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Stabilization of basic fibroblast growth factor with dextran sulfate

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Dextran sulfate protected bFGF from heat and acid inactivation and from proteolytic degradation. The protective effect was stronger than that of heparin which is known as a stabilizer of bFGF. Dextran sulfate and bFGF formed a high molecular weight complex via ionic interaction when mixed together in aqueous solution. The complex was dissociated when the ionic strength was increased and the protective effect was completely abolished. Successive digestion of bFGF with *Staphylococcus aureus* V8 protease and pepsin followed by affinity chromatography on an immobilized dextran sulfate column and reversed-phase high performance liquid chromatography yielded three positively charged fragment peptides, Tyr^{24}-Phe^{30}, Tyr^{105}-Trp^{114} and Tyr^{124}-Leu^{138}. These results suggest that dextran sulfate stabilizes bFGF by binding close to the putative heparin binding sites of the bFGF molecule.

Basic fibroblast growth factor; Dextran sulfate; Complex formation

1. INTRODUCTION

Basic fibroblast growth factor (bFGF) belongs to a group of growth factors that have mitogenic, neurotrophic and angiogenic activities [1–3]. Because of its high affinity for heparin, bFGF is referred to as one of the heparin binding growth factors. Heparin protects bFGF from heat and acid inactivation [4]. It also protects bFGF from degradation by trypsin and chymotrypsin [5].

E. coli-derived recombinant bFGF and its genetically engineered acid-stable mutein CS23 (rbFGF-CS23) [6] have been demonstrated to significantly accelerate the healing of chronic duodenal ulcers produced by cysteamine in rats [7]. Oral administration of rbFGF-CS23 caused a significant increase in angiogenesis in the ulcer bed compared with that in untreated control rats [8]. These findings demonstrate the important role(s) of bFGF in the healing of duodenal ulcers and imply a novel pharmacological modulation of gastrointestinal ulcers.

Since orally administered rbFGF-CS23 was found to be degraded by the digestive enzymes in the gastrointestinal tract, we examined the use of heparin-related reagents derived from natural and synthetic sources to protect rbFGF-CS23 from proteolytic degradation. Of them, we found that dextran sulfate was most potent. We report here that dextran sulfate has high affinity for bFGF and that it protects bFGF from heat, acid and proteolytic inactivation.

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2. MATERIALS AND METHODS

2.1. *Materials*

Dextran sulfate with an average molecular weight of 7,500 was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Heparin was purchased from Roussel Uclaf (Paris, France). Pepsin, TPCK-treated trypsin and chymotrypsin were obtained from Sigma Chemical Co (MO, USA). *S. aureus* V8 protease was obtained from ICN Immuno Biologicals (IL, USA).

2.2. *bFGF*

rbFGF-CS23 is a mutein of human bFGF derived from recombinant *E. coli* and in which Cys^{69} and Cys^{87} in the original bFGF have both been replaced by serine residues [6]. The amount of molecules with an amino terminal Met was less than 0.1% in our preparation. The amino acid residues are numbered starting from the amino-terminal Pro of the mature molecule in this paper. rbFGF-CS23 was purified to homogeneity (99.8% pure as determined by densitometry) and used throughout the work.

2.3. *Bioassay*

The proliferation-stimulating activity of rbFGF-CS23 was determined with fetal bovine heart endothelial (FBHE) cells ATCC CRL 1395 by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described [9].

2.4. *Dextran sulfate affinity chromatography*

rbFGF-CS23 (400 μg/ml) was incubated at 37°C for 16 h with *S. aureus* V8 protease at an E/S ratio of 1:20 (w/w). The digest was applied to a dextran sulfate high performance liquid chromatography (HPLC) column (Shodex AF pak DS-894, 0.8 × 5 cm, Shoko Co Ltd., Tokyo, Japan), and the fragment peptides were eluted with a linear gradient of NaCl concentration from 0 to 2 M. The fragment peptides having affinity for dextran sulfate were pooled and digested further with pepsin at 37°C for 16 h. The peptic digest was applied to the dextran sulfate HPLC column, and the peptides were eluted as described above. The fragment peptides thus obtained were pooled and

