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<p><b>(54) Title:</b> HEPARIN-COPPER BI-AFFINITY CHROMATOGRAPHY OF FIBROBLAST GROWTH FACTORS</p> <p><b>(57) Abstract</b></p> <p>Methods for separating, identifying and purifying various forms of fibroblast growth factors with a novel bi-affinity chromatography technique based upon the interaction of fibroblast growth factors with heparin and copper.</p>		

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## HEPARIN-COPPER BI-AFFINITY CHROMATOGRAPHY OF FIBROBLAST GROWTH FACTORS

### BACKGROUND OF THE INVENTION

This invention relates to methods for separating, purifying and/or identifying a compound from a sample, where the compound has an affinity for at least two ligands. The method, hereinafter referred to as "bi-affinity chromatography", is particularly useful for the separation, identification and purification of species of fibroblast growth factor through use of a heparin-copper bi-affinity chromatography column.

Affinity chromatography is a well known process which enables the efficient isolation of biological macromolecules or biopolymers by making use of a feature unique to these substances. For example, biopolymers such as enzymes and antibodies are capable of recognizing certain chemical structures with a high degree of selectivity and then binding to them. The process of affinity chromatography typically involves a selective adsorbent which is placed in contact with a solution containing several kinds of substances including the desired species, the ligate. The ligate is selectively adsorbed to the ligand, which is attached to an insoluble support or matrix. The nonbinding species are removed by washing. The ligate is then recovered by eluting with a specific desorbing agent.

While affinity chromatography has been widely used in recent years in connection with a number of

biological compounds, it has only recently found use in the study of angiogenic factors. The term "angiogenesis" was coined earlier in this century to describe the formation of new blood vessels in the placenta. Early experiments involving observation of angiogenesis in wound healing and tumor growth led to speculation that tumor growth might be dependent upon capillary growth. Not until the late 60's, early 70's was it demonstrated that tumors induce capillary growth by the release of a diffusible tumor-derived angiogenic factor.

Purification of these factors was hampered, however, by the lack of suitable bioassays as well as inadequate knowledge of the components of the angiogenic process.

Advances in mammalian cell culture technique as well as an appreciation that endothelial cell locomotion and proliferation were major components of angiogenesis led to the isolation and purification of angiogenic factors. Among the first such factors (also referred to as endothelial cell growth factors) to be isolated was basic fibroblast growth factor.

A major breakthrough in the purification of such factors came in 1983 as a result of the observation that endothelial cell growth factors had a marked affinity for heparin. Heparin affinity chromatography has been widely used for the purification of endothelial cell growth factors from a variety of tissue sources since first reported by Shing et al., J. Cell Biol. 97, 395a (1983)), Shing et al., Science 223, 1296-1298 (1984)), the disclosures of which are hereby incorporated by reference herein.

Since the development of heparin-affinity chromatography, it has been found that virtually all

endothelial cell growth factors bind avidly to heparin. Moreover, on the basis of their respective affinities for heparin and isoelectric points, the heparin-binding endothelial cell mitogens can be subdivided into two classes of fibroblast growth factor (hereinafter "FGFs") which are typified by basic fibroblast growth factor (hereinafter "bFGF") and acidic fibroblast growth factor (hereinafter "aFGF"), (See, for example, Schreiber et al. J. Cell Biol. 101, 1623-1626 (1985)), and Lobb et al. J. Biol. Chem. 261, 1924-1928 (1986)). More recently, the purification and amino acid sequences of bFGF and aFGF have been established (See for example, Esch et al. Proc. Natl. Acad. Sci. U.S.A. 82, 6507-6511 (1985)) and Gimenez-Gallego et al. Science 230, 1385-1388 (1985)), Esch et al. Biochem. Biophys. Res. Comm. 133, 554-562 (1985)), Burgess et al. Proc. Natl. Acad. Sci. U.S.A. 7216-7220 (1986)), Strydom et al. Biochemistry 25, 945-951 (1986)).

