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The Stability of bFGF Against Thermal Denaturation

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Abstract—The influence of sulphated ligand and pH on thermal denaturation of basic fibroblast growth factor (bFGF) was investigated by differential scanning calorimetry (DSC), and verified by fluorescence spectrophotometry. Purity of bFGF before and after heat denaturation was assessed by SDS-PAGE analysis. In DSC studies the samples were heated to 95°C. The midpoint of the temperature change in the thermogram was designated as T_m. Sulphated ligand experiments were undertaken in potassium phosphate (pH 6·5) and sodium acetate buffers. Control thermograms (with no ligand) showed a T_m at 59°C in potassium phosphate buffer. Higher T_m values were noted as sulphated ligand concentration was increased. Similarly when heparin was added, the T_m moved to a higher temperature. A ratio as low as 0·3 : 1 of heparin to bFGF, increased the T_m to 90°C, which is a 31°C shift in T_m. The effect of pH on thermal denaturation of bFGF was studied in a citrate-phosphate-borate buffer system. A shift in T_m from 46 to 65°C was observed as the pH is changed from 4 to 8. Changes in protein conformation as a function of pH were monitored by fluorescence spectroscopy. It was found that a pH range from 5 to 9 is optimal for the stability of bFGF formulations. In a stability study it was noted that heparin protected bFGF from thermal denaturation only at high temperature.

Fibroblast growth factors (FGFs) are heparin binding proteins having mitogenic and neurotrophic properties (Burgess & Maciag 1989; Baird & Bohlen 1990). FGFs induce the proliferation of a variety of skin-derived cells and the angiogenic properties (Folkman & Klagsburn 1987) suggest several possible roles in tumour growth. Isolation of cDNA clones encoding both bovine and human basic fibroblast growth factors (bFGFs) has been reported (Abraham et al 1986a, b). The bFGF is a single chain molecule, with a mol. wt of 17·1 kDa and pI of 9·8. Its wound healing properties have made it an attractive candidate as a therapeutic drug (Tsuboi & Rifkin 1990).

Studies have demonstrated that topical applications of bFGF promote wound healing in healthy animals and in an animal model of impaired healing (Sprugel et al 1987; McGee et al 1988; Hebda et al 1990; Klingbeil et al 1991). Currently, bFGF is being investigated for potential clinical indications such as treatment of pressure sores, diabetic ulcers and healing of bone fractures.

In living tissue, bFGF is found in extra-cellular matrix in association with heparin which perhaps stabilizes bFGF in-vivo (Sakaguchi et al 1991). Heparin protects the FGFs from high temperature, pH variations, and proteolysis (Gospodarowicz & Cheng 1986). It has been suggested that polyanions other than heparin also protect bFGF through receptor dimerization (Yayon et al 1991; Ornitz et al 1992). Therefore, heparin and related compounds can potentially be used as the ligands in a pharmaceutical preparation to stabilize bFGF against denaturation.

In-vitro stabilization of proteins is an important practical concern, since an unstable protein formulation may impair product appearance, purity, potency and ease of handling. Chemical and physical modifications could affect product stability. Foster et al (1991) have reported that they were successful in stabilizing bFGF against oxidation and metal ion-induced aggregation by including chelating agents in the formulation. Protein denaturation is a physical change. The occurrence of protein denaturation may be attributed to a variety of factors, including change in pH, buffer species, temperature, and ionic strength which in turn may cause conformational changes and protein precipitation.

The purpose of the present work was to investigate some of the factors which may influence the stability of bFGF. In particular, this study addressed the effects of changes in pH, buffer species, heparin, and other sulphated molecules on thermal denaturation of bFGF.