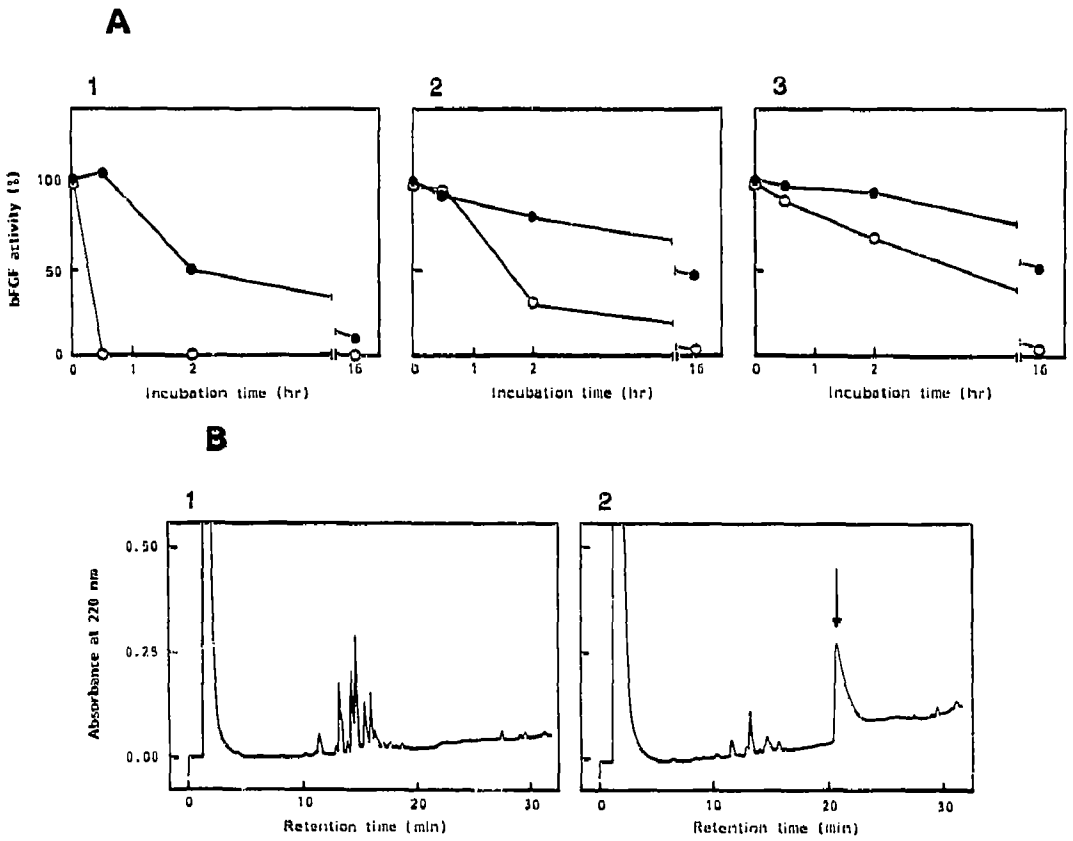

Fig. 1. Inactivation of rbFGF-CS23 by pepsin, trypsin and chymotrypsin in the presence or absence of dextran sulfate. (A) Time courses of inactivation. rbFGF-CS23 (200 μg/ml) was incubated at 37°C for 16 h with one-fiftieth, by weight, the amount of pepsin, trypsin or chymotrypsin in the presence (●) or absence (○) of an equimolar concentration of dextran sulfate. pH: 2.0 for pepsin and 7.4 for trypsin and chymotrypsin. (1) pepsin; (2) trypsin; and (3) chymotrypsin. (B) RP-HPLC pattern of rbFGF-CS23 incubated with pepsin in the absence or presence of dextran sulfate. Aliquots of the 30 min-reaction mixtures used for Fig. 1A, were subjected to RP-HPLC using an Ultrapore RPSC column (0.46 × 7.5 cm; Beckman Instruments Inc., CA, USA). Elution was performed with a linear gradient of acetonitrile concentration in the presence of 0.05% trifluoroacetic acid. (1) incubated in the absence of dextran sulfate; and (2) in the presence of dextran sulfate. The arrow indicates the rbFGF-CS23 peak.

applied to a reversed-phase (RP) HPLC column (ODS 120T, 0.46 × 25 cm, Tosoh, Tokyo, Japan). Elution was performed with a linear gradient of acetonitrile concentration in the presence of 0.05% trifluoroacetic acid. Peptide peaks were analyzed with a PICO TAG amino acid analyzer (Millipore, MA, USA) and gas phase protein sequencer 470A (Applied Biosystems Inc., CA, USA).

3. RESULTS AND DISCUSSION

3.1. *Protection from heat and acid inactivation*

Stabilizers for rbFGF-CS23 were screened from among heparin-related natural and synthetic substances with respect to protection from acid and heat inactivation. Of them, dextran sulfate was found to be most potent. When rbFGF-CS23 was incubated at 37°C and at pH 3.0, the biological activity was rapidly decreased with time and was almost completely abolished after 2 h. However, in the presence of an equimolar concentration of dextran sulfate, the biological activity was protected, and 89% of the initial activity remained even after 2 h of incubation (Table I). It is worth noting that heparin was much less effective than dextran sulfate when used with rbFGF-CS23 at an equimolar concentration under the experimental conditions.

