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Primary structure of ovine pituitary basic fibroblast growth factor

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The complete amino acid sequence of basic FGF (146 residues) from ovine pituitary glands has been established. This has been achieved by the sequence analysis of subnanomole amounts of the intact molecule and of peptides derived by enzymatic digestions with clostripain, chymotrypsin, pepsin and *Staphylococcus aureus* V8 protease. Microbore HPLC, employing 1–2 mm i.d. columns, was used to purify, concentrate and buffer-exchange the FGF peptides. A novel application of ion-pairing chromatography was employed to isolate peptides which were not retained on conventional reversed-phase systems. There is only one positional difference between the ovine and bovine basic FGFs, but there are 3 positional differences between ovine and human basic FGFS.

Fibroblast growth factor; Microsequencing; Primary structure; Ion-pair chromatography; Microbore HPLC

1. INTRODUCTION

Basic fibroblast growth factor (bFGF), a cationic mitogen (p*I* 9.6), was first isolated by Gospodarowicz from bovine pituitary and brain [1]. bFGF has been shown in vitro to be a very potent mitogen for vascular endothelial cells and a wide variety of mesodermal or neuroectodermal-derived cells [2,3]. Although structural studies on bovine bFGF reveal that it is a single-chain polypeptide of 146 amino acids [4], the recent characterization of cDNA clones that encode human [5] and bovine [6] bFGF suggests that both growth factors are synthesized with amino-terminal 9-residue extensions that are not found in the isolated forms of bovine [4,7] and human [8,9] bFGFs. We have now determined the complete amino acid sequence of bFGF from ovine pituitary glands.

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2. MATERIALS AND METHODS

2.1. *Basic FGF isolation*

Ovine bFGF was prepared from pituitary glands as in [4,10]. bFGF mitogenic activity was determined using mouse 3T3 fibroblasts as described [11].

2.2. *Preparation of* S-*carboxymethyl (SCM) bFGF*

Pure bFGF (12 μg in 420 μl) recovered from a Brownlee RP-300 column (30 × 2.1 mm i.d., Brownlee Laboratories, Santa Clara, CA) developed with a 0.15% (v/v) TFA/acetonitrile solvent system was diluted with 3 ml of 0.2 M Tris-HCl buffer, pH 8.3, containing 6 M guanidine hydrochloride, 4 mM EDTA and 0.02% (v/v) Tween 20 (Pierce). Dithiothreitol was added to a

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final concentration of 0.7 mM and reduction was allowed to proceed for 3 h at 45°C. A 4.3-fold molar excess of iodoacetic acid over total sulfhydryl groups was added and the incubation was continued at 25°C for 30 min in the dark. The alkylation was terminated by the addition of a large excess of mercaptoethanol over iodoacetic acid and SCM-bFGF was purified by reversed-phase HPLC.

2.3. *Proteolytic digestion of basic SCM-bFGF*

SCM-bFGF (10 μg in 500 μl of 36% acetonitrile/0.15% aqueous TFA containing 0.02% Tween 20) was first concentrated 5-fold in a polypropylene tube (Eppendorf) in a Speed Vac concentrator (Savant) to reduce the acetonitrile concentration and then diluted to 1.3 ml with 0.07 M sodium phosphate buffer, pH 7.6, containing 8 mM dithiothreitol, 1 mM $CaCl_2$ and 0.02% Tween 20. Clostripain (Boehringer), 3.3 μg, was added and the mixture incubated overnight at 37°C. Prior to use clostripain was dissolved in the same buffer and incubated at 25°C for 2 h. Digestions with chymotrypsin (Worthington) or *S. aureus* V8 protease (Miles Co.) were performed overnight in 1% (w/v) ammonium bicarbonate containing 0.02% Tween 20, whereas digestion with pepsin (Sigma) was performed in 5% formic acid for 65 min.

2.4. *Separation of peptides by HPLC*

Chromatography was performed using a Hewlett-Packard model 1090 liquid chromatograph fitted with a model 1040A diode array detector as described in [11,12].