Materials and Methods

Materials

Recombinant human bFGF was expressed in *E. coli* B cells and purified using chromatographic techniques. The purity of bFGF was greater than 98%. Protein concentration in experimental solutions was determined spectrophotometrically using an extinction coefficient of $E^{1\,cm}_{0\cdot01\%} = 0\cdot84$ at 277 nm. Heparin (approx. mol. wt 16000) and low mol. wt heparin (approx. mol. wt 5000) were purchased from Celsus Labs Inc., Cincinnati, OH. Sucrose octasulphate potassium salt (mol. wt 1286) was purchased from Toronto Research Chemicals, Downs View, Ontario, Canada, and inositol hexasulphate hexapotassium salt (mol. wt 890) from Sigma Chemical Co., St Louis, MO. All other reagents were obtained commercially and were of the highest grade available.

Sample preparation

For sulphated ligand studies, concentrated bFGF stock solution was exchanged into 100 mM potassium phosphate, 1 mM EDTA, pH 6·5, or into 100 mM sodium acetate, 1 mM EDTA, pH 5 buffers by ultrafiltration using Amicon Centricon 10 microconcentrators (mol. wt cut-off 10 kDa). Samples that were used for DSC, fluorescence and SDS-PAGE analysis were prepared with and without heparin and other sulphated compounds by combining buffer exchanged

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bFGF stock solution, sulphated compound stock solution in corresponding buffer and the buffer itself to obtain 1 mg mL^{-1} bFGF. The solutions were stored at 4°C for no longer than three days. The concentrations for heparin, low mol. wt heparin, sucrose octasulphate, inositol hexasulphate and sodium sulphate were varied as described in the text.

For pH-dependence studies, bFGF solutions were prepared at 1 mg mL^{-1} in phosphate-citrate-borate buffers, pH range 2–11 using the above described buffer exchange technique followed by dilution. The ionic strength of the buffers varied between 0·07–0·10. The pH was adjusted for each of the buffers with 0·1 M HCl.

The protein concentration in the prepared samples was measured by UV spectrophotometry.

Thermal denaturation of protein by DSC

Samples (500 μL) containing bFGF at 1 mg mL^{-1} and the corresponding buffer alone were transferred into the sample and reference cells, respectively, and hermetically sealed. Cells were placed in the chamber of a calorimeter (Model 7708, Hart Scientific, Pleasant Grove, UT). Samples were initially equilibrated at 20°C for 10 min before heating, then were heated at 1°C min^{-1} to 95°C. In a few cases, the samples were heated only to 90°C. Samples were then cooled to 20°C and reheated at the same rate to 90 or 95°C to obtain the baseline.

Fluorescence spectra for protein denaturation

Fluorescence spectra were obtained with a Shimadzu RF-540 spectrophotometer. The slit width for both excitation and emission was maintained at 5 nm. Excitation wavelength was set at 277 nm and emission scans were obtained between 285 and 400 nm at ambient temperature (23–25°C). A tenfold dilution of the sample with corresponding buffer was required to obtain fluorescence spectra. For each sample a blank was run using the corresponding buffer. The fluorescence intensity ratio for bFGF at 342/307 was calculated.

SDS-PAGE for protein purity

The gel-electrophoresis technique of Laemmli (1970) was used to assess the purity of bFGF. In brief, SDS-PAGE was carried out on a 15% gel cast and run in a Mini Protein II Cell (Bio-Rad 165-2940) apparatus. Samples were prepared (non-reduced) and loaded on the gel after heating at 37°C for 30 min. Protein loads on the gel were maintained at approximately 10 μg per lane. When the electrophoresis was completed the gels were stained for 30 min in about 40 mL Coomassie blue stain solution and then destained for 5–12 h.

HPLC analysis for bFGF in stability samples

Concentrated bFGF stock solution was exchanged into 100 mM potassium phosphate buffer by ultrafiltration using Centriprep-10 devices (Amicon), and stored at −80°C if not used immediately. Stability samples were prepared with and without heparin by diluting bFGF stock solution in phosphate buffer to 1 mg mL^{-1}. The samples were filtered (0·2 μm) into 2-mL sterile glass vials, capped with Teflon-lined stoppers, and incubated at 25, 35 or 50°C. At specified time intervals, samples were filtered into 200-μL glass HPLC vials for heparin affinity chromatography analysis. Chromatographic analyses were performed on a Hewlett Packard 1090 HPLC using a refrigerated autosampler, a 1-μm-on-line filter and a 250-μL injection loop. Stability samples were injected on a TOSO Haas heparin TSK-gel column (7·5 mm × 7·5 cm) and eluted with 100 mM potassium phosphate, 1 mM EDTA disodium salt, pH 6·5 using a linear NaCl gradient of 0–3 M (1 mL min^{-1}, 25°C, detection at 277 nm).

