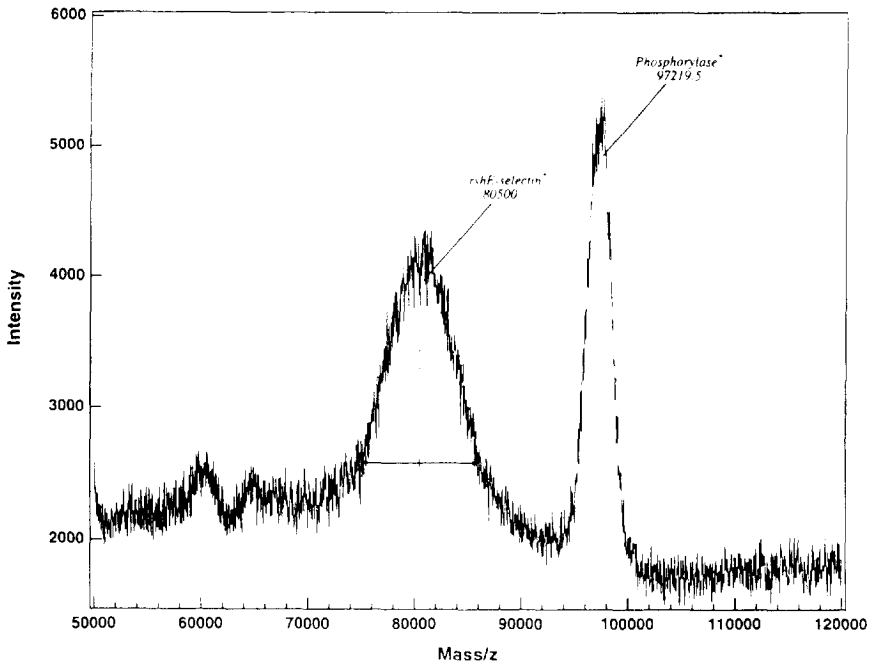

Fig. 3. MALD-MS of rshE-selectin. Measurements demonstrate the protein to be highly glycosylated causing broadening of the peak. The calculated mean molecular mass for rshE-selectin was 80 500 ± 3500 Da. The internal calibrant used in these measurements, phosphorylase b (MH^+ = 97219), is also shown.

[15]. Pre-treatment of the rshE-selectin coated wells (Fig. 5B) with 0.6 m*M* 3′-sfl reduced HL60 adhesion to 38 ± 11% of control. Comparable levels of inhibition were seen using up to 2.5 m*M* while 0.15 m*M* had no effect. The asialo analog of 3′-sfl, 3′-fucosyl lactose (3′-fl), had no effect on adhesion at concentrations up to 2.5 m*M*. These results demonstrate specific inhibition of E-selectin mediated cell adhesion by oligosaccharides, as has been previously reported.

Specific inhibition of rshE-selectin/HL60 cell adhesion was also demonstrated using monoclonal antibodies. When rshE-selectin coated wells were pretreated with 50 μg/ml of monoclonal antibody 1.2B6, a neutralizing anti-E-selectin antibody, the adhesion of HL60 cells was reduced by approximately 50%. A reduction in adhesion is also seen using 10 μg/ml of MoAb 1.2B6. However, no inhibitory effect was seen when a neutralizing anti-ICAM-1 monoclonal antibody, 6.5B5, of identical isotype to MoAb 1.2B6, was added to rshE-selectin coated wells. Addition of anti-E-selectin MoAb 1.2B6 at concentrations greater than 50 μg/ml did not result in increased inhibition of HL60 cell adhesion, i.e. we were unable to reduce adhesion by greater than 50% using this and other monoclonal antibodies (data not shown). The reason for the inability to completely block HL60 cell adhesion to rshE-selectin coated plates is curious but not unprecedented.