Both bFGF and aFGF have been shown to exist in multiple molecular weight forms. (See Ueno et al. Biochem. Biophys. Res. Comm. 138, 580-588 (1986)), Klagsbrun et al. Proc. Natl. Acad. Sci. U.S.A. 84, 1839-1843 (1987)), Gospodarowicz et al. Endocrinology 117, 2383-2391 (1985)), Baird et al. Regulatory Peptides 12, 201-213 (1985)), Gospodarowicz et al. Endocrinology 118, 82-90 (1986)), Thomas et al. Proc. Natl. Acad. Sci. U.S.A. 81, 357-361 (1984)), Burgess et al. Proc Natl. Acad. Sci. U.S.A. 83, 7616-7220 (1986)), Gautschi et al. Eur. J. Biochem. 160, 357-361 (1986)). All forms of FGFs, however, have been found to be heat and acid labile. This precludes the use of reverse-phase HPLC for their isolation in their native form (Gospodarowicz et al. Mol. Cell. Endocrinol. 46,

187-204 (1986)). Fibroblast growth factors which have a broad spectrum of target cells in culture have been reported to induce vascular growth (angiogenesis) in vivo (Esch et al. Proc. Natl. Acad. Sci. U.S.A. 82, 6507-6511 (1985)), Thomas et al. Proc. Natl. Acad. Sci. U.S.A. 82, 6409-6413 (1985)). There has been speculation that the binding of FGFs to heparin might be copper-dependent (Folkman et al., Science 235, 442-447 (1987)). This idea was based on several reports in the literature indicating that copper levels in tissue could somehow modulate the intensity of the neovascular response to a given angiogenic stimulus. Such reports included the observation that (i) copper ions could augment endothelial locomotion in vitro (McAuslin et al. Exp. Cell Res. 130, 147-157 (1980)); (ii) rabbits on a copper deficient diet are unable to mount an angiogenic response to prostaglandin E<sub>1</sub> (Ziche et al. J. Nat. Canc. Inst. 69, 475-482 (1982)); (iii) both ceruloplasmin and heparin become angiogenic when complexed to copper, but not when deprived of copper (Raju et al. Cancer Res. 44, 1579-1584 (1984)); and (iv) heparin can act as a copper chelator (Grushka et al. Analyt. Lett. 15, 1277-1288 (1982)) and Stivala et al. Fed. Proc. 36, 83-88, (1977)).

Despite the apparent importance of FGF, its exact structure has been the subject of some controversy since its identification. Due to the differences in tissue and species sources as well as purification procedures, it has been difficult to make direct comparisons of the various forms of FGFs isolated in separate laboratories. Furthermore, although it has been well established that both basic and acidic FGFs exist in multiple forms, it has been difficult to isolate them in their native forms in a reproducible

manner. To date the physiological significance of these structurally similar FGFs in various tissues is not clear and until the present invention it has been difficult to isolate them in sufficient quantities for further investigation.

#### SUMMARY

In accordance with the present invention there is provided methods for separating, purifying and identifying compounds which have an affinity for at least two ligands from a sample. In its simplest form, the method comprises the steps of (a) contacting the sample with a bi-affinity column comprising two ligands for which the compound of interest has affinity, (b) contacting the column with an eluate which elutes compounds which do not bind to at least one of the two ligands, and (c) contacting the column with an eluate which elutes compounds which have affinity for both of the ligands.

The method of the present invention has been particularly useful in the separation, identification and purification of various forms or species of fibroblast growth factors in a simple, rapid manner based on the interaction of these growth factors with heparin and copper.

In one preferred embodiment, the method for separating, identifying and purifying the various forms or species of FGFs comprises contacting a sample containing FGFs with a bi-affinity chromatography column comprising (i) heparin-Sepharose and (ii) a copper-saturated chelating-Sepharose, both commercially available from Pharmacia AB, Uppsala, Sweden. The column is thereafter alternately rinsed with (A) an

eluate, such as a concentrated salt solution, which will remove compounds which removably bind to heparin, but not to copper; and (B) an eluate which will remove compounds which removably bind to copper, but not to heparin, e.g. a weak saline (NaCl) solution containing a relatively high concentration of imidazole. This step removes proteins which do not bind to heparin or copper, and also removes proteins which have affinity to heparin, but not to copper, and proteins which have affinity to copper, but not to heparin. Elution with eluate A can follow elution with eluate B, but it is preferred that elution with eluate B follow elution with eluate A.

The FGF proteins, which have affinity to both heparin and copper, are removed from the column by elution with an eluant which will remove such proteins from both the heparin and copper binding sites. Preferably, the eluate contains a combination of materials, which serve to remove the proteins from both the heparin and the copper, depending on the concentration of the materials in the eluate. Most preferably, the eluate used to remove the FGF's from the column is a linear NaCl/imidazole gradient. Fractions eluted from the column are collected, analyzed and characterized as to their molecular weight, acidic or basic nature and other properties of interest.