Results and Discussion

Effect of pH on thermal denaturation

The pH-dependence of thermal denaturation of bFGF was studied in a phosphate-citrate-borate buffer system. The pH-dependent changes in DSC endothermic peaks of bFGF are shown in Fig. 1. The apex of the temperature-induced endothermic change is designated as T_m and the peak is attributed to the denaturation of the bFGF. It is generally accepted that T_m can be used as an indicator for stability of proteins against thermal denaturation (Privalov & Khechinashvili 1974).

An upward shift in T_m from 40 to 64°C was observed as the pH was changed from 4 to 9 (Fig. 2). Endothermic change in

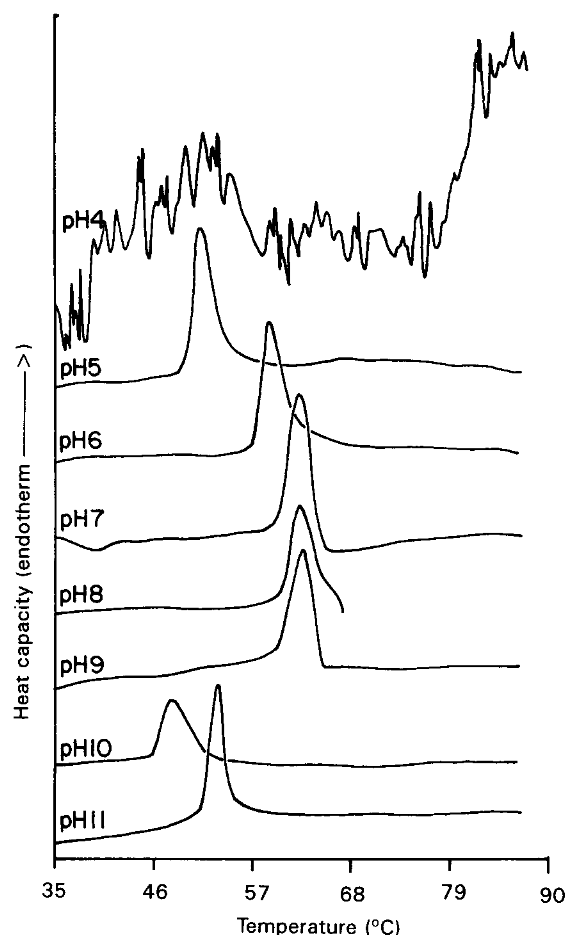

FIG. 1. pH-Dependent change in DSC endothermic peak of bFGF. Experiments were performed at 1 mg mL^{-1} bFGF in phosphate-citrate-borate buffer.

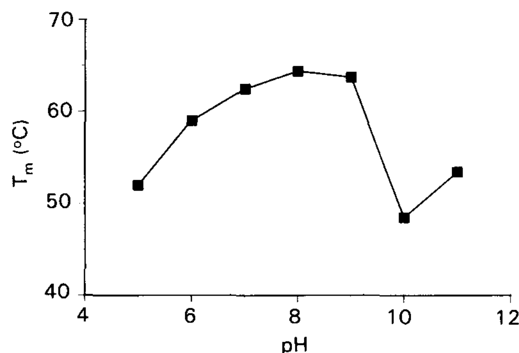

FIG. 2. pH-Dependent T_m of bFGF in phosphate-citrate-borate buffer.

