DARU Vol. 19, No. 2 2011

# Effects of temperature and additives on stability and spectrum of a therapeutic fibroblast growth factor

[1]Zhou CY. , [1]Guo X., [2]Wang ST., [*2]Zhu YP., [1]Mu DZ.

*[1]Department of Pediatrics, West China Second University Hospital [2]Institute for Nanobiomedical Technology and Membrane Biology, Sichuan University, Chengdu, P. R. China.*

Received 14 Sep 2010; Revised 22 March 2011; Accepted 23 March 2011

**ABSTRACT**

*Background and the purpose of the study*: Human fibroblast growth factor 20 (FGF20) is a 16.5 kDa protein containing 154 amino acid residues with reportedly poor thermal stability, and low stability, which are considered to be major factors that can limit its pharmacological applications. Thus, the aim of this study was to enhance the thermal stability and bio activity of a therapeutic FGF20 by addition of sucrose or heparin as additives and also at different temperatures.

*Methods*: A variety of biophysical techniques such as far-UV circular dichroism (CD), fluorescence and high resolution derivative UV absorption spectroscopy, were employed to characterize FGF20 and study the effects of heparin and sucrose on its thermal stability and bio activity at pH 7.0.

*Results*: Results of this study suggest that human FGF20 is significantly unstable and induction of heat by increased temperatures results in aggregation and precipitation at pH 7.0. Great changes in the fluorescence intensity and shape were achieved by addition of heparin and sucrose at different temperatures compared to the control. From 10 °C to 60 °C, no significant changes were observed in far-UV CD spectrum compared to the control, but significant changes were observed by adding sucrose when these temperatures are above 45 °C. Upon addition of heparin and sucrose, the mitogenic activity increased significantly at all tested temperatures, and these changes may be related to the roles of heparin and sucrose on the structure and conformation of FGF20.

*Conclusion*: Results of this study suggest that heparin and sucrose as additives seems to benjm sufficient to prevent thermal inactivation of FGF20 and also maintain its conformation stability and bio activity.

Keywords: Human fibroblast growth factor 20, Thermal stability, Circular dichroism, Fluorescence and absorption spectroscopy.

## INTRODUCTION

Fibroblast growth factors (FGFs) constitute a family of heparin binding proteins involved in the regulation of cell growth and differentiation, and angiogenesis (1). FGF20 is a member of this family that is expressed preferentially in dopaminergic neurons of the substantia nigra pars compacta (SNPC) of rat brain and also expressed in human cerebellum and SN tissue (2). Evidences have suggested that the expression of FGF20 significantly enhances the survival of midbrain dopaminergic neurons, and monkey stem cells are differentiated in vitro into dopaminergic neurons after treatment with exogenous FGF20. In addition, FGF20 have been transplanted into a primate Parkinson's disease model, alleviated some symptoms (3, 4).Therapeutic potential of FGF20 has also been investigated for the treatment of acute intestinal inflammation, parkinson's disease susceptibility and alleviating oral mucositis (5, 6).

Recently, recombinant technology has been utilized to produce growth factor drugs massively. However, a major problem in the utilization of these peptides is their thermal stability and enhanced thermal stability is a desirable feature for therapeutic uses of wound healing and tissue repair. Several members of FGF family have been shown to exhibit marginal stability with disrupted secondary and tertiary structure under physiological conditions (7). Earlier results suggested that heparin and sugar lock the molecules in their native conformations and protect them from thermal unfolding in some growth factors (8, 9). FGF20 is a potent mitogen and angiogenic factor, with reportedly poor thermal stability and a relatively short in vivo half-life (10). The present work was conducted to study the effects of heparin

*Correspondence:* yipingzhu09@gmail.com

and sucrose on the thermal stability and activity of FGF20 using high resolution UV absorption spectroscopy, intrinsic and extrinsic fluorescence spectroscopy, and far-UV circular dichroism (CD) spectroscopy.

