Chromatographic Methods for Quantitative Analysis of Native, Denatured, and Aggregated Basic Fibroblast Growth Factor in Solution Formulations

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High-performance liquid chromatography (HPLC) methods were developed for evaluating stability of human recombinant basic fibroblast growth factor (bFGF) against denaturation and aggregation in solution formulations. Reversed-phase chromatography (RP-HPLC)—insensitive to bFGF tertiary structure—was used to measure total soluble protein; heparin affinity chromatography (HepTSK) provided quantitative analysis of native bFGF species. The folding state of bFGF was determined by fluorescence spectroscopy: Tryptophan emission, which was quenched in native protein, increased upon unfolding. Slow unfolding/refolding kinetics of bFGF in 2 *M* guanidine hydrochloride made possible the separation of native from denatured species by size exclusion chromatography (SEC). Although the tertiary structure affected bFGF retention times, it did not change the sample recovery by SEC. These chromatographic techniques, which quantitatively measure physical and chemical changes taking place in solution formulations, can be used in future investigations of bFGF stability.

KEY WORDS: fibroblast growth factor; protein stability; formulation; HPLC; denaturation kinetics; fluorescence.

INTRODUCTION

Stability of protein-based pharmaceuticals is an issue of major importance for the biotechnology industry. Rapid decomposition via physical and chemical pathways often precludes the use of solution formulations, making lyophilization a frequent method for prolonging protein shelf life (1). At the preformulation stage, the choice of appropriate analytical tools is a critical part of rational formulation design. Currently available bioassays have a large margin of error (±10–50%) and cannot be relied upon for accurate determination of protein stability. UV spectroscopy can be used to evaluate total protein content, and high-performance liquid chromatography (HPLC) to determine chemical integrity; tertiary structure can be assessed by affinity HPLC, circular dichroism (CD), or fluorescence spectroscopy; and aggregation state can be monitored by size-exclusion chromatography (SEC) and/or polyacrylamide gel electrophoresis (PAGE). Clearly, no single analytical technique provides comprehensive characterization of a protein solution; in fact, valuable information may be lost under the particular conditions of an assay. Reversed-phase (RP)-HPLC eluants are often denaturing (low pH, organic solvents), making this method unsuitable for measuring the extent of protein unfolding (2). Similarly, chemical modifications with little effect on tertiary structure may go undetected by affinity chromatography or fluorescence assays.

Basic fibroblast growth factor (bFGF) is a mitogenic, heparin-binding growth factor (3,4), currently undergoing clinical trials for treatment of impaired wound healing (5). Development of stable bFGF solution formulations requires rapid and quantitative characterization of physicochemical changes leading to degradation. Chemical modifications are readily detected by chromatographic methods, but physical decomposition presents additional complications. While direct biophysical measurements provide qualitative evaluations of overall physical stability, they lack specificity: Changes in CD or fluorescence spectra are indicative of conformational perturbations but do not identify the affected species. For optimization of formulation parameters, quantitative HPLC techniques (heparin affinity and SEC) with separation based on tertiary structure were developed.

MATERIALS AND METHODS

Reagents. Recombinant human bFGF (MW 17.1 kD; pI = 9.8) was produced at Scios Nova in an *Escherichia coli* expression system described by Abraham *et al.* (6). Buffer reagents and excipients were of reagent or higher grade and were obtained as follows: KH_2PO_4 and K_2HPO_4 (Fisher), Na_2EDTA (Spectrum), dithiothreitol (DTT; Research Organics), trifluoroacetic acid (TFA; Pierce), acetonitrile (ACN; Baxter), NaCl (Baker), guanidine hydrochloride (GdnHCl; ultra-pure, ICN), and gel filtration standards (Bio-Rad).

Analytical Methods. All chromatographic analyses were performed on a Hewlett Packard HP 1090, using a refrigerated autosampler, a 1-μm in-line filter, and a 250-μL injection loop. All mobile-phase buffers and samples were filtered through 0.22-μm cellulose acetate filters.