Proteolytic digests of SCM-bFGF (typically 10 μg in 1 ml) were loaded, at a flow rate of 1 ml/min, onto a Brownlee RP-300 column which had been previously equilibrated with aqueous 0.1% (v/v) TFA. The column was developed, at a flow rate of 100 μl/min, with a linear 60-min gradient from 0 to 100% B, where secondary solvent B was 60% acetonitrile/40% aqueous 0.09% (v/v) TFA. Selected peak fractions were rechromatographed on the same column previously equilibrated with aqueous 0.9% (w/v) NaCl and eluted with a linear 50-min gradient from 0 to 50% B, where solvent B was acetonitrile.

Non-retained peptides which eluted in the column breakthrough fraction from the first chromatographic dimension were rechromatographed on an ODS-Hypersil column (Shandon Southern, Runcorn, England; 12 nm pore size, 5 μm C18 packed into a 50 × 1 mm i.d. column as described in [11]) previously equilibrated with aqueous 20 mM sodium phosphate, 0.1% phosphoric acid, pH 2.5 and 30 mM sodium octylsulfonate (BDH, Poole, England) (solvent A) and eluted, at a flow rate of 50 μl/min, with a linear 50-min gradient from 0 to 100% B, where solvent B was 50% acetonitrile in solvent A. In all cases, column temperature was maintained at 45°C and fractions were monitored by absorbance at 215 nm before being collected manually.

2.5. *Structural analysis*

Sub-50 pmol amino acid analysis of clostripain peptides of SCM-bFGF were performed using the DABS-Cl procedure described by Knecht and Chang [13]. Automated amino acid sequence analysis of protein and peptides was performed using an Applied Biosystems sequencer (model 470) as in [11,12].

3. RESULTS AND DISCUSSION

During the early phases of this work severe sample losses were encountered when manipulating HPLC-derived bFGF for proteolytic digestion (e.g. reduction of organic solvent concentration and buffer-exchange). Such losses, presumably due to the irreversible absorption of bFGF to the polypropylene HPLC collection tubes, were circumvented by the inclusion of a nonionic detergent (0.02% Tween 20) during all manipulations. Microbore column technology [11,12] was employed throughout this study to trace-enrich protein and polypeptides in large volumes (>1 ml) onto short (<10 cm) microbore columns (1–2 mm i.d.) and, subsequently, recover them in high yield (>90%) in small volumes (20–60 μl) suitable for microsequence analysis.

The sequence of ovine bFGF was determined by automated Edman degradation of the intact protein, and of peptides produced by clostripain, chymotrypsin, *S. aureus* V8 protease and pepsin digestion. The strategy by which the complete sequence of ovine bFGF (146 residues) was derived from these data is summarized in fig.1. Much of the sequence was determined by analysis of the 13