## MATERIAL AND METHODS

*Chemicals and protein purification*

Heparin sodium and sucrose were purchased from Sigma (St. Louis, USA). Chemicals were analytical grade. All solutions were made in Milli Q water. A synthetic gene for the 140 amino acid form of human acidic fibroblast growth factor was inserted into the pET-32a(+) vector (Novagen, Madison, WI, USA) and introduced into *Escherichia coli* strain BL21(DE3). The transformed *E. coli* was grown at 37°C in Lysogeny broth (LB) media to an optical density of $A_{600}$ = 1.0. At this point 1 mM of isopropyl *α*-D-thiogalactopyranoside (IPTG) was added and the incubation temperature was shifted to 28 °C. The cells allowed to grow for an additional 4 hrs and were then harvested by centrifugation (10000 rpm) for 10 min and stored frozen (-20 °C) prior to use. Protein was purified to apparent homogeneity, as judged by Coomassie Brilliant Blue-stained sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS-PAGE), by a combination of Ni-NTA column and heparin affinity chromatography using AKTA Explorer system (Amersham Biosciences, Uppsala, Sweden). Protein purity of >95% was confirmed by silver-stained SDS-PAGE and high performance liquid chromatography (HPLC, Agilent 1100, Agilent Technologies, Palo Alto, CA, USA). The stock human FGF20 samples were dialyzed against 10 mM phosphate/10 mM citrate buffer containing 200 mM sodium sulfate at pH 7.0. The samples were filtered through a 0.23 μm filter (Whatman Ltd., Maidstone, United Kingdom) to remove insoluble aggregates and stored at -80 °C for analyses. Samples were diluted to final concentrations of 0.2 mg/ml. Protein concentration was determined using an experimentally determined extinction coefficient ($E^{0.1\%}$ 1cm) of 0.97 at 280 nm.

*Assay of FGF20 activity*

The mitogenic activity of FGF20 was assessed by methylthiazoletetrazolium (MTT) assay using NIH 3T3 cells (11). Viable cells were assessed through colorimetric changes using a Model 550 microplate reader at 570 and 630 nm (Bio-Rad, Cambridge, MA, USA).

*Turbidity measurement*

UV absorbance spectra of FGF20 were collected as a function of temperature from 10 to 60 °C with a Beckman Coulter DU800 UV-visible spectrophotometer (Beckman Coulter Inc., Fullerton, USA) at 2.5 °C interval.

*Fluorescence spectroscopy*

Fluorescence spectra of human FGF20 were obtained via F-7000 spectrofluorometer (Hitachi High-Technologies, Tokyo, Japan), equipped with a temperature controlled cell sample holder. For extrinsic ANS fluorescence experiments, an optimal molar ratio of 15:1 (ANS: protein) was used. Both Trp and ANS fluorescence emission spectra were collected from 10 to 60 °C at 5 °C intervals with a 5 min equilibration time at each temperature point.

*Circular dichroism spectroscopy*

Far-UV CD studies were performed with an AVIV model 400 spectrometer (AVIV Associates, Lakewood, NJ, USA). All spectra were corrected by subtracting the baseline and the data were expressed as mean residue ellipticity, [θ], which was given the units of [deg·cm$^2$·dmol$^{-1}$]. The CD signals were monitored as a function of temperature from 10 to 60 °C at 5 °C intervals for samples at pH 7.0, with a resolution of 0.2 °C and a heating rate of 20 °C per min. Quantitative estimations of the secondary structure content were made with the aid of the programs CDSSTR, CONTIN and SELCON3 included in the CDPro software package (http://lamar.colostate.edu/~sreeram/CDPro) (12).

*Effects of heparin and sucrose on CD and fluorescence spectroscopy*

In this experiment, heparin used at a concentration of 4-fold weight excess (solute: protein, w/w), and sucrose was used at a concentration of 50-fold weight excess in a pH 7.0, 100 mM phosphate buffer. FGF20 concentration of 0.2 mg/ml was used for all the experiments described below. The same instrument and experimental procedure that are described above were used for CD and fluorescence spectroscopy analysis.

## RESULTS AND DISCUSSION

*Effects of different temperatures on aggregation of FGF20*

The optical density of human FGF20 solutions at 350 nm ($OD_{350}$) was measured to monitor the protein's aggregation behavior induced by increasing temperature (Fig. 1). Increases of optical density at high temperatures may be related to the increased particle size due to aggregation, while a later drop in the signal reflects gross settling of the protein above 55 °C.