If HL60 cells are allowed to adhere to cytokine stimulated human umbilical vein endothelial cells (HUVEC's) under ice cold conditions, adhesion can be competed entirely by a sialyl-Lewis^x analog [15]. We have used this observation to establish an E-selectin specific cell-cell adhesion assay and show that rshE-selectin can be used as a soluble antagonist of the adhesion of HL60 cells to TNF treated HUVEC's, which

Table 1
Amino acid composition analysis of RP-HPLC purified rshE-selectin

Amino acid	Theoretical	Observed
ALA	33	31.6
ARG	14	13.5
ASX	41	42.1
GLX	72	74.6
GLY	42	41.0
HIS	8	7.2
ILE	15	13.6[b]
LEU	25	25.0
LYS	24	22.9
MET	9	8.2
PHE	20	21.1
PRO	20	21.2
SER	55	51.2
THR	39	35.9
TYR	17	N/D[a]
VAL	29	26.1[b]

rshE-selectin was hydrolyzed *in vacuo* under 6 *M* HCl for 24 or 72 h at 110°C. The resulting hydrolyzate was analyzed by ion-exchange amino acid analysis using post-column ninhydrin detection. All values are expressed on a mol amino acid/mol protein basis.

[a] Value not determined due to coelution of hexosamine sugars.

[b] Values reported for 72 h hydrolysis.

is an E-selectin driven phenomenon. As shown in Fig. 5C, antagonism of HL60 cell/HUVEC adhesion by rshE-selectin is dose-dependent and is specific for rshE-selectin in that antagonism is not seen using comparable amounts of purified rs-ICAM-1.

Table 2
Comparative carbohydrate analysis of RP-HPLC purified and conventionally purified rshE-selectin

Monosaccharide	Conventional purification[a] (mol/mol)	RP-HPLC method[a] (mol/mol)
Glucosamine	32.6	27.7
Galactosamine	1.2	1.9
Galactose	17.7	17.9
Mannose	65.5	23.6
Fucose	6.8	5.8

[a] rshE-selectin purified from HFM by conventional low-pressure chromatography and by RP-HPLC. Molar ratios calculated using theoretical molecular mass of 58 600 Da for rshE-selectin.

4. Conclusions

Based on the following lines of evidence we conclude that rshE-selectin purified by the RP-HPLC method described herein is biologically active. First, rshE-selectin coated wells are able to support the adhesion of HL60 cells in a dose and calcium dependent manner. Secondly, the adhesion of HL60 cells to rshE-selectin coated wells can be inhibited by a carbohydrate analog of the E-selectin ligand, i.e. 3′-sialyl-fucosyl-lactose, and by a neutralizing anti-E-selectin monoclonal antibody. Finally, rshE-selectin was demonstrated to be a soluble antagonist of the adhesion of HL60 cells to cytokine stimulated human umbilical vein endothelial cells. From these data it is clear that rshE-selectin purified using RP-HPLC is biologically indistinguishable from the same molecule purified via conventional low-pressure chromatographic procedures. Further, we have demonstrated that from a biophysical standpoint, these two molecules are also indistinguishable. The advantages of the RP-HPLC purification protocol, as described here, over the conventional scheme are those of speed, yield and indeed, integrity of the final product. Yields of >98% were achieved with the present protocol resulting in purities in excess of 98%, as determined by analytical RP-HPLC. In addition, greater than 30 mg rshE-selectin could be purified in a 45-min purification cycle. It was also demonstrated that the carbohydrate composition of the RP-HPLC purified material was reflective of typical biantennary oligosaccharides whereas conventionally purified material was shown to contain excess mannose. The source of this mannose contamination is unknown but it is clearly removed by the present procedure.

It is important to note that the present experiments also demonstrate the robustness of rshE-selectin. Not only does this molecule survive the rigor of RP-HPLC, but we have shown that rshE-selectin can be dialyzed against water, lyophilized and subsequently re-constituted to