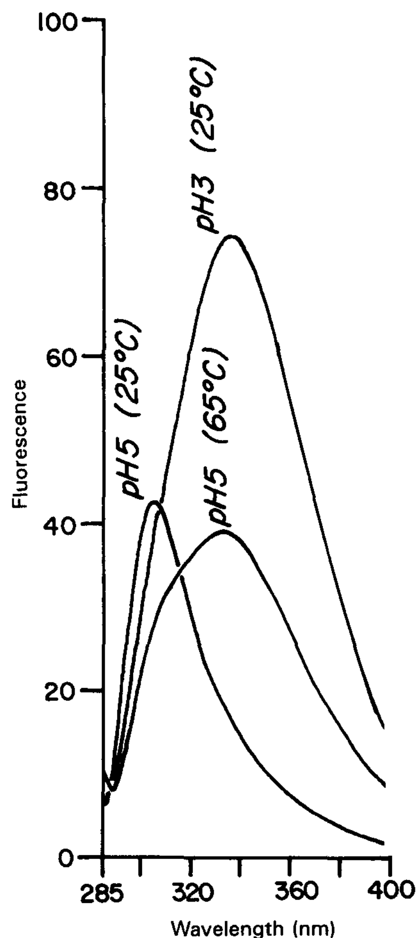

FIG. 3. Fluorescence spectra of bFGF in phosphate-citrate-borate buffer. The 0·1 mg mL^{-1} bFGF samples were excited at 277 nm. The emission spectrum at pH 5 and 25°C showed a maximum at 307 nm, but no evidence of tryptophan emission at 340 nm. At 65°C the emission peak shifted to 342 nm and slightly decreased in intensity. In pH 3 buffer, the fluorescence spectrum showed a great increase in intensity with a maximum at 342 nm.

the thermogram became apparent above pH 5. As it is evident from Fig. 2, T_m reached a plateau between pH 7 and 9. The data suggest that the bFGF is most thermally stable between pH 7 and 9. Samples of bFGF at pH 2, 3, and 4 were also run on DSC. Repeated attempts to obtain phase transition data in the pH range 2–4 yielded several non-discernable peaks and the experiments were not successful. This phenomenon is illustrated in the thermogram of bFGF at pH 4. Samples of bFGF in the pH range of 2–4 showed several faint bands of protein on SDS-PAGE gel which is an indication of protein fragmentation.

Fluorescence spectroscopy was chosen as an alternative to study the protein denaturation in the acidic pH range. The fluorescence emission spectra of bFGF (pH 5) at room temperature showed a single peak at 307 nm. Fig. 3 illustrates the emission spectra of bFGF at pH 5 and 25°C, at pH 5 after exposure to 65°C, and at pH 3 and 25°C. When the temperature of the sample was raised to 65°C for 30 min beyond its T_m value (i.e. 61°C) the emission peak shifted to 342 nm. Similarly when the pH of the bFGF solution was adjusted to 3, the fluorescence spectrum was affected and showed increased fluorescence intensity with a maximum at 342 nm. These observations suggest that the protein denaturation induced by heat and pH causes shifts in fluorescence emission. A ratio of fluorescence intensity 342/307 was used as an index to follow the stability of bFGF under stress conditions.

Fig. 4 shows the pH-dependent fluorescence intensity ratio of bFGF. As is evident from this figure, the fluorescence intensity ratio increased as the pH was reduced from 5 to 2. When the pH was raised from 5 into the alkaline region, there was no change in fluorescence intensity ratio up to pH 8. A slight increase in fluorescence intensity ratio was noted at pH 9. The increased fluorescence intensity ratio in the acidic region is attributed to the unfolding of protein.

Effect of sulphated ligand

Since bFGF is a heparin-binding protein, we examined the influence of heparin (a sulphated polysaccharide) and other sulphated ligands on the thermal stability of bFGF. DSC scans were obtained for the samples at a bFGF concentration of 1 mg mL^{-1} while the weight ratio of heparin to bFGF was varied from 0 to 3. The effect of varying the heparin to