3.2. *Protection from proteolytic degradation*

rbFGF-CS23 was treated with digestive enzymes

Table I

Residual activity of rbFGF-CS23 incubated at 37°C and at pH 3.0 in the presence of dextran sulfate or heparin

Addition	Incubation time (h)	Residual activity (%)
None	0	100
	2	10
	20	2
Dextran sulfate	2	89
	20	34
Heparin	2	20
	20	4

rbFGF-CS23 (100 μg/ml) was incubated at 37°C and at pH 3.0 for 20 h with an equimolar concentration of dextran sulfate or heparin.

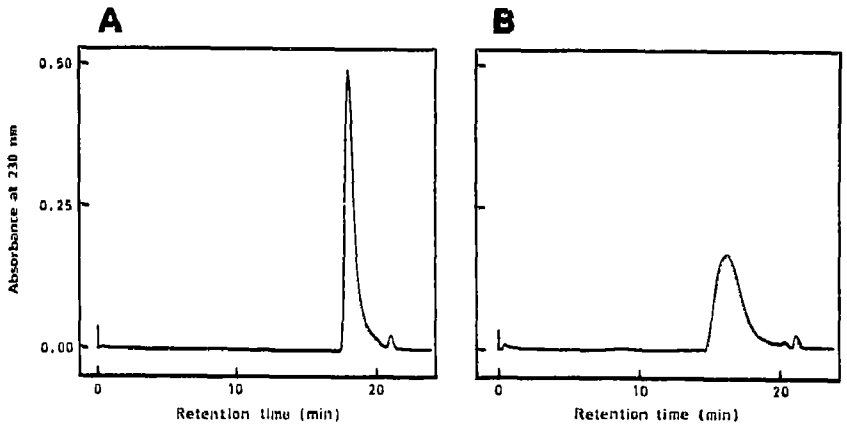

Fig. 2. GPC patterns of rbFGF-CS23 (A) and rbFGF-CS23–dextran sulfate complex (B). (A) rbFGF-CS23 (20 μg) was subjected to GPC using a TSK gel 3000 SW column (0.75 × 60 cm; Tosoh). Elution was performed with 0.1 M phosphate buffer (pH 6.0) containing 0.1 M Na_2SO_4 at a flow rate of 1.0 ml/min. (B) rbFGF-CS23 (100 μg/ml) was incubated with an equimolar concentration of dextran sulfate (46 μg/ml) at room temperature for 30 min, and an aliquot of the reaction mixture (20 μg as rbFGF-CS23) was subjected to GPC as described above.

such as pepsin, trypsin and chymotrypsin in the presence or absence of dextran sulfate (Fig. 1A). Samples were taken at the indicated times, and the remaining activity was determined. rbFGF-CS23 lost its biological activity within 30 min when incubated with pepsin at 37°C and at pH 2.0 in the absence of dextran sulfate (Fig. $1A_1$). The addition of an equimolar concentration of dextran sulfate protected rbFGF-CS23 from the inactivation by pepsin. About 50% of the initial activity remained after 2 h incubation, and about 15% of the initial activity was still detected even after 16 h. Consistently with the loss of biological activity, rbFGF-CS23 was rapidly degraded by pepsin into fragment peptides in the absence of dextran sulfate as evidenced by the elution profile on a RP-HPLC column (Fig. $1B_1$). The fragmentation was inhibited in the presence of dextran sulfate (Fig. $1B_2$). Dextran sulfate also protected rbFGF-CS23 from inactivation by trypsin and chymotrypsin (Fig. $1A_2$ and A_3). Sommer and Rifkin reported that complete protection of bFGF from tryptic digestion was achieved when heparin and bFGF were mixed in a molar ratio of 10:1 [5].

3.3. *Complex formation*

Dextran sulfate was mixed and incubated with rbFGF-CS23 in a molar ratio slightly in excess of 1:1, and the mixture was applied to a gel permeation chromatography (GPC) column (TSK gel 3000SW, 0.75 × 60 cm; Tosoh). The mixture gave a single but much broader peak having an apparent M_r of about 45,000 as compared with the sharp peak having a M_r of 17,000 obtained for rbFGF-CS23 alone (Fig. 2), and the biological activity coincided exactly with the peak. This indicates that rbFGF-CS23 and dextran sulfate formed a complex in the aqueous solution and that the biological activity migrated with the complex. The broader peak of the complex may be a reflection of the broader size distribution of the dextran sulfate preparation used in this study.