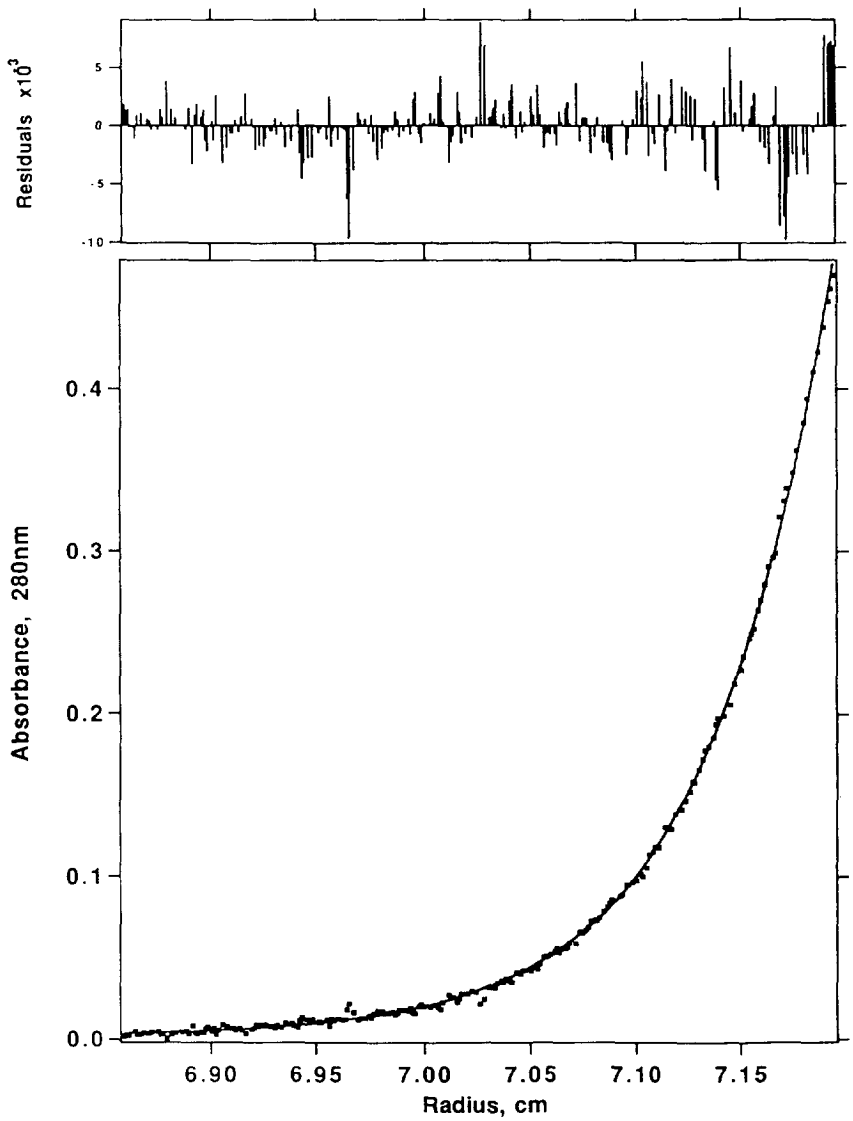

Fig. 4. Equilibrium analytical ultracentrifugation of rshE-selectin. Data fit to Eq. (1) describe a single molecular species and result in a molecular mass of 77 700 ± 3300 Da. The loading concentration of rshE-selectin was 1.05 μM. Experiments were performed at 298 K (25°C) and at a rotor speed of 15 000 rpm. The upper trace is a plot of the residuals between calculated and experimental data. The lower plot shows the calculated fit (solid line) to the raw data (■).

concentrations in excess of 10 mg/ml with no detectable loss in biological activity. This bodes well for the large-scale production and long-term storage of this critical reagent. Given the apparent similarities between E-selectin and P-selectin reported in the literature, it is envisaged that a similar RP-HPLC procedure would be applicable to the purification of a recombinant, soluble form of human P-selectin, and possibly L-selectin as well.