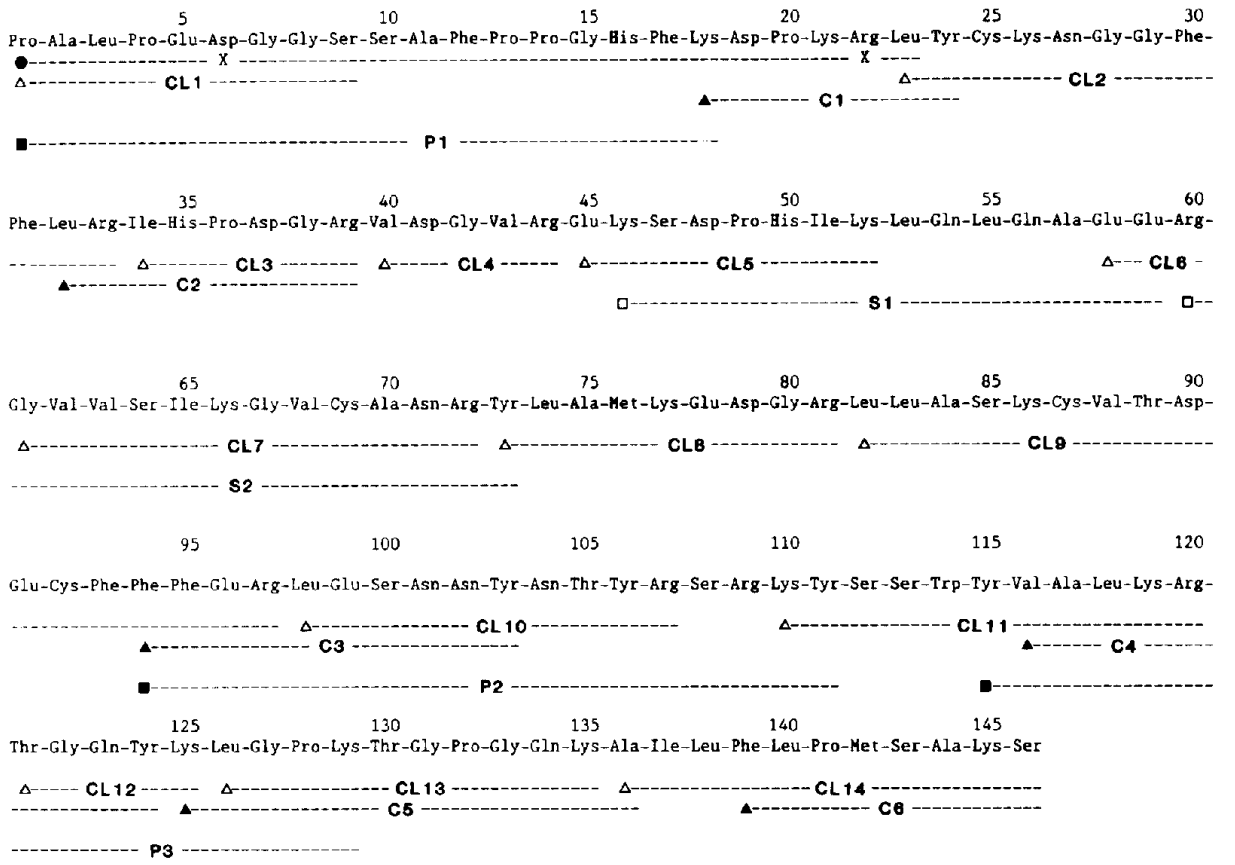

Fig.1. Primary structure of ovine basic FGF. Sequence data obtained from intact FGF (●), clostripain peptides (△), chymotryptic peptides (▲), *S. aureus* V8 protease peptides (□) and peptic peptides (■) are marked as indicated.

clostripain peptides derived from SCM-bFGF. The clostripain digest of SCM-bFGF was fractionated by RP-HPLC employing a low pH TFA (pH 2.1) mobile-phase buffer (fig.2). Peptides CL 1,2,7–11,13 and 14 were purified to homogeneity by rechromatography on the same support but employing unbuffered saline (0.9% NaCl) in the mobile phase (fig.3).

The remaining clostripain peptides (CL 3–6,12) which were non-retained and eluted in the column breakthrough of fig.1 were rechromatographed on ODS-Hypersil. An anionic ion-pairing reagent (octylsulfonate) was added to the mobile phase buffer to enhance the chromatographic retention of these peptides and thereby facilitating their purification (fig.4). Paired-ion complexes of peptides CL 3–6 and 12 were successfully sequenced without further manipulation.

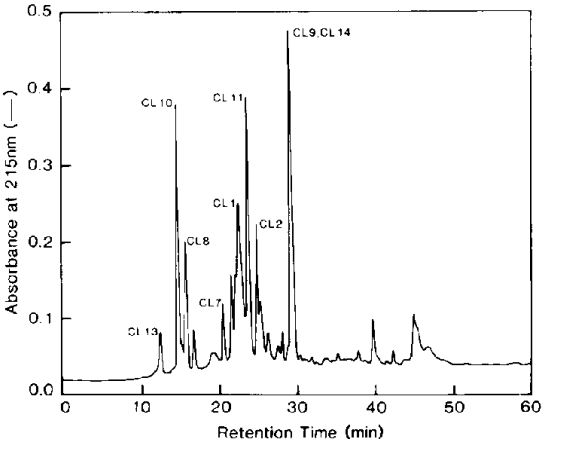

Fig.2. HPLC separation of clostripain peptides of SCM-bFGF on Brownlee RP-300. Mobile phase, 0.15% (v/v) aqueous TFA.

