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Separation of interleukins by a preparative chromatofocusing-like method

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ABSTRACT

A chromatofocusing-like method used in the large-scale separation of deamidated from amidated recombinant human interleukin-1α (amino acids 117–271), derived from *Escherichia coli*, is described. Two major protein species having isoelectric points (p*I*) of approximately 5.3 and 5.1 were separated by high-performance liquid chromatography using a sulfopropyl strong cation-exchange column. Unlike standard chromatofocusing technique, this method does not use carrier ampholytes during gradient separation of proteins, nor does it employ increased ionic strength for protein elution, the usual method for performing standard ion-exchange chromatography. N-Terminal sequence analysis of the protein with a p*I* of 5.3 revealed an Asn residue at position 32 as predicted by the cDNA sequence. The p*I* 5.1 species showed an Asp residue at the same position as a result of deamidation of Asn. This method was also used in the large-scale separation of N-Met from des-Met recombinant human interleukin-1β.

INTRODUCTION

The separation of proteins using chromatofocusing technique is a widely accepted method because of its excellent resolving power. This technique has been predominantly used on an analytical basis in the conventional mode on glass columns [1–4], but can also be adapted to the high-performance liquid chromatographic (HPLC) mode and subsequently modified to preparative scale.

The isolation and characterization of recombinant human interleukin-1α (rhIL-1α) from *Escherichia coli* cells was previously described [5]. Recently, two major isoelectric species of purified rhIL-1α were detected on an analytical isoelectric focusing (IEF) gel following the previous observation of a single protein band in sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE) [6]. A scaleable, "chromatofocusing-like" technique was developed in an attempt to separate and isolate these species for further characterization. The term "chromatofocusing-like" is suggested to describe a distinct branch of ion-exchange chromatography. Unlike standard chromatofocusing technique, this method does not use carrier ampholytes during gradient separation of proteins, nor does it employ high concentrations of counter-ions for protein elution, the usual method for performing standard ion-exchange chromatography. The basis for this powerful separation technique is in the titration of chemical functional groups attached to an ion-exchange column, in this case the sulfopropyl (SP) functionality of a strong cation-exchange HPLC column, with low-buffering-capacity buffers.

We have also applied our method to resolve two forms of recombinant human interleukin-1β (rhIL-1β) in a manner similar to the rhIL-1α separation.

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EXPERIMENTAL

Apparatus

The chromatographic system consisted of the following from Waters Chromatography Division (Millipore, Milford, MA, USA): Delta Prep 3000 HPLC system, Lamda-Max Model 480 variable-

wavelength detector, Model 740 data module. The preparative fraction collector used was a Model PF-30 unit (Pharmacia, Piscataway, NJ, USA). The peak detector (Hoffmann–La Roche Instrument Support Section, Nutley, NJ, USA) activated a collection valve (Valcor Engineering, Springfield, NJ, USA) for automated peak collection.

Columns

The stainless-steel HPLC columns were all silica-based SP-strong cation-exchange columns (Separation Industries, Metuchen, NJ, USA; NuGel P-SP).

Buffers

Buffers for rhIL-1α separation consisted of the following: buffer A was 3.7 m*M* acetic acid, adjusted to pH 4.8, with sodium hydroxide; buffer B was 10 m*M* potassium phosphate, dibasic, adjusted to pH 6.8, with hydrochloric acid.

Buffers for rhIL-1β separation consisted of the following: buffer A was 20 m*M* acetic acid, adjusted to pH 5.0 with sodium hydroxide; buffer B was the same as that for the rhIL-1α separation, except adjusted to pH 7.1 with hydrochloric acid.

Filtration of buffers and samples was done using a combination 0.8–0.2 μm filter (Sartorius Filters, Hayward, CA, USA).

All buffers were sparged continuously with ultra-high-purity helium (Linde Specialty Gases, Danbury, CT, USA).

Separation of isoelectric species of rhIL-1α

Initially, an eight-fold dilution of purified rhIL-1α, amino acids 117-271 (0.8 mg protein per ml of 0.1 *M* sodium chloride), with buffer A for rhIL-1α separation was made, the pH adjusted to 4.8 and the sample filtered.

