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Analysis of recombinant human growth hormone in *Escherichia coli* fermentation broth by micellar high-performance liquid chromatography

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Abstract

A method for the reversed-phase high-performance liquid chromatographic (HPLC) determination of recombinant methionylaspartyl-human growth hormone (MD-HGH) in *Escherichia coli* fermentation broth is described. The technique utilizes mobile phases containing *n*-propanol and the anionic surfactant sodium dodecyl sulfate (SDS) under micellar conditions at pH 6.4. The methodology is directly applicable to the analysis of samples solubilized via sulfitolysis in the presence of SDS, and offers superior resolution in comparison with chromatography in the absence of the surfactant. Using this method, acceptable precision (day-to-day R.S.D. = 4.9%), accuracy, selectivity, range, linearity and ruggedness were achieved.

1. Introduction

Advances in recombinant DNA technology have provided a variety of host–vector systems for the cloning and expression of heterologous proteins in microorganisms. The production and isolation of these recombinant proteins has typically been monitored through the use of analytical techniques such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [1,2], immunoassays [3,4], biological activity assays [4–6] and high-performance liquid chromatography (HPLC) [3,6–9]. However, these methods have been limited by non-specificity, labor-intensive sample manipulation or the use of harsh conditions such as high concentrations of formic acid [8], or are useful for only properly folded, soluble target protein. There have been few reports of the determination of recombinant proteins in fermentation broth matrices, where the target molecules frequently exist inside the microorganism as inclusion bodies in an insoluble denatured state.

The analysis of heterologous proteins in recombinant hosts such as *E. coli* presents many challenges to the analytical biochemist. The cells must be lysed and the inclusion bodies solubilized prior to quantification. Cell lysis and protein solubilization can be accomplished chemically through the use of SDS, an anionic surfactant [9]. As the proteins in inclusion bodies can exist as a distribution of forms, such as covalent and non-covalent polymers, it is crucial to convert the target protein into a single molecular entity prior to analysis. This can be achieved by unfold-

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ing the proteins and disrupting the inter- and intramolecular disulfide bonds via reduction [10] or sulfitolysis [11]. As both the whole cell and the inclusion bodies can also contain nucleic acids, salts, lipids and other host molecules in addition to proteinaceous material, the complexity of the matrix adds to the difficulty of the determination of the recombinant protein.

The employment of ionic surfactants such as SDS has traditionally been avoided in the HPLC of proteins owing to the generation of a strongly denaturing environment. However, for the determination of recombinant proteins in fermentation broth, the characteristics of ionic surfactants are in fact useful for the enhancement of the solubility of the unfolded denatured proteins, the elimination of irreversible adsorption on the stationary phase and the facilitation of unique separation selectivity.

In this paper, a method for the determination of a protein in *Escherichia coli* fermentation broth is described. Recombinant human growth hormone (HGH) is synthesized in *E. coli* as methionylaspartyl-HGH (MD-HGH), a hydrophobic protein which readily polymerizes and is not easily solubilized in its sulfitolyzed form. The method described utilizes isocratic reversed-phase micellar HPLC at near neutral pH to determine sulfitolyzed MD-HGH.

2. Experimental

MD-HGH reference material and fermentation broth were provided by Eli Lilly (Indianapolis, IN, USA). SDS and potassium tetrathionate were purchased from Fluka (Ronkonoma, NY, USA), and *n*-propanol (HPLC grade) from Baxter Scientific Products (McGaw Park, IL, USA) and tris(hydroxymethyl)-aminomethane (Tris) from Fisher Scientific (Springfield, NJ, USA). Human carbonic anhydrase I and bovine carbonic anhydrase were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of at least analytical-reagent grade and were supplied by EM Science (Gibbstown, NJ, USA). Purified water was obtained via a Milli-Q reagent water system (Millipore, Millford, MA).