Heparin Affinity Chromatography. A Toso Haas heparin TSK-gel column (7.5 mm × 7.5 cm) was equilibrated with 100 m*M* potassium phosphate, 1 m*M* Na_2EDTA, pH 6.5 (Buffer A), and eluted using a linear NaCl gradient of 0–3 *M* in the same buffer (400 mmol/min from 4 to 7 min, 150 mmol/min from 7 to 17 min, 1 mL/min, 25°C, detection at 215 and 277 nm).

RP-HPLC. A Vydac C4 column (4.6 mm × 15 cm), equilibrated with 0.1% TFA/water, was eluted using an acetonitrile gradient created by 0.08% TFA/ACN (2 min at 11.5%/min, 5 min at 1%/min, 20 min at 0.5%/min, 1 mL/min, 40°C, detection at 215 and 277 nm).

SEC. Analysis was done on Bio-Rad Bio-Sil Sec-125 or Toso Haas TSK-Gel G2000 SWXL columns (7.8 mm × 30 cm), using isocratic elution (0.75 mL/min, 25°C, detection at 215 and 277 nm) with appropriate on-line mixing of Buffer A with 3 *M* NaCl in Buffer A (pH 6.5) and 4 *M* GdnHCl in Buffer A (pH 6.5). For on-line fluorescence detection of tryptophan emission, a Shimadzu fluorescence HPLC monitor (Model RF-530) was attached in series with the UV detector; the excitation wavelength was set at 290 nm, and

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detection at 350 nm. The delay time between UV and fluorescence detectors was 0.15 min.

Fluorescence Spectroscopy. bFGF solutions (diluted to 0.1 mg/mL) were analyzed in quartz cuvettes (4- and 10-mm excitation and emission pathlength, respectively) using either a Schimadzu RF540 or a Perkin Elmer LS50B fluorimeter. Emission spectra (290–420 nm) were recorded at 400 nm/min for excitation at 277 nm (5-nm excitation and emission slit widths). Background buffer intensity corrections were done manually. Kinetics of unfolding and refolding were determined by automated scanning of emission spectra between 290 and 390 nm at 1.5-min and 40-sec intervals, following 30-fold dilution of 3 mg/mL bFGF solution with appropriate buffer. In native bFGF, fluorescence arose from tyrosine residues (308 nm), while tryptophan emission (350 nm) was quenched; upon unfolding, tryptophan emission increased. F_r, or the ratio of emission at 350 nm to that at 308 nm (which, on the Perkin Elmer fluorimeter, changed from 0.25 in native to 2.5 in unfolded bFGF), was used to determine the extent of denaturation (7).

UV Spectroscopy. Samples were filtered (0.2 μm, PVDF Millipore or Polysulfone Acrodisc syringe filters) into quartz cuvettes (5-mm width × 1-cm pathlength) and scanned from 200 to 500 nm on a Cary 3 spectrophotometer. bFGF concentrations were determined using $E_{1\%}^{1\ cm} = 8.4$ at 277 nm.

Sample Preparation. Concentrated bFGF stock solutions were exchanged into appropriate buffers by ultrafiltration using Centriprep-10 (Amicon), diluted to 2–4 mg/mL, and stored at −80°C if not used immediately. Samples for denaturation studies were prepared by combining bFGF stock (2 mg/mL) with 8 *M* GdnHCl or 8 *M* urea and diluting to appropriate chaotrope and protein concentrations. Prior to HPLC analyses, bFGF concentrations in all samples were determined by UV absorbance at 277 nm.

RESULTS AND DISCUSSION

In development of bFGF solution formulations, the following HPLC techniques were evaluated for use as stability indicating methods.

RP-HPLC

RP-HPLC is a conventional method for assessing chemical integrity of proteins. During RP-HPLC analysis, bFGF was denatured by the mobile phase (F350/F308 ratio of 1.7 in 0.1% TFA/50% ACN) so that unfolding by chaotropes had no effect on its interactions with the RP column (Fig. 1). Since it was insensitive to tertiary structure, this RP-HPLC technique provided quantitation of total soluble protein. The ability to analyze denatured bFGF was of particular importance for mass balance determination and characterization of degradation products (e.g., precipitates containing bFGF aggregates which can be resolubilized by chaotropes).