After thorough column equilibration with buffer A, the diluted sample was applied to the column. A steady baseline was established by equilibration with buffer A washing off any unabsorbed contaminants. A linear, ascending pH gradient (4.8–6.8) was performed from 0 to 80% buffer B in 120 min, and monitored at 280 nm absorbance.

Separation of two forms of rhIL-1β

Twenty-fold dilution of purified rhIL-1β (4 mg protein per ml of 0.1 *M* sodium chloride) was made with buffer A for rhIL-1β separation, the pH adjusted to 5.0 and the sample filtered. Column equilibration with buffer A for rhIL-1α separation was established. A linear, ascending pH gradient (5.0–7.1) was performed from 0 to 100% buffer B in 100 min, and monitored at 280 nm absorbance.

Analytical evaluation of purity

IEF of rhIL-1α was performed on a LKB Model 2117 Multiphor horizontal unit using pH 4.0–6.5 and rhIL-1β using pH 5.5–8.5 gradient polyacrylamide gels as described by the manufacturer (Ampholine PAGplates, pH 4–6.5, Part No. 80-1124-81 and pH 5.5–8.5, Part No. 80-1124-82; Pharmacia). Proteins on the gel were fixed in a fresh solution containing 5% (w/v) sulfosalicylic acid and 10% (w/v) trichloroacetic acid for 30 min at room temperature. Gels were stained in 0.025% Coomassie blue R-250 (BioRad Labs., Richmond, CA, USA; Cat. No. 161-0500) in destain solution (acetic acid–ethanol–water; 9.5:30:60.5, v/v/v) for 40 min and destained overnight with several additional changes. Calibration standards for IEF (Sigma, St. Louis, MO, USA) were run simultaneously with samples and p*I* values calculated by plotting the standard p*I* values *versus* the distance from the cathode (−) in mm. The unknown p*I* values were then measured in millimeters from the cathode and extrapolated from the standard curve to determine their approximate p*I*.

Total rhIL-1α concentration was obtained by performing the automated immunoaffinity chromatographic assay [7]. Each of the species reacts equally with antibody. Protein concentration was monitored using the BCA protein assay (Pierce, Rockford, IL, USA).

RESULTS AND DISCUSSION

Two major isoelectric species were observed in the purified rhIL-1α preparation by analytical IEF (Fig. 1, lane 2), one having a p*I* of 5.1 and the other a p*I* of 5.3. These species were indistinguishable from each other and appeared as a homogeneous band on SDS-PAGE. Two proteins were separated on the SP-strong cation-exchange HPLC column (Fig. 1, peaks 1 and 2) and reanalyzed for purity by analytical IEF (Fig. 1, lanes 3 and 4). An Asp residue at position 32 in the IL-1α with a p*I* of 5.1 (Fig. 1, lane 3, peak 1) and Asn residue at the identical posi-

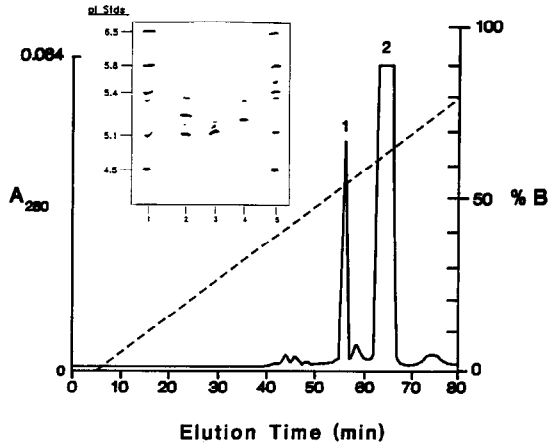

Fig. 1. Separation of deamidated (peak 1, p*I* 5.1) from amidated (peak 2, p*I* 5.3) rhIL-1α (amino acids 117–271) performed on SP-strong cation-exchange HPLC. Separation conditions were as follows: column size, 250 mm × 50 mm I.D.; particle size, 15–20 μm; pore size, 300 Å; sample load, 250 mg; flow-rate, 75 ml/min; gradient, 0–80% B in 120 min, buffer A (3.7 m*M* acetic acid, pH 4.8), buffer B (10 m*M* potassium phosphate, dibasic, pH 6.8). Inset: analytical isoelectric focusing of rhIL-1α (amino acids 117–271) performed on pH 4.0–6.5 IEF gel. Samples derived from SP-HPLC separations. Lanes: 1, 5 = standard proteins, p*I* values indicated; 2 = SP-HPLC sample load, 3 = peak 1, deamidated species (p*I* 5.1); 4 = peak 2, amidated species (p*I* 5.3).