Chromatography was achieved through the use of a Gilson Medical Electronics (Middleton, WI, USA) two-pump system composed of a Model 305 and a Model 303 pump, a Model 805 manometric module, a Model 811 dynamic mixer, a Model 231 autosampler equipped with a Model 401 diluter and a 20-μl injection loop and a Model 116 variable-wavelength UV detector. The column temperature was maintained at 60°C with a Model CHM column oven equipped with a Model TCM control module (Waters, Millford, MA, USA). A 25 cm × 0.46 cm I.D. Nucelosil C_4 packing (particle size 5 μm, pore size 300 Å) (Phenomenex, Torrance, CA, USA) was employed as an analytical column following a 1.5 cm × 0.32 cm I.D. Brownlee RP-4 guard cartridge (Munhall, Worthington, OH, USA) for all the experiments unless noted otherwise. A saturation precolumn was prepared by packing a 7.5 × 0.75 cm I.D. stainless-steel column with 100–200-μm silica (Universal Scientific, Atlanta, GA, USA), and was incubated inside the column oven in-line between the dynamic mixer and the injection valve to presaturate the mobile phase with dissolved silica to preserve the analytical resin. Mobile phase A [50 m*M* ammonium phosphate (pH 6.4)–20% *n*-propanol–1.0% SDS] was prepared by dissolving 3.2 g of monobasic ammonium phosphate, 1.6 g of dibasic ammonium phosphate and 10.0 g of SDS in 800 ml of water and adding 200 ml of *n*-propanol. The preparation of mobile phase B [50 m*M* ammonium phosphate (pH 6.4)–30% *n*-propanol–1% SDS] was identical with that of A with the exception that the salts were dissolved in 700 ml of water and 300 ml of *n*-propanol. The pH of the mobile phases was 6.3–6.5 (if necessary, adjustments were made using either concentrated phosphoric acid or 6 *M* sodium hydroxide solution). Other parameters included a flow-rate of 0.5 ml/min and UV detection at 214 nm. Analog data (1.0 Hz sampling rate) were collected directly from the detector on an in-house centralized chromatography computer system based on the Hewlett-Packard Model 1000

minicomputer. Retention time, efficiency, normalized peak width, tailing and resolution were calculated by the minicomputer.

The sulfitolysis solubilization reagent was prepared in the following manner. SDS (20.0 g) was dissolved in 700–800 ml of water. To this solution was added 6.1 g of Tris, 12.0 g of anhydrous sodium sulfite, 3.7 g of anhydrous potassium tetrathionate and 0.37 g of disodium ethylenediaminetetraacetate dihydrate. The pH of the solution was adjusted to 8.5–8.7 using 6 *M* hydrochloric acid and the solution was diluted to 1 liter.

Fermentation broth samples were diluted with an equal volume of methanol prior to preparation for analysis. An appropriate aliquot of the broth–methanol suspension was transferred into a 125 mm × 16 mm I.D. centrifuge tube. Following centrifugation at 3000 *g* for 15 min, the supernatant was discarded and the pellet was resuspended in 10.0 ml of solubilization reagent. The sample was cooled on ice while sonicated for 30 s using a Vibra Cell sonicator (Sonics and Materials, Danbury, CT, USA) equipped with a microtip and set at a power setting of 5 in the continuous mode. The solution was then stirred for 10–16 h at room temperature (an investigation of the kinetics of protein sulfitolysis in the presence of SDS at ambient temperature revealed that sulfitolysis under these conditions is complete within 6–8 hours [12]). On completion of stirring, the sulfitolysis reaction was quenched and the solubilized protein stabilized by adjusting the pH of the solution to 5.8–6.2 using 1 *M* maleic acid. The samples were then filtered (0.45-μm nylon membranes; Alltech, Deerfield, IL, USA) prior to analysis.