Heparin Affinity Chromatography

bFGF is a heparin-binding protein whose complex with sulfated polysaccharides is disrupted at >1 *M* NaCl concentrations (8). Because of this unique feature, bFGF has been analyzed on heparin affinity columns (9). Interaction with

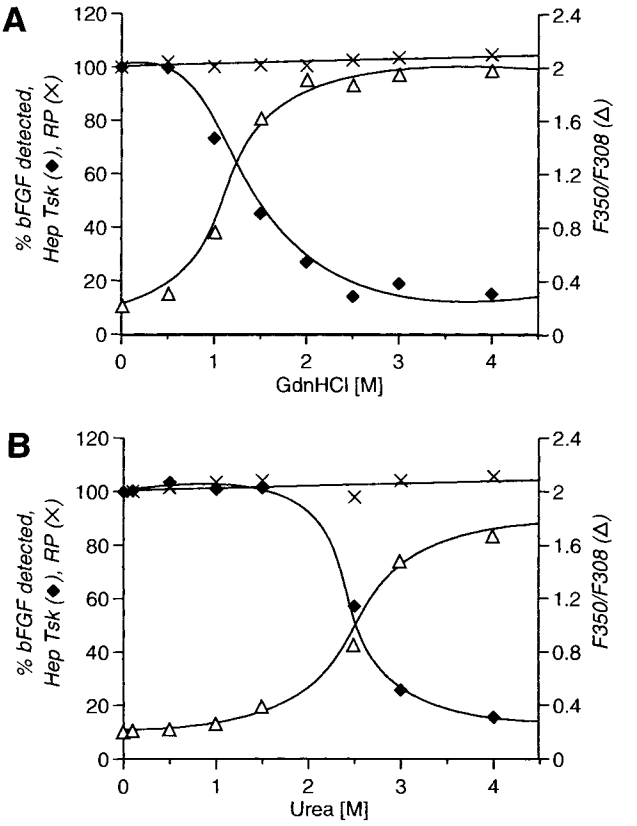

Fig. 1. Chaotrope-dependent bFGF denaturation: degree of unfolding expressed as the ratio of fluorescence intensities at 350 to 308 nm (△) and bFGF recovery from HepTSK (◆) and RP (×) columns following denaturation in (A) GdnHCl and (B) urea.

heparin-bound column packing material relies on maintenance of bFGF tertiary structure (or at least that of its heparin-binding domain) because it involves bringing into proximity residues distant from each other in the sequence (10,11). To evaluate HepTSK sensitivity to conformational changes, the degree of bFGF unfolding in the presence of urea or GdnHCl was determined by fluorescence spectroscopy prior to HPLC analysis. Figure 1 clearly illustrates that only native bFGF was retained on HepTSK, and the extent of binding was inversely proportional to the extent of denaturation. GdnHCl and urea had similar effects on bFGF affinity for heparin, suggesting that increased ionic strength of GdnHCl was not solely responsible for disrupting the heparin-bFGF complex. (Refolding of bFGF was not possible because, during analysis, denatured bFGF eluted with the injection front, where it remained exposed to high local concentrations of GdnHCl or urea.)

As shown previously (12), disulfide-linked multimers of bFGF (which retain native folding and biological activity) have a stronger affinity for heparin (multiple binding sites) and elute at higher NaCl concentrations. Thus, HepTSK analysis provided a rapid and accurate method for determining concentrations of native bFGF monomers and multimers (up to pentamer) remaining in solution. Aggregation of denatured protein appeared to be the main route of bFGF solution degradation (13), making HepTSK-HPLC an appropriate method for evaluating bFGF stability against denaturation.