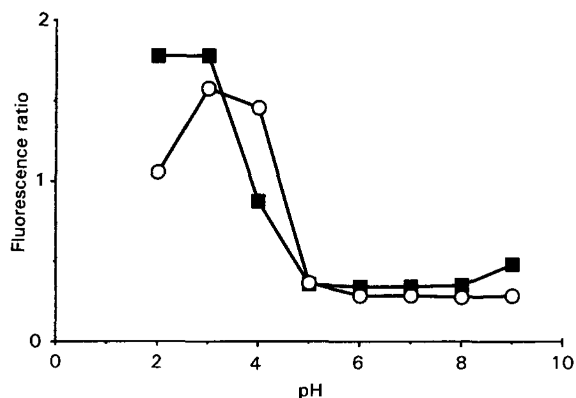

FIG. 4. pH-Dependent fluorescence intensity ratio of bFGF with and without heparin. ○ bFGF alone (control), ■ bFGF heparin at 1:0·3 weight ratio.

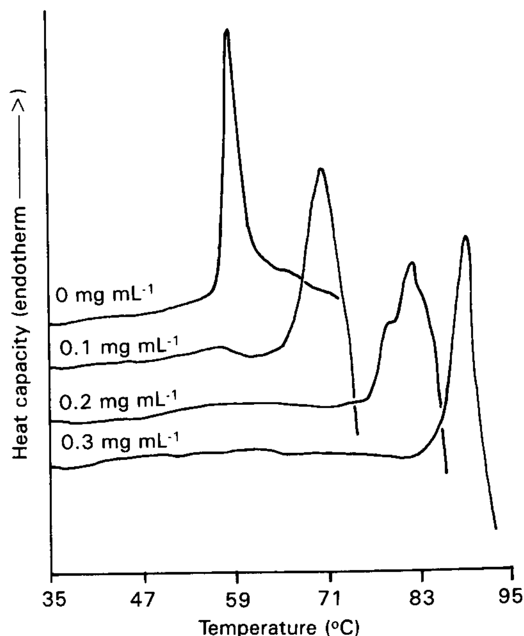

FIG. 5. Effect of heparin concentration on the DSC endotherm of bFGF. Experiments performed at 1 mg mL^{-1} of bFGF and various weight ratios of heparin to bFGF. An increase in T_m as a function of heparin concentration is shown.

bFGF ratio on the thermal denaturation (T_m) of bFGF is shown in Fig. 5. As this ratio was increased the T_m shifted to a higher temperature. The effect of the increasing weight ratio of heparin to bFGF on thermal denaturation of bFGF is shown in Fig. 6A. A ratio as low as 0·3 : 1 of heparin to bFGF shifted the endothermic peak from 61 to 90°C. Copeland et al (1991) showed a T_m shift of about 25°C by the addition of heparin to a formulation of acidic FGF. Similar experiments carried out with low mol. wt heparin corroborated the above finding (Fig. 6D). Since heparin and low mol. wt heparin offered protection against thermal denaturation, we extended the study to follow the effects of low mol. wt sulphated compounds on bFGF thermal stability. Sucrose octasulphate (Fig. 6B), and inositol hexasulphate (Fig. 6C) produced effects similar to that of heparin. These findings suggest that bFGF can be protected from thermal denaturation at high temperatures (change in protein conformation due to heat) either by heparin or by low mol. wt polyanions such as sucrose octasulphate and inositol hexasulphate. The common ion in all these compounds is sulphate and thus the effect of sulphate ions (i.e. 45 mM sodium sulphate) on thermal denaturation was investigated and an increase of 6°C in T_m value of bFGF was found. At the highest concentration of 250 mM sodium sulphate, the T_m of bFGF increased to 68°C which is 9°C higher than the T_m of bFGF alone in phosphate buffer.

In the absence of heparin, bFGF denatures at 59°C in phosphate buffer, pH 6·5, and at 54°C in acetate buffer, pH 5·0. The lower T_m value for bFGF in acetate buffer (Fig. 6A, B), as compared with phosphate buffer, is related to physicochemical interactions of protein with the buffer species (Privalov et al 1986). Further, the T_m values of bFGF in acetate buffer at pH 5 and 6·5 were different. This is perhaps due to the fact that the enthalpies of the protonation of protein groups and of the acetate buffer are similar. When the heat effects of protonation and deprotonation of protein and buffer compensate for each other there was no change in T_m. This observation supports the argument that the noted difference in T_m in acetate vs phosphate is due to buffer species.