The complex was dissociated into rbFGF-CS23 and dextran sulfate when NaCl was added at a concentration higher than 0.75 M, and the activity peak on gel filtration shifted to a lower M_r (data not shown). The protective effect of dextran sulfate at pH 3.0 and 37°C on rbFGF-CS23 was completely abolished in the presence of >0.75 M NaCl. These results indicate that the association of bFGF and dextran sulfate is due to ionic interactions and that the association is essential for the protection of the biological activity of bFGF.

3.4. *Binding sites on bFGF*

To study the binding site(s) on rbFGF-CS23 for dextran sulfate, rbFGF-CS23 was digested with *S. aureus* V8 protease, and the fragment peptides having affinity for dextran sulfate were obtained by affinity chromatography on an immobilized dextran sulfate column (data not shown). The obtained fragments, Asp^{6}-Glu^{45} and Ser^{100}-Ser^{146}, were pooled and subjected further to peptic digestion. The peptic digest was then applied to the same dextran sulfate column and peptides were eluted with a linear gradient of NaCl concentration from 0 to 2 M. The peptides having affinity for dextran sulfate thus obtained were pooled and subjected to RP-HPLC to give three positively charged fragment peptides (Fig. 3): Tyr^{106}-Trp^{114} (peak 1:Tyr-Arg-Ser-Arg-Lys-Tyr-Thr-Ser-Trp), Tyr^{124}-Leu^{138} (peak 2: Tyr-Lys-Leu-Gly-Ser-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-Ile-Leu) and Tyr^{24}-Phe^{30} (peak 3: Tyr-Cys-Lys-Asn-Gly-Gly-Phe).

Baird et al. reported several synthetic peptides, FGF-(24–68)-NH_2 and those containing residues 106–120 of bFGF, bind to heparin [10]. Seno et al. reported that deletion of the carboxyl-terminal 40–42 residues of bFGF greatly diminishes heparin affinity [11]. Consistently with their observations for heparin, our finding

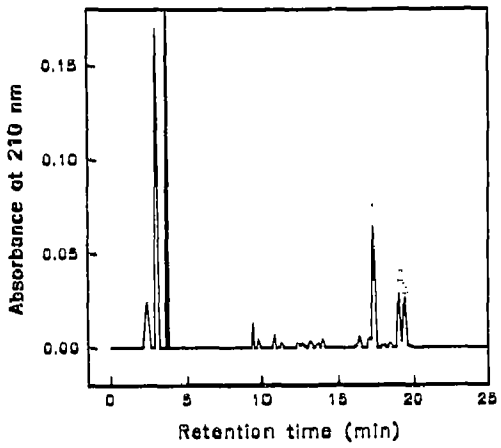

Fig. 3. RP-HPLC pattern of fragment peptides derived from rbFGF-CS23 having affinity for dextran sulfate. A mixture of peptic fragments derived from rbFGF-CS23 which had been obtained by affinity chromatography on a dextran sulfate column was subjected to RP-HPLC as described in Materials and Methods. Peaks (1) Tyr^{106}-Trp^{114}; (2) Tyr^{124}-Leu^{138}; and (3) Tyr^{24}-Phe^{30}.The peaks observed between 0 and 5 min are those derived from the solvent and the reagents.

that Tyr^{24}-Phe^{30}, Tyr^{106}-Trp^{114} and Tyr^{124}-Leu^{138} bound to dextran sulfate indicates that the basic residues located in these peptides play an important role(s) in the binding of bFGF to dextran sulfate. Recently, based on three-dimensional structure analysis of bFGF, a cluster of several basic amino acid residues was found on the surface of the bFGF molecule and suggested to constitute binding sites for heparin and other sulfated substrates [12–14]. Despite limitations of our approach to identify the dextran sulfate-binding sites of bFGF, it is worth noting that the four basic residues, Lys^{26}, Lys^{125}, Lys^{129} and Lys^{135}, are included in our fragment peptides having affinity for dextran sulfate. Eriksson et al. showed that four sulfate oxygens of heparin are hydrogen-bonded by the side chains of Asn^{27}, Arg^{120} and Lys^{125} as well as the main-chain amide of Arg^{120} [12]. Asn^{27} and Lys^{125} are also included in our fragment peptides.

In conclusion, our results presented in this paper suggest that dextran sulfate stabilizes bFGF by binding close to the putative heparin binding sites of bFGF. The effect of orally administering dextran sulfate together with bFGF on the acceleration of the healing of chronic duodenal ulcers in rats remains to be elucidated.

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