3. Results and discussion

Micellar mobile phases have been employed successfully for the chromatography of small biomolecules, and these studies have demonstrated the potential advantages offered by systems of this nature, such as enhanced fluorescence [13] and improved selectivity [14]. These advantages may also be characteristic of the micellar chromatography of macromolecules such as proteins. The unique selectivity of the micellar HPLC of MD-HGH is demonstrated in Fig. 1, where the elution profiles of a 467 μg/ml MD-HGH standard are displayed. Fig. 1c, obtained through the use of isocratic elution with *n*-propanol in the absence of SDS (SDS was omitted from the mobile phases, which were otherwise identical with those described under Experimental), revealed the presence of a contaminant in the purified reference material which eluted within the front shoulder of the MD-HGH peak. The contaminant was also visible in the profiles of solubilized fermentation broth samples analyzed under the same conditions (data not shown). This peak did not appear on the shoulder of the main peaks generated by the same samples when they were analyzed using mobile phases containing SDS (see the chromatogram of the standard in Fig. 1b). Approximately 30 μg of the contaminant (determined by dry mass relative to that of an equivalent volume of mobile phase following freeze-drying) was isolated via fraction collection in the absence of SDS and then dried and reconstituted in 1.0 ml of solubilization reagent. Analysis of the resolubilized isolated contaminant by micellar HPLC revealed that the material generated a peak at ca. 540 s, an elution time much earlier than that of MD-HGH (see Fig. 1a). Also, the analysis of a solubilization reagent blank (Fig. 1d) demonstrated that the peak of interest had not been generated by a component present in the reagent. Because it offered superior resolution between MD-HGH and this contaminant, micellar HPLC in SDS was the method of choice for the analysis of the protein in *E. coli* fermentation broth.

Fig. 2b shows a typical chromatogram of MD-HGH in solubilized fermentation broth obtained via micellar HPLC. Under the chromatographic conditions described, the values of the asymmetry and theoretical plate count were 1.2 and 1569 theoretical plates/m, respectively [15]. A test of precision with a fermentation broth sample containing ca. 740 μg/ml of MD-HGH (five injections analyzed on five different days) generated a relative standard deviation (R.S.D.)