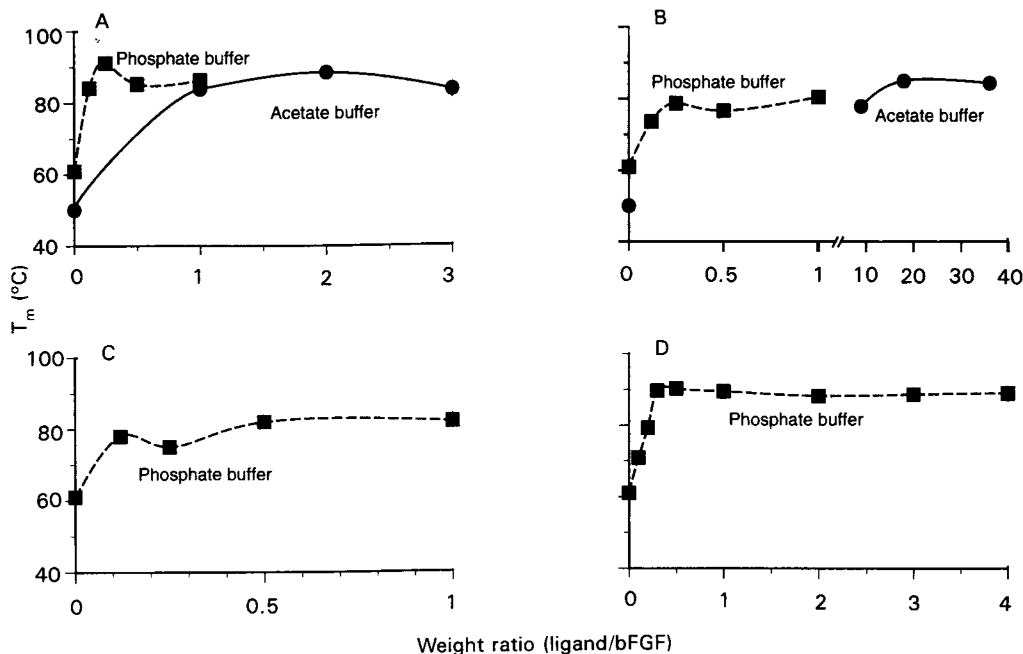

FIG. 6. Effect of weight ratio of various ligands to bFGF on thermal denaturation of bFGF as measured by DSC. A. Heparin, B. sucrose octasulphate/sucralphate, C. inositol hexasulphate, D. low mol. wt heparin.

In an effort to correlate protein denaturation determined by DSC with fluorescence intensity ratio, freshly prepared samples of bFGF with heparin in phosphate-citrate-borate buffers of various pH values were used in the study. Fluorescence intensity ratios at various pH values with and without heparin are similar from pH 5 onwards (Fig. 4). At pH 4, bFGF with heparin showed greater change in intensity ratio compared with the sample without heparin. This observation suggests that heparin enhances bFGF stability within the pH range 5–8.

Degradation products

In almost all the experiments, aggregates of bFGF were observed visually after heat exposure in DSC. All samples were analysed by SDS-PAGE for qualitative assessment of degradation products. Fig. 7 shows SDS-PAGE analysis of the samples with various heparin to bFGF weight ratios. The post DSC samples that had gone through the electrophoresis process showed more intense dimer bands, and faint trimer bands (not illustrated). SDS-PAGE analysis of bFGF at different pH values showed two primary bands, which represent monomer and dimer, while the pH 9 sample showed three bands. After thermal exposure the samples at all pH values showed excessive aggregation and the large aggregates remained on the stacking gel. The disulphide-linked covalent bFGF dimers and trimers are detected on non-reducing SDS-PAGE as different bands, while monomeric bFGF is observed on reducing SDS-PAGE gel (not illustrated).