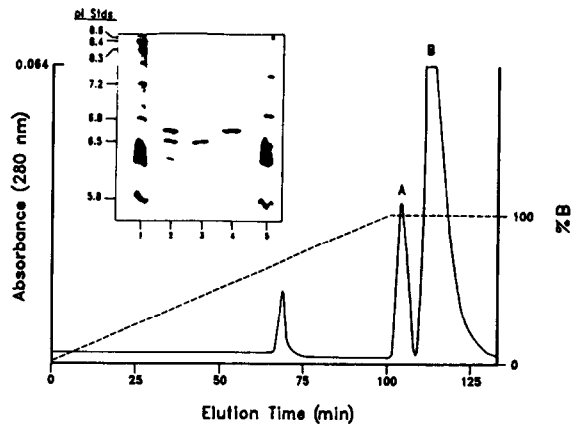

Fig. 2. Separation of N-Met (peak A, p*I* 6.5) from des-Met (peak B, p*I* 6.7) rhIL-1β performed on SP-strong cation-exchange HPLC. Separation conditions were as follows: column size 250 mm × 25 mm I.D.; particle size, 15–20 μm; pore size, 300 Å; sample load, 125 mg; flow-rate 20 ml/min; gradient, 0–100% B in 100 min, buffer A (3.7 m*M* acetic acid, pH 4.8), buffer B (10 m*M* potassium phosphate, dibasic, pH 7.1). Inset: analytical isoelectric focusing of rhIL-1β performed on pH 5.5–8.5 IEF gel. Samples derived from SP-HPLC separations. Lanes: 1, 5 = standard proteins, p*I* values indicated; 2 = SP-HPLC sample load; 3 = peak A, N-Met form (p*I* 6.5); 4 = peak B, des-Met form (p*I* = 6.7).

tion in the species with a p*I* of 5.3 (Fig. 1, lane 4, peak 2) was determined by N-terminal protein sequence analysis of up to the first 74 residues for each of the proteins. These determinations were confirmed by comparison of each sequence to the predicted cDNA precursor gene sequence [8]. Results indicated that the Asn–Asp difference at residue 32 is due to the conversion of the amide sidechain of the Asn (p*I* 5.3) to a carboxyl group in Asp (p*I* 5.1), also known as deamidation, as previously reported [9–11].

A rhIL-1α sample (250 mg total protein) loaded on the 250 mm × 50 mm I.D. SP-HPLC column typically yielded recoveries of 85% amidated species (p*I* 5.3), 10% deamidated species (p*I* 5.1) and 5% consisting of other minor contaminants.

Similar deamidated and amidated species of rhIL-1α from *E. coli* were previously isolated by chromatofocusing by other investigators [12].

Two forms of the rhIL-1β were observed during its large-scale purification by analytical IEF (Fig. 2, lane 2), the des-Met form having a p*I* of 6.7 and the N-Met form having a p*I* of 6.5. These appeared as a single band on SDS-PAGE. Separation was made possible by our HPLC method (Fig. 2, peaks A and B) and subsequent analysis by analytical IEF (Fig. 2, lanes 3 and 4). The presence of an incompletely processed N-terminal methionine (N-Met) in the earlier-eluting HPLC peak A and that of a correctly processed physiological N-terminal alanine (des-Met) form, the later-eluting peak B on HPLC, was established by comparison of each sequence to the predicted cDNA precursor gene sequence [8].

Mixtures of similar forms were previously isolated from recombinant-derived interleukin-1β by chromatofocusing and characterized by others on an analytical scale [13].

There are several advantages to using our method for the separation of closely related isoelectric species. First, expensive ampholytes are not needed which are impractical for scale-up. Also, because ampholytes are not used, their somewhat difficult removal from protein samples is not an issue. The

presence of sodium chloride in the elution buffers for both rhIL-1α and rhIL-1β in the separations we performed resulted in an extreme loss in resolution, or even co-elution of species or forms. For other proteins which may be highly charged, salt may be required in the elution buffer to resolve species of interest. In any case, salts should be used sparingly to avoid interference in protein charge differences which normally enhance resolution between peaks.

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