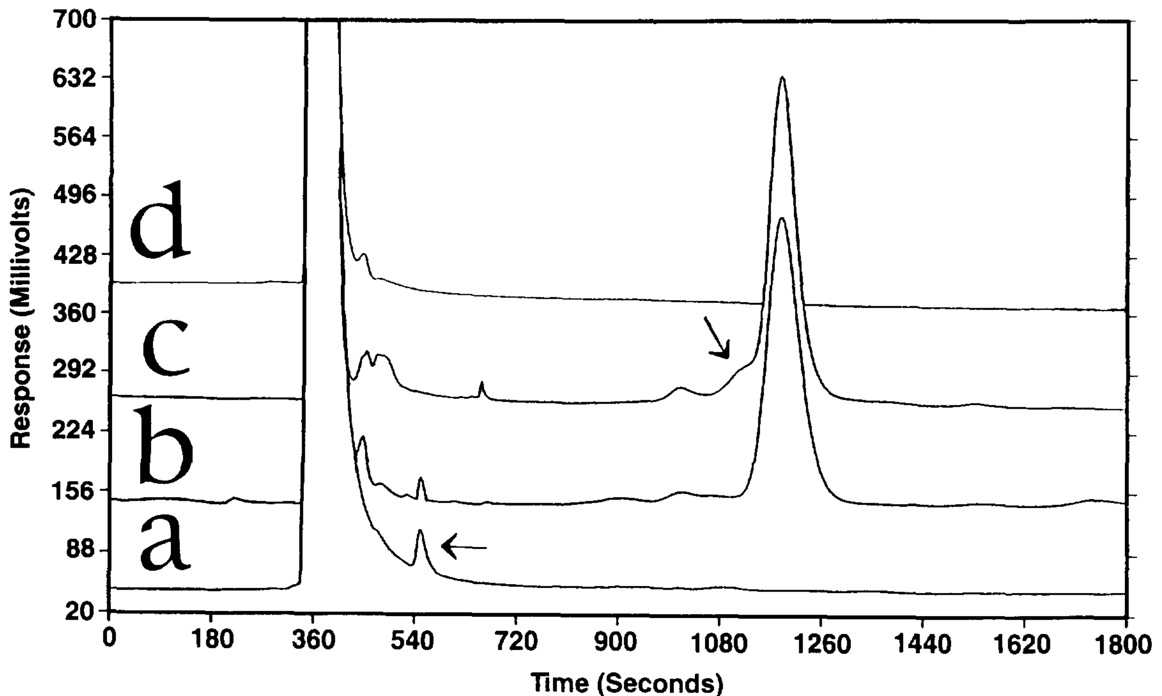

Fig. 1. HPLC profiles of 20-μl injections of (a) a 30.0 μg/ml solution of contaminant peak (isolated using chromatography in the absence of SDS) obtained via isocratic elution at 47.5% B in 50 m*M* ammonium phosphate (pH 6.4)–1.0% SDS, (b) a 467 μg/ml MD-HGH standard obtained using isocratic elution at 47.5% B in 50 m*M* ammonium phosphate (pH 6.4)–1.0% SDS, (c) a 467 μg/ml MD-HGH standard obtained using isocratic elution at 45.0% B in 50 m*M* ammonium phosphate (pH 6.4) in the absence of SDS and (d) a reagent blank obtained using isocratic elution at 47.5% B in 50 m*M* ammonium phosphate (pH 6.4)–1.0% SDS, using a 250 cm × 0.46 cm I.D. Nucleosil C_8 packing, a flow-rate of 1.0 ml/min and UV detection at 214 nm. The contaminant in the elution profiles of the solubilized standard material and the re-chromatographed fraction of the isolated contaminant are indicated by arrows.

of 4.46% (the within-day RSD ranged from 0.50 to 1.88%). The method was determined to be linear over the range 50–800 μg/ml (correlation coefficient = 0.999) using peak area for quantification.

To evaluate the possibility of matrix effects on the assay, a series of standards ranging from 0 to 800 μg/ml MD-HGH were prepared in a control fermentation broth containing cells in which MD-HGH had not been expressed. The slope of a plot of peak area versus concentration for this standard series was 45.06 arbitrary units. The slope of a calibration plot for standards prepared in solubilization reagent alone was 45.20 units, i.e., only a 0.3% difference. In addition, the former plot passed through the origin. Further evidence for the absence of matrix interference was provided by an overlay of the chromatographic profiles of the control broth and a 467 μg/ml standard (see Fig. 2a and c).

The fermentation broth sample recovery was evaluated by spiking a known volume of fermentation broth with a non-sulfitolyzed MD-HGH standard solution at four levels ranging from 100 to 800 μg/ml. The recovery of MD-HGH at these levels averaged 103.5%, indicating complete extraction of the sulfitolyzed protein from the broth.

To determine the magnitude of the sensitivity of the assay to changes in environmental and operational parameters, analyses of an MD-HGH standard, fermentation broth sample and a resolution mixture containing human carbonic anhydrases II and bovine carbonic anhydrase