Stability studies

Formulations of bFGF (1 mg mL^{-1}) with and without heparin were prepared in 100 mM potassium phosphate buffer pH 6·5 at 35 and 50°C. The amount of monomeric, native bFGF remaining in the sample was determined by HPLC. Solution stability studies indicated that while heparin protected bFGF against aggregation caused by thermal denaturation, it had no stabilizing effect at or below 35°C (Fig. 8). Room temperature (25°C) solutions containing heparin degraded faster than those formulated in phosphate buffer alone (data not shown). It appears, at lower temperatures, oxidative degradation occurred much faster than thermal denaturation and aggregation (which appeared to be the predominant degradation pathway at 50°C). Thus, while heparin may have stabilized the bFGF tertiary structure, it did not increase formulation stability at temperatures below the T_m value of bFGF.

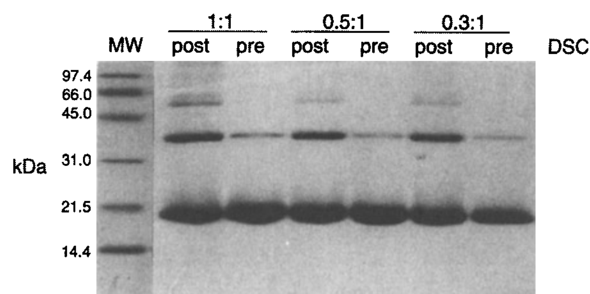

FIG. 7. Non-reducing SDS-PAGE analysis of bFGF-heparin samples before and after exposure to DSC scanning. After exposure samples showed trimers and increased dimer concentration.

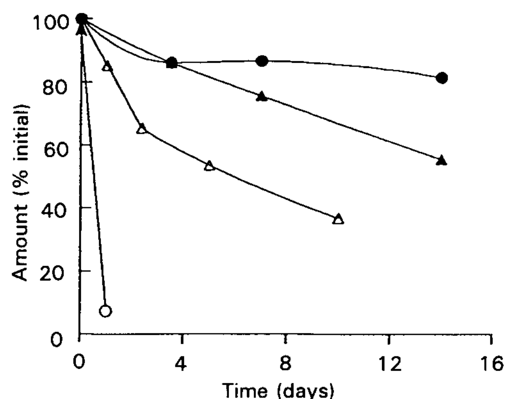

FIG. 8. Stability of bFGF in potassium phosphate buffer, pH 6·5, shown as percent remaining bFGF in solution as a function of time. ● bFGF alone at 35°C, ▲ bFGF with heparin (1:0·3 weight ratio) at 35°C, ○ bFGF alone at 50°C, △ bFGF with heparin (1:0·3 weight ratio) at 50°C.

Conclusions

Heparin, low mol. wt heparin, inositol hexasulphate, and sucrose octasulphate were used in this study to stabilize bFGF against thermal denaturation. All studied molecules offered protection against thermal denaturation of bFGF. Two of these stabilizers are small organic molecules. It appears that there is low structural specificity for protection, as long as it is a sulphated molecule (negatively charged). The T_m values of bFGF in the presence of sodium sulphate were elevated by 6°C to 9°C, but this change is not as significant as those with other sulphated compounds. This observation suggests that a high degree of sulphation is needed to result in an increased thermal stability of bFGF. It has been reported that human bFGF in solution with heparin is protected from degradation by pH, proteolysis and temperature (Saksela et al 1988; Sommer & Rifkin 1989). This study confirms that bFGF can be protected from thermal denaturation by heparin. Further, our study identified an optimal pH range for the bFGF formulations. The protection of bFGF from thermal denaturation is shown as increased T_m values. This work is further supported by the tertiary structure conformation studies. The fluorescence spectroscopy studies showed that there was no change in fluorescence intensity ratio at and above 0·3:1 weight ratio of heparin to bFGF, which can be assessed as a minimal change in protein conformation. Real-time stability studies carried out in our laboratories with and without heparin in bFGF formulations indicated that heparin indeed offered protection against aggregation caused by thermal denaturation when the samples were stored at 50°C (Fig. 8), but it had no stabilizing effects at or below 35°C.

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