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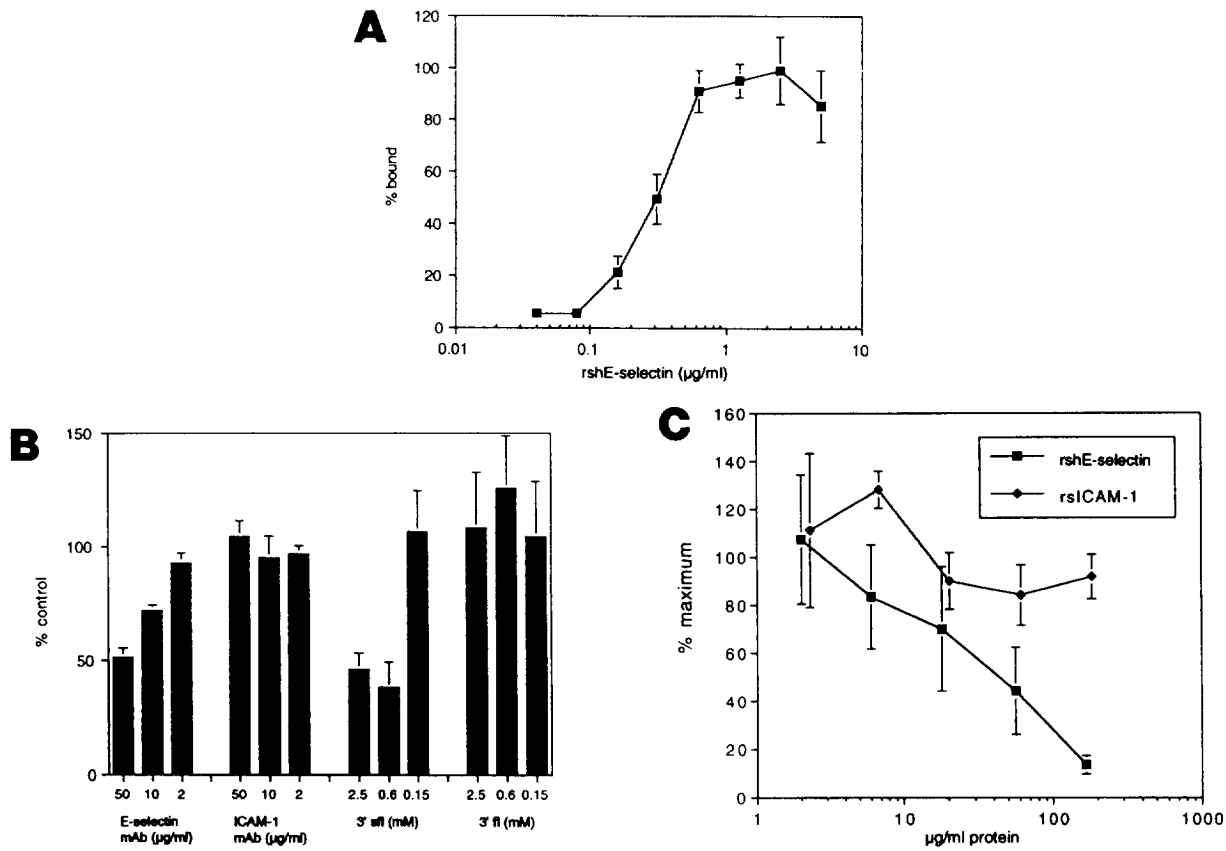

Fig. 5. Biological activity of purified rshE-selectin. (A) Adhesion of HL60 cells to microtiter plates coated with purified rshE-selectin. Various concentrations of RP-HPLC purified rshE-selectin were added to wells of 96-well plates and adhesion of HL60 cells assessed as described under Experimental. A clear dose-response is observed. Data represent the mean and standard deviation of triplicate wells at each rshE-selectin concentration. (B) Inhibition of HL60 cell adhesion to rshE-selectin coated 96-well plates. HL60 cell adhesion to rshE-selectin is inhibited by pretreatment of the coated plates with anti-E-selectin monoclonal antibody but not with the anti-ICAM-1 monoclonal antibody. Adhesion can also be inhibited by pretreatment with a sialyl-Lewis^x analog, 3'-sialyl-fucosyl-lactose (3'-sfl) whereas the asialo sugar, 3'-fucosyl-lactose (3'-fl), has no effect. (C) HL60 cell adhesion to cytokine stimulated human umbilical vein endothelial cells (HUVECs) is inhibited by pretreatment of the HL60 cells with RP-HPLC purified rshE-selectin but not by purified rs-ICAM-1 (assay performed at 4°C).

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