*Effects of different temperatures and additives on fluorescence spectrum of FGF20*

Changes in fluorescence spectra at different temperatures and additives are shown in figures 2 and 3. A single trp of FGF20 showed fluorescence spectrum with $\lambda_{max}$ at 337 nm indicating trp residue in the hydrophobic environment. The λmax was

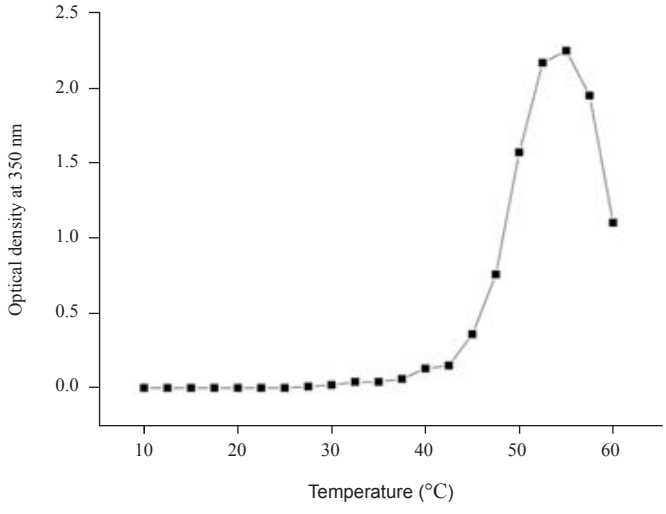


**Figure 1.** Derivative of UV absorbance spectroscopy. Optical densities at 350 nm ($OD_{350}$) are recorded from 10 to 60 °C for FGF20 at pH 7.0.

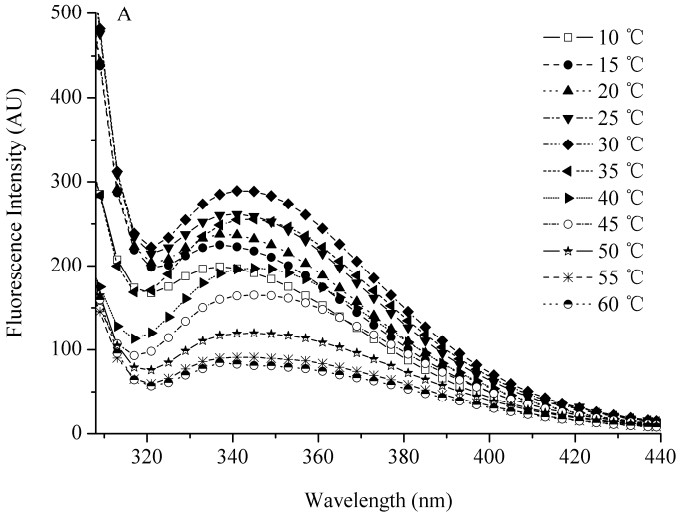


**Figure 2.** Intrinsic fluorescence of human FGF20 from 10 to 60 °C at pH 7.0. The excitation wavelength is set at 295 nm.

gradually blue shifted by increasing temperatures compared to the control (Fig. 2). The blue shift in tryptophan fluorescence probably reflects occupying a less polar environment within the protein, and is corrected with the changes of secondary structure components (Fig. 4). The fluorescence emission by addition of heparin had a very low quantum yield, and the fluorescence intensity decreased significantly by increase in temperature up to 60 °C (Fig. 3A). These decreases may partly results from the conformation changes of active center in the FGF20 molecule. In addition, the λmax showed a small red-shift to 331 nm, and maintained a stable value up to 40 °C, and then showed a small blue shift (2 or 4 nm) by increase in temperatures up 60 °C in comparison to the control. Upon addition of sucrose, the fluorescence intensity was about 0.03 times of those without additive, and the emission λmax showed red shift from 2 to 6 nm compared to the control (Figs. 2 and 3B). Fluorescence quenching may be explained by occurrence of interaction between sucrose and conformation center of tryptophan in the molecule. These results indicate that addition of heparin and sucrose may change the micro-environment conformation of trp center and decrease the fluorescence intensities of FGF20 significantly, but they showed different effects.

*Effects of different temperatures and additives far-UV CD spectra of FGF20*

Results of far-UV CD spectra exhibited features typical for proteins containing *b-trefoil* structure,