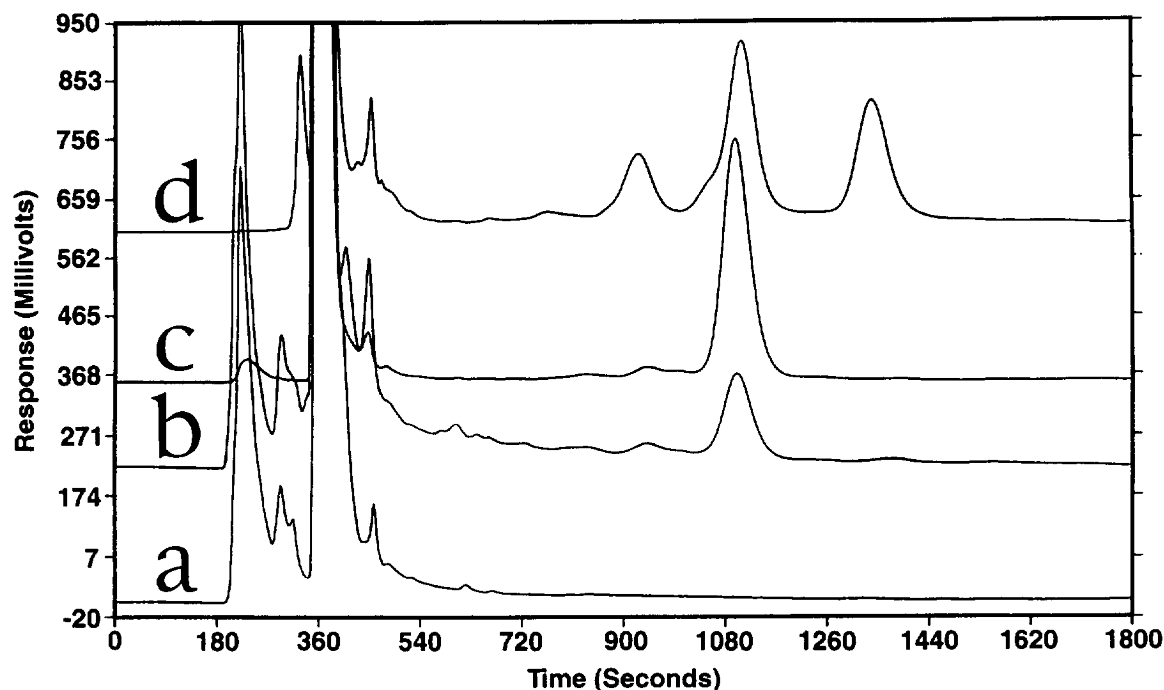

Fig. 2. Micellar HPLC profiles of 20-μl injections of (a) solubilized control fermentation broth, (b) solubilized fermentation broth, (c) 467 μg/ml MD-HGH standard and (d) resolution control containing 500 μg/ml each of bovine carbonic anhydrase and human carbonic anhydrase IIa obtained using isocratic elution at 47.5% B in 50 m*M* ammonium phosphate (pH 6.4)–1.0% SDS using a 250 cm × 0.46 cm I.D. Nucleosil C_8 packing, a flow-rate of 1.0 ml/min and UV detection at 214 nm.

[chosen because of their elution time proximity to MD-HGH in this assay (see Fig. 2d)] were obtained in systems where the organic modifier concentration, column temperature, mobile phase pH and column packing were varied. The two latest eluting peaks within the elution profile of the resolution control were utilized for the calculation of resolution values.

The effects of *n*-propanol concentration in the mobile phase on the isocratic elution of MD-HGH and the resolution control are displayed in Table 1, where the retention time, plate count, normalized peak width, tailing and resolution are listed for analyses obtained at 45.0, 47.5, 50.0, 52.5 and 55.0% B (a 1% change in B corresponded to a 0.1% change in the *n*-propanol

Table 1
Effects of mobile phase organic composition on the retention time, efficiency, normalized peak width, peak tailing and resolution of MD-HGH

B (%)	Retention time (s)	Efficiency (plates)	Peak width (s)	Asymmetry	Resolution
45.0	1319	1170	111.7	1.30	–[a]
47.5	1190	1465	103.9	1.19	1.73
50.0	1085	1569	100.0	1.20	2.64
52.5	1000	1636	98.4	1.19	1.74
55.0	927	1670	98.4	1.16	2.33

All data are mean values from three replicate injections.
[a] Peaks did not elute within a 30-min run time.

Table 2
Effects of column temperature on the retention time, efficiency, normalized peak width, peak tailing and resolution of MD-HGH

Temperature (°C) retention	Time (s)	Efficiency (plates)	Peak width (s)	Asymmetry	Resolution
54.0	1262	1679	99.8	1.12	2.60
57.0	1174	1710	97.8	1.15	2.61
60.0	1099	1934	92.3	1.14	2.75
63.0	1043	2114	88.2	1.14	2.75
66.0	996	2111	88.3	1.14	2.99

All data are mean values from three replicate injections.

concentration in the mobile phase). The retention time, peak width and asymmetry all appeared to decrease in response to an increase in mobile phase organic content. There was no apparent decrease in resolution with these changes in B content.