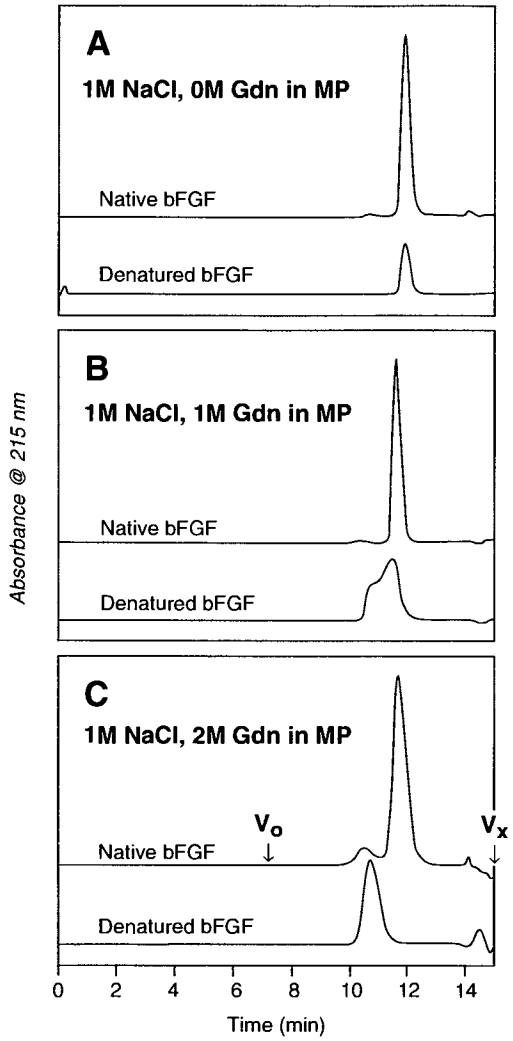

Fig. 2. SEC analysis of native and denatured bFGF under various mobile phase (MP) conditions (as indicated). (A) Under native elution conditions, a fraction of denatured bFGF refolded and eluted at the same retention time as native bFGF monomer; (B) in the presence of 1 *M* GdnHCl in the mobile phase, the bimodal population distribution suggested simultaneous detection of denatured and refolded bFGF species; (C) in the presence of 2 *M* GdnHCl in the mobile phase, denatured bFGF remained unfolded and eluted at a retention time close to that of native bFGF dimer (the small peak eluting before the native monomer peak). All chromatograms were obtained on a Bio-Rad column; V_o and V_x are void and excluded volumes, respectively.

The combination of HepTSK and RP-HPLC provided an indirect measurement of denatured bFGF concentrations. However, a single method for separation and direct quantitation of unfolded and native bFGF was not available.

Size Exclusion HPLC

A SEC method (1 *M* NaCl in Buffer A) was developed for characterization of bFGF multimers. Native bFGF monomer eluted at an apparent molecular weight (MW) of 14.0 ± 0.6 kD (~80% of actual MW). Similar nonideal elution from SEC columns (caused by nonspecific ionic and/or hydrophobic interactions with column-packing materials) has been observed for many proteins, requiring modification of mobile phase conditions to minimize nonspecific interactions (14). Higher (1.5 *M*) NaCl concentrations in the mobile phase resulted in even longer bFGF retention times (corresponding to lower apparent MWs). Little change in retention times was observed for NaCl concentrations ranging from 0.5 to 1 *M*, but adequate recovery and resolution could not be obtained with less than 0.5 *M* NaCl in the mobile phase (not shown). The inability to eliminate the discrepancy between apparent and real MWs by varying ionic strength of the eluants strongly suggested hydrophobic interactions between bFGF and column packing material.

When eluted with 1 *M* NaCl, fully denatured bFGF samples (incubated overnight at 4°C in 4 *M* GdnHCl) had the same retention time as native protein (Fig. 2A); this suggested that refolding was taking place during analysis. Confirming evidence came from fluorescence spectroscopic observation of rapid refolding of denatured bFGF upon 30-fold dilution with nondenaturing buffer (t_{50} of 35 sec at ambient temperature; Fig. 3). To prevent such refolding, various concentrations of GdnHCl were added to the mobile phase (15). With 1 *M* GdnHCl, two populations were observed, one peak eluting at the position of native monomer and the other eluting 1 min earlier (Fig. 2B). The bimodal distribution suggested that only a fraction (~60%) of bFGF refolded under these conditions, while unfolded species eluted 1 min earlier than native monomer. This explanation was consistent with the midpoint of bFGF denaturation at 1.2 *M* GdnHCl (Fig. 1A). Further evidence for this explanation was provided when GdnHCl concentration was raised to 2 *M*, and fully denatured species eluted as a single peak 1 min earlier than native monomer (Fig. 2C).