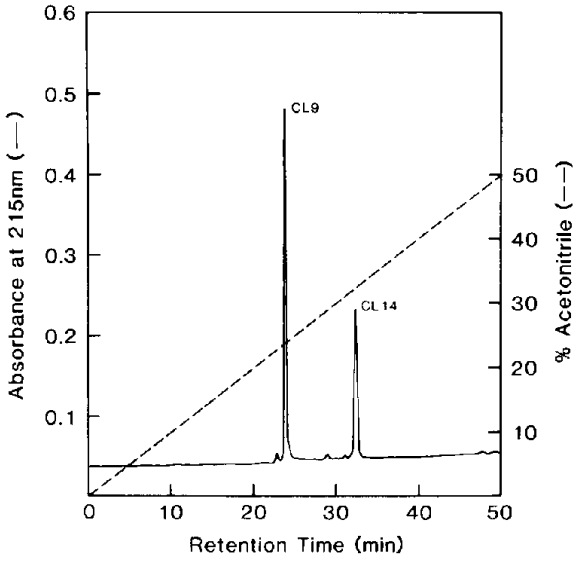

Fig.3. HPLC purification of clostripain peptides CL9 and CL14. Same chromatographic conditions as in fig.2 except that mobile phase is 0.9% (w/v) aqueous NaCl.

Automated Edman degradation of the intact molecule established the sequence of the first 21 residues while the remainder of the sequence was established, primarily, by sequence analysis of clostripain peptides CL 2–14. Confirmatory sequence data and information necessary to order some of the clostripain peptides were derived from chymotryptic, *S. aureus* V8 and peptic peptides (fig.1). Amino acid compositions of peptides CL 7–11,13 and 14 provided confirmatory evidence of the sequence of these peptides. The order of clostripain peptides CL 3,4 and CL 8,9 was determined by their corresponding position in bovine bFGF [4].

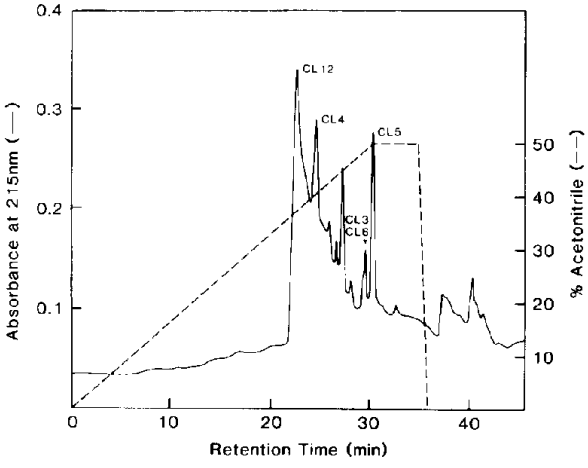

Fig.4. Ion-pair chromatography on ODS-Hypersil of clostripain peptides not retained on a conventional reversed-phase HPLC system (eluent breakthrough in fig.2).

Table 1

Summary of amino acid differences between sequences of ovine, bovine [5] and human [6] bFGFs

	Position in sequence		
	10	112	128
Ovine	Ser	Ser	Pro
Bovine	Gly	Ser	Pro
Human	Gly	Thr	Ser

The results presented here reveal that the primary structure of bFGF has been highly conserved between species. Only three differences are apparent when the amino acid sequence of ovine bFGF shown in fig.1 is compared with that of bovine bFGF [4,6] and the cDNA-deduced human bFGF [5]. These differences are summarized in table 1. The high degree of structural homology (97.9%) thus far observed for bFGFs implies a strong evolutionary selection pressure for maintenance of biological function and is consistent with the biological cross-reactivity across species of bFGF [3,9,10]. In contrast to the cDNA-deduced primary structures reported for bovine [6] and human bFGF [5], the ovine bFGF structure reported in fig.1 lacks a 9-residue amino acid terminal extension. This finding is in agreement with the automated Edman degradation of the isolated forms of bovine [4,7] and human bFGF [8,9] but differs from the bFGF-related hepatoma-derived growth factor from human tumor cells [14].

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