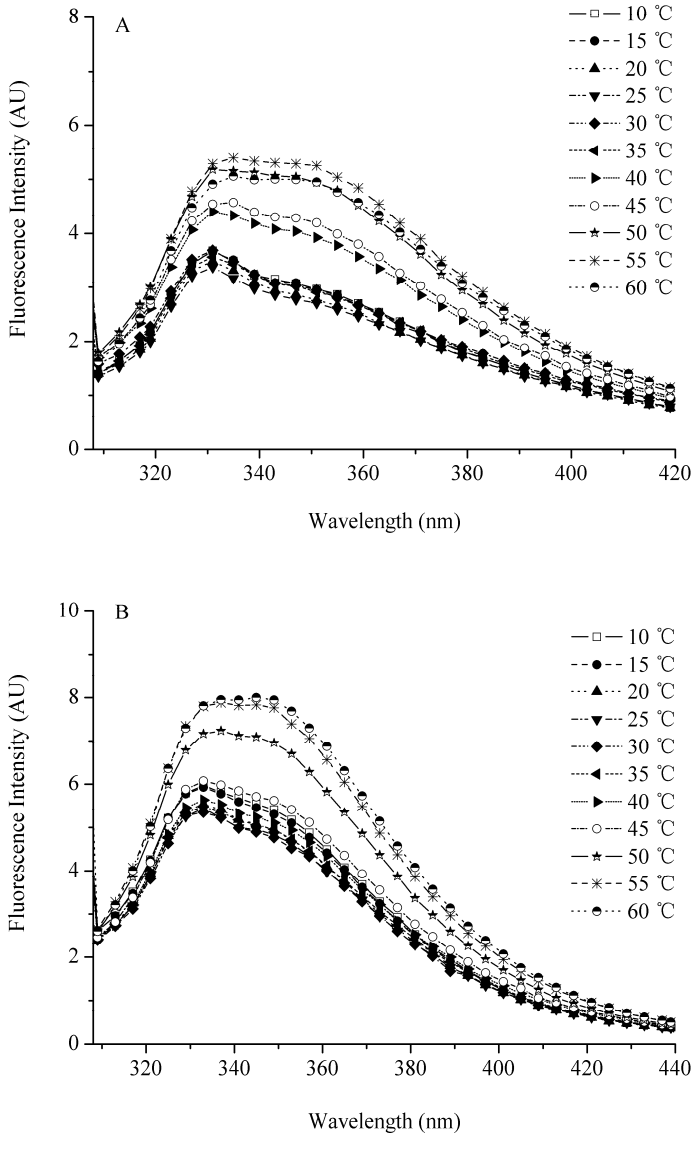


**Figure 3.** Effects of heparin and sucrose on the fluorescence spectra of FGF20 from 10 to 60 °C at pH 7.0. A: 4-fold weight excess of heparin. B: 50-fold weight excess of sucrose.

and CD curves manifest a positive peak at 227 nm and a negative signal near 207 nm, which clearly indicates a mixed α-helix and b-trefoil structure (Patterns are no shown). These results are consistent with pervious reports and appear to be a common feature of the FGF family (13, 14). The relative percentages of secondary structure elements were analyzed using CDPro package (Table 1), and results suggest that values of 13.9% α-helix, 37.2% β-sheet, 22.4% β-turn, and 48.8% random coil are calculated at 10 °C. This protein showed slight variations in the percentage of calculated secondary structure from 10 to 45 °C, such as increased values of α-helix as well as reduced values of β-sheet, β-turn and random coil. However, the percentage of random coil increased significantly at temperatures higher than 50 °C which indicate a gradually unfolding process of this protein above 45 °C. As shown in figure 4A, strong negative peaks with a minimum at 213 nm and weak positive peaks at 227 nm in far-UV CD spectrum of FGF20 were detected by addition of heparin. In addition, no significant change was observed by increase in temperatures compared to that of the addition of heparin at 10°C. These strong negative peaks at 213 nm presented a much higher content of α-helical. Upon addition of heparin, the structure calculations indicated that this protein was formed by 34.6% α-helix, 16.8% β-sheet, 17.2% β-turns, and 29% random coil at pH 7.0 and 10 °C. Addition of additives resulted in significant increase in values of α-helix, reduced values of β-sheet, β-turns and random compared to those without additives (Table 1). These results suggest that conformational transition from β-sheet,