While native bFGF monomer eluted mainly at its expected MW, fully denatured bFGF eluted at a MW closer to that of its dimer. To confirm that changes in retention time were due to unfolding, not disulfide-linked multimerization, chaotrope-treated samples were uncubated for 3 hr at 25°C with 50 m*M* DTT. DTT treatment had no effect on bFGF

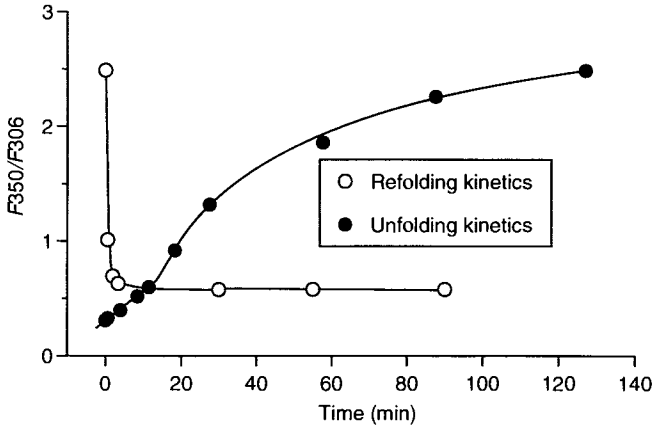

Fig. 3. Kinetics of bFGF unfolding (●) at 25°C following 30-fold dilution from native buffer to a final concentration of 0.1 mg/mL bFGF and 2 *M* GdnHCl). Kinetics of refolding (○) at 25°C following 30-fold dilution from 4 *M* GdnHCl with buffer A to a final concentration of 0.05 mg/mL and 0.12 *M* GdnHCl. Sample dilutions reflected SEC elution conditions in the presence and absence of GdnHCl in the mobile phase.

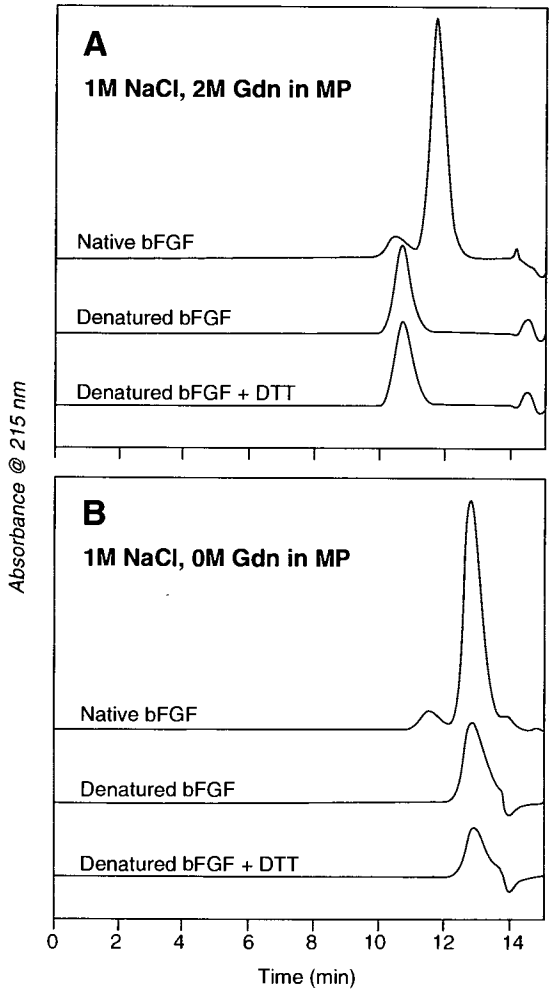

Fig. 4. SEC analysis of native and denatured bFGF ± DTT (as indicated) under (A) denaturing and (B) native mobile-phase conditions. The species eluting at 10.7 min (2 *M* GdnHCl in mobile phase) was not a disulfide-linked multimer (A) and eluted at the position of native monomer under nondenaturing mobile-phase conditions (B). All chromatograms were obtained using a Bio-Rad column.

retention time under both denaturing (Fig. 4A) and native (Fig. 4B) elution conditions, indicating that the apparent high MW of denatured bFGF was not due to formation of intermolecular disulfide bonds.