The effect of temperature on the chromatography of MD-HGH was investigated by performing analyses at five different column oven settings ranging from 54 to 66°C. The chromatographic parameters determined in this set of experiments are listed in Table 2. The effects of temperature on the mass transfer kinetics appear to be evidenced by the decrease in retention time and peak width and the enhanced efficiency as the column over temperature was adjusted to higher levels. Peak asymmetry and resolution were not influenced by temperature changes within the range studied.

Mobile phase pH can have strong effects on the elution characteristics of proteins. In general, acidic proteins such as MD-HGH ($pI = 5.9$) will display a tendency toward greater hydrophobicity as the pH is lowered, since acidic residues become protonated under these conditions. Evidence for this occurrence is provided by the data in Table 3. The optimum mobile phase pH for the determination of MD-HGH appears to be near 6.4. At pH 5.76 and 6.08, MD-HGH does not elute within 1800 s. As the mobile phase pH was increased from 6.40 to 7.04, the retention time and peak width increased by ca. 10%, while the efficiency decreased by 20%. Also, at pH > 6.40, the carbonic anhydrase resolution control generated a profile of multiple partially resolved peaks, which prevented the calculation of resolution for this sample under these conditions. The extra peaks which appear at the higher pH may represent contaminants present in the commercially available carbonic anhydrase material which are not resolved at pH 6.40. The effect of temperature on mobile phase pH was determined to be minimal, as the pH of 50 m*M* ammonium phosphate, which measured pH 6.4

Table 3
Effects of mobile phase pH on the retention time, efficiency, normalized peak width, peak tailing and resolution of MD-HGH

pH	Retention time (s)	Efficiency (plates)	Peak width (s)	Asymmetry	Resolution
5.76	–[a]	–[a]	–[a]	–[a]	–[a]
6.08	–[a]	–[a]	–[a]	–[a]	–[a]
6.40	990	1563	101.0	1.19	2.18
6.72	1020	1298	108.0	1.25	–[b]
7.04	1058	1223	111.0	1.25	–[b]

All data are mean values from three replicate injections.
[a] Peaks did not elute within a 30-min run time.
[b] Multiple (>4) peaks were generated, which prevented determination of resolution.

Table 4
Effects of column packing on the retention time, efficiency, normalized peak width, peak tailing and resolution of MD-HGH chromatography

Packing	Retention time (s)	Efficiency (plates)	Peak width (s)	Asymmetry	Resolution
Nucleosil	1099	1934	92.3	1.14	2.75
Vydac	1108	1338	111	1.14	2.35

All data are mean values from three replicate injections.

at room temperature (26°C), rose to only 6.5 on being heated in a water bath to 60°C to emulate the environment present inside the column oven.

To identify an alternative column for use in the micellar HPLC determination of MD-HGH, a Vydac C_4 packing [15 cm × 0.46 cm I.D. Vydac C_4 packing, particle size 5 μm, pore size 300 Å (Munhall)] was evaluated. Although the experimental data suggested that the Vydac packing was slightly inferior to the Nucleosil packing for use in this application (see Table 4), the performance of both columns appeared to be acceptable for the determination of the recombinant protein in fermentation broth.

4. Conclusions

A micellar HPLC assay for the determination of MD-HGH in *E. coli* fermentation broth has been described. For this application, micellar chromatography was directly compatible with samples solubilized in SDS sulfitolysis reagent and offered a resolution superior to that achievable via reversed-phase chromatography in the absence of surfactant. The method is rugged in response to variations in mobile phase elution strength, temperature and column packing, but not to mobile phase pH changes. Analytical column lifetimes have been found routinely to correspond to at least 400 injections under the conditions specified in the method. The linear dynamic range of the method is particularly useful for monitoring fermentation processes.

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