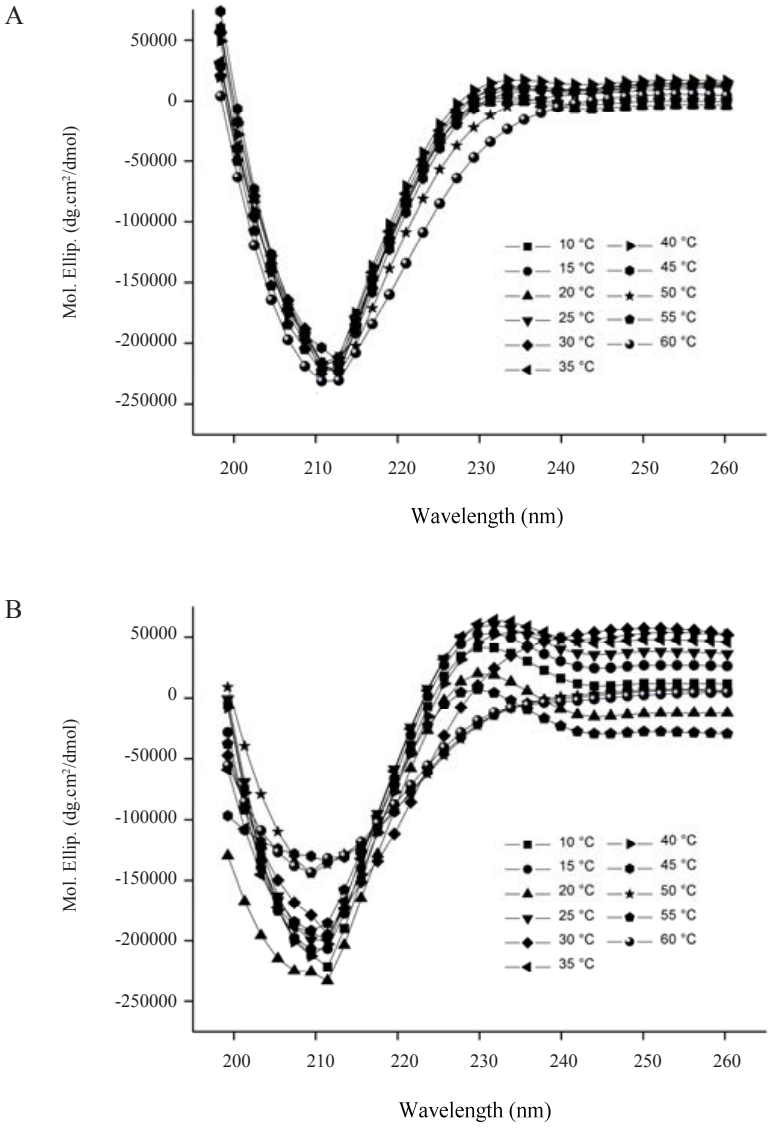


**Figure 4.** Effects of heparin and sucrose on the CD spectra of FGF20 at pH 7.0.
A: a 4-fold weight excess of heparin
B: 50-fold weight excess of sucrose.

β-turns and random coil to α-helix structure was observed when heparin was added as a stabilizing reagent during unfolding process. Figure 4B shows changes in far-UV CD spectrum of FGF20 from 10 °C to 60 °C upon addition of sucrose. Results suggest that no significant change were observed when these temperatures are below 45 °C compared to that of the addition of sucrose at 10°C. However, spectrum shows significant changes when the temperatures are higher than 60 °C. Upon addition of sucrose, the percentages of the secondary structures were calculated to be 20.8% α-helix, 26.3% β-sheet, 20.2% β-turns, and 39.9% random coil at 10 °C, indicating that the addition of sucrose may increase the percentage of α-helix structure by reducing some percentages of β-sheet, β-turns and random coil structure compared to those without additives. Moreover, the percentages of α-helix structure at different temperatures were higher than those without additives, and the percentages of β-sheet, β-turns and random coil were less than those without additive. Based on these results, it may suggested that part of secondary structure elements in the FGF20 molecules, like β-sheet, β-turns and random coil, may transform to α-helix upon addition of heparin and sucrose. Thus, these conformational transitions may maintain the structure stability and activity of FGF20 (Figs 4 and 5). Earlier results have shown that heparin and sucrose are required to control and maintain the stability of FGF family proteins (13, 15). In addition, studies also show that the presence of heparin in the solution has a much

**Table 1.** Analysis of secondary structure (%) of CD Spectrum of FGF20 at various temperatures and in the presence of additives.