The shift to a higher apparent MW upon unfolding is well documented in the literature for other proteins (15,16); separation of native from denatured conformations by SEC for proteins undergoing slow denaturation has also been reported (17). An important feature of our analysis is that while elution with 2 *M* GdnHCl prevented refolding of denatured bFGF, a short exposure to GdnHCl was not sufficient to denature native bFGF. This stemmed from kinetics of bFGF unfolding in 2 *M* GdnHCl: refolding of denatured bFGF did not occur at this GdnHCl concentration, whereas unfolding of native protein was slow (t_{50} of 25 min; Fig. 3) relative to the time required for elution (12 min). Under native mobile phase conditions, denatured bFGF monomer rapidly refolded and eluted at the retention time of native monomer. Low recovery of denatured bFGF under native elution conditions (Table I) suggested that refolding was not complete; the unfolded fraction probably underwent hydrophobic association with column packing material. This interaction could not be disrupted without GdnHCl in the mobile phase.

To illustrate that the SEC method with 2 *M* GdnHCl in the mobile phase can be used for quantitative analysis of mixtures of native and unfolded protein, bFGF was incubated with 2 *M* GdnHCl at 4°C (where the rate of unfolding was slow enough for detection of both native and denatured subpopulations) and analyzed at various time intervals by RP-HPLC and SEC. Direct fluorescence measurements were made concurrently with HPLC analysis to determine the extent of unfolding. (These measurements were made using the Schimadzu RF540 fluorimeter, where fluorescence ratios F_r for fully native and fully denatured samples were 0.21 and 1.9, respectively.) At t_0, the GdnHCl-treated sample showed minimum unfolding by SEC; its fluorescence ratio of 0.32 was comparable to that of native bFGF. Following 2.5 hr of incubation, the F_r of the total sample increased to 1.18, which corresponded to ~40% denatured protein. By SEC, two peaks were observed: 38% of the total area was in a peak eluting at the position of denatured bFGF (10.1 min); the rest eluted at the position of native species (Fig. 5). The population distribution was reversed after 24 hr: ~94% of the protein which was fully unfolded by direct fluorescence determination (F_r of 1.78) eluted at 10.1 min, and a ~5% folded fraction eluted at the retention time of native monomer (Fig. 5). On-line fluorescence measurements showed higher tryptophan emission of the early-eluting peak, directly confirming that it was the denatured species.

Although a bimodal distribution was obtained at 2.5 and 24 hr by SEC, RP-HPLC analysis yielded only one major peak with an elution profile identical to that of bFGF monomer (not shown). This observation confirmed that while RP-HPLC could be used to analyze denatured bFGF samples, it could not distinguish between native and unfolded bFGF. Thus, in one analytical step, our SEC method combined the RP-HPLC ability to analyze denatured bFGF and the HepTSK ability to analyze native and multimeric species quantitatively.

On SEC columns, retention times of native dimer and denatured monomer were close to each other (<0.5-min difference), making adequate baseline resolution of the two species unlikely. However, with on-line fluorescence detection (in series with UV), it was possible to determine relative

Table I. Recovery of Native and Denatured bFGF from SEC Columns Under Various Mobile-Phase Conditions

Mobile phase (Buffer A, 1 *M* NaCl)	Native bFGF		Denatured bFGF	
	R^a	F_I^b	R^a	F_I^b
No GdnHCl	245 ± 1	0.013	103 ± 3	0.016
1 *M* GdnHCl	229 ± 1	0.016	240 ± 1	0.071
2 *M* GdnHCl	225 ± 1	0.020	255 ± 1	0.111

[a] Response factor at 215 nm as mV · sec · min/μg · mL (peak areas normalized by μg injected).

[b] Fluorescence index, calculated as the ratio of peak areas of fluorescence at 350 nm to absorbance at 215 nm; higher peak areas at 350 indicate unfolding of bFGF, so a higher fluorescence index corresponds to a greater extent of denaturation.

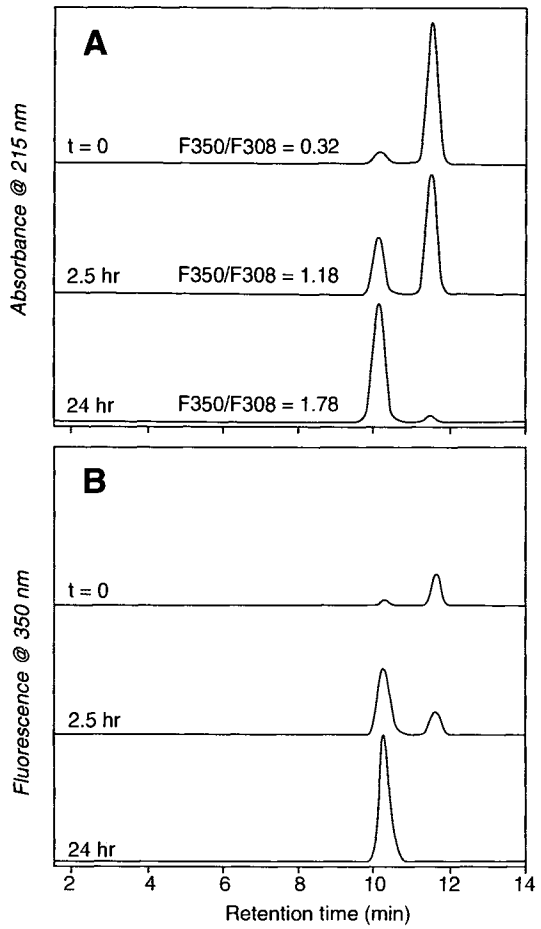

Fig. 5. SEC analysis of partially denatured bFGF solutions (2 *M* GdnHCl, 1 *M* NaCl in the mobile phase, TosoHaas column). Separation of native from denatured bFGF (A) detected at 215 nm was verified by (B) on-line fluorescence measurements. The extent of unfolding determined by SEC was in good agreement with that concurrently determined by direct fluorescence spectroscopy (F_r; ratio of emission at 350/308 nm).

amounts of native and denatured species coeluting in a single peak. A fluorescence index (F_I) defined as the ratio of peak areas detected at 350 and 215 nm was calculated for fully native and fully denatured bFGF (Table I). The F_I of native bFGF increased slightly with increasing concentrations of GdnHCl in the eluant, indicating that a minor fraction of the protein became unfolded during analysis. Changes in F_I of denatured bFGF were much greater, as the eluting species changed from refolded bFGF (found under native mobile phase conditions) to fully denatured species observed in the presence of 2 *M* GdnHCl. For analysis of a mixture of native and denatured protein under denaturing elution conditions, F_I could be expressed as

$$F_I = (X) \cdot (F_I)_{native} + (1 - X) \cdot (F_I)_{denatured} \quad (1)$$

where X is the fraction of native protein, $(1 - X)$ is the denatured fraction, and $(F_I)_{native}$ and $(F_I)_{denatured}$ are fluorescence indices of native and denatured bFGF, respectively. Once the F_I of a peak is determined experimentally, the fraction of native protein can be calculated from

$$X = [(F_I) - (F_I)_{denatured}]/[(F_I)_{native} - (F_I)_{denatured}] \quad (2)$$

Native bFGF dimers have the same fluorescence emission as native monomers, so this simple relationship can be used to measure the extent of unfolding and multimer content in various bFGF formulations.

Finally, the SEC method developed here allows future investigation of aggregation in bFGF formulations: Such denatured aggregates cannot be detected by HepTSK and may be disrupted by RP-HPLC solvents.

CONCLUSIONS

The investigation of protein stability requires a variety of analytical methods for comprehensive characterization of chemical and physical properties of these complex macromolecules. Many techniques are perturbing, so it is important to consider how the analytical processes themselves affect protein structure and function. Once appropriate criteria for stability are established, those analytical tools which minimize interference with the parameters of interest can be selected. Rapid aggregation of bFGF in solution formulations required the development of an SEC method for quantitation and characterization of native, aggregated, and denatured bFGF. Due to the unique unfolding/refolding kinetics of bFGF, this method can be used to directly measure the extent of denaturation in our formulations. The versatility of this technique makes it a particularly useful tool for future investigations of bFGF stability.

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