| Structures | | 10°C | 15°C | 20°C | 25°C | 30°C | 35°C | 40°C | 45°C | 50°C | 55°C | 60°C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No additive | α-helix | 13.9 | 13.9 | 14.5 | 14.5 | 15.2 | 14.9 | 15.3 | 15.4 | 14.1 | 14 | 13.9 |
| | β-sheet | 37.2 | 37.3 | 36.3 | 36.1 | 34.9 | 35.4 | 34.6 | 34.3 | 36.7 | 36.9 | 37.1 |
| | β-turn | 22.4 | 22.4 | 22.1 | 22.1 | 21.8 | 21.9 | 21.6 | 21.5 | 21.9 | 21.9 | 21.9 |
| | Random Coil | 48.8 | 48.7 | 48.4 | 48.1 | 47.8 | 47.8 | 47.7 | 47.6 | 50.4 | 50.6 | 51.2 |
| Heparin | α-helix | 34.6 | 34.6 | 34 | 33.9 | 33.3 | 33.9 | 33.9 | 34.7 | 35.2 | 35.4 | 39 |
| | β-sheet | 16.8 | 16.8 | 17.2 | 17.2 | 17.5 | 17.3 | 17.2 | 16.7 | 16.5 | 16.3 | 14.5 |
| | β-turn | 17.2 | 17.2 | 17.4 | 17.4 | 17.5 | 17.5 | 17.3 | 17.3 | 17 | 17 | 16.4 |
| | Random Coil | 29 | 29 | 28.9 | 29 | 29.2 | 28.8 | 29.4 | 28 | 29 | 28.4 | 26 |
| Sucrose | α-helix | 20.8 | 20.3 | 20.8 | 20.1 | 19.6 | 21 | 20.6 | 19.4 | 17.8 | 18.9 | 18.3 |
| | β-sheet | 26.3 | 27.9 | 27.2 | 28.2 | 28.7 | 27.1 | 27.3 | 28.8 | 30.5 | 29.4 | 30.1 |
| | β-turn | 20.2 | 20.4 | 20.3 | 20.4 | 20.6 | 20.2 | 20.1 | 20.5 | 20.7 | 20.3 | 20.6 |
| | Random Coil | 39.9 | 40.2 | 38.9 | 40.7 | 40.7 | 39.7 | 40.7 | 41.9 | 44.4 | 44 | 44 |

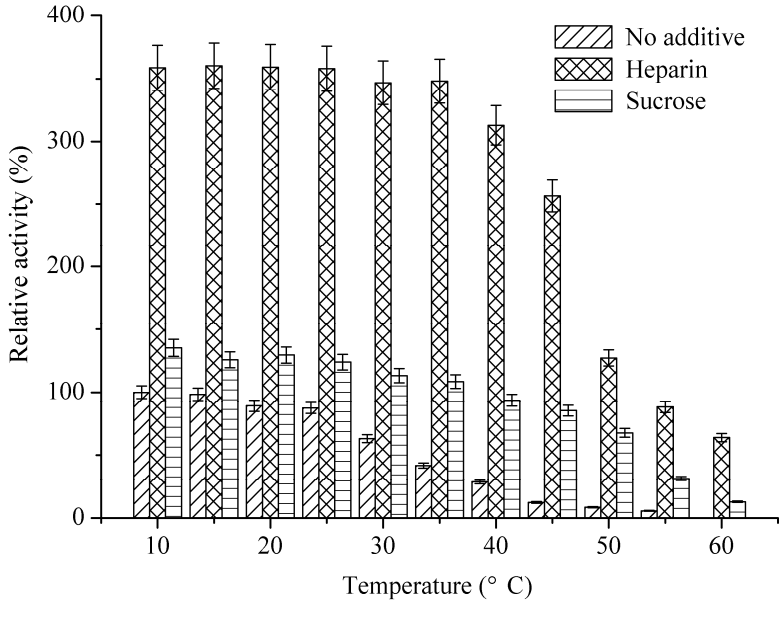


**Figure 5.** Effects of different temperatures and additives on the mitogenic activity of FGF20 at pH 7.0. Values are average of three independent determinations. Full (100%) activity corresponds to the protein concentration of 0.2 mg/ml.

greater stabilizing effects than that of sucrose.

*Effects of different temperatures and additives on mitogenic activity of FGF20*

Results suggest that the activity of FGF20 gradually decrease by increase in temperature up to 60 °C. A complete loss of the mitogenic activity was observed at 60 °C for 5 min. In contrast, the activity was significantly enhanced when heparin was added in the solution before increase in temperature compared to those without additives. Thus, the highest activity was observed between 3.46 and 3.6 times of the control when the temperature was below 35 °C. However, the activity decreased gradually by increase in temperature up to 60 °C. These results suggest that heparin binding sites might locate in the activity site (7, 13, 15). Although addition of sucrose may increase the activity of FGF20, its stabilizing effects of sucrose on the activity were lower than those which were formed by addition of heparin. FGF has been shown to possess structurally localized heparin and polyanion binding sites on its surface. It has been reported that heparin binding has significant

effects on the biological activity of the protein and provides dramatic thermal stabilization (7). In conclusion, the present findings suggest that heparin and sucrose play important roles in maintenance of the conformation and activity of FGF20.

It seems that addition of stabilizers might be useful for the formulation design of therapeutic proteins.

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