While not wishing to be bound by theory, separation of the various species of FGFs may be accomplished because FGFs may have separate binding sites for copper and for heparin. At any rate, it has been discovered that, when a sample containing FGFs is contacted with a bi-affinity column containing both heparin and copper on the chromatography media, treatment of the column

singly with an eluate which would remove the FGF's from a chromatographic media containing heparin, or with an eluate which would remove the FGF's from a chromatographic media containing copper, leaves FGF's on the bi-affinity heparin/copper media. Yet when the right eluate is applied, e.g. an increasing concentration gradient of both NaCl and imidazole, not only are FGF's removed from the column, but they are clearly separated in accordance with type (aFGF, bFGF) and size.

By taking advantage of the unique interactions between heparin, copper and FGFs, the present invention permits resolution of the various multiple forms of FGFs, based upon bi-affinity chromatography.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of a heparin/copper bi-affinity chromatography column useful in the separation, purification and identification of fibroblast growth factors.

Figure 2 illustrates application of heparin/copper bi-affinity chromatography to a sample isolated from bovine hypothalmi. Fig. 2A shows the presence and activity of various species of FGFs collected in fractions 6 - 20. The inset to Fig. 2A is a depiction of a silver stained gel showing the protein profile prior to heparin/copper bi-affinity chromatography. Fig. 2B is depiction of the silver stained fractions collected in Fig. 2A, which samples were previously subjected to SDS gel electrophoresis. Fig. 2C and 2D are Western blots of the fractions probed with antibodies raised against basic FGF and acidic FGF respectively.

## DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there is provided a novel method for separating, identifying, and/or purifying a compound from a sample, where the compound releasably binds or otherwise has an affinity for at least two ligands.

In general, the method comprises contacting a sample containing the compound of interest with a bi-affinity column comprising two ligands to which the compound releasably binds. The column is then contacted with a first eluate which elutes those compounds which releasably bind to one of the two ligands. The column is thereafter contacted with a second eluate which elutes compounds which releasably bind to the other ligand. Preferably this process is continued in an alternating fashion for a sufficient number of times until the undesirable components of the sample have been removed. The compound of interest is then recovered by contacting the column with an eluate which elutes compounds which releasably bind to both ligands.

As will be appreciated by the skilled artisan, there are many compounds, particularly biological molecules, which releasably bind or otherwise have an affinity for two or more ligands. For example, there are a number of biological molecules, such as enzymes, which are known to bind to metals such as iron, zinc, copper and the like. These enzymes also, by definition, interact, typically by binding with its particular enzyme substrate. Tissue plasminogen activator (TPA) is another example of a biological

molecule which is known to bind to at least two ligands. TPA has been shown to bind to zinc (Radcliffe et al., Arch. Biochem. Biophys. 189, 185-194 (1978)) as well as to the amino acid lysine (Rijken et al., J. Biol. Chem. 256, 7035-7041 (1981)).

In accordance with the present invention, such compounds can be separated, purified and/or identified by (i) contacting a sample containing the compound of interest with a bi-affinity column comprising both ligands to which the compound has an affinity, (ii) alternately contacting the column with eluates which elute compounds which bind to one ligand but not the other, and (iii) contacting the column with an eluate which elutes compounds which have an affinity for both ligands.

The eluates which may be used in practicing the present invention will vary depending on the compound of interest, and more particularly on the ligands used to construct the bi-affinity column. For example, where one of the ligands is a metal such as copper or zinc, the eluate used should be one which releases the compound of interest from the metal without breaking the bond between the metal and the matrix used to support the metal. Weak chelating agents such as imidazole may be used where the matrix is a metal saturated chelating resin such as Pharmacia's chelating-sepharose. In many instances, the alternate eluate used will be a strong ionic solution such as NaCl. The skilled artisan will appreciate, however, that the eluates chosen will depend on the ligands used to construct the bi-affinity column as well as the relative affinity of those ligands for the compound of interest. Recovery of the compound from the column may be achieved with a gradient of the eluates used in the

alternate eluting steps. Fractions are then collected and analysed for the compound of interest.

The method of the present invention has been particularly useful in the separation, identification and purification of FGFs. The method preferably comprises the steps of: (i) contacting a sample containing FGFs with a bi-affinity column comprising heparin and copper on a suitable support; and (ii) eluting the species of FGFs with a suitable eluate such as an increasing concentration gradient of materials which are effective to remove the FGF's from the heparin and the copper. Preferably the column is rinsed singly with eluates for heparin-binding proteins and for copper-binding proteins prior to the elution of the FGF's themselves. Preferably the method also involves collecting fractions containing the separated species of FGFs.

The heparin and copper components of the bi-affinity column are preferably in the form of a mixture or blend of heparin-Sepharose (Pharmacia Fine Chemicals, Sweden) and a copper-saturated chelating-Sepharose (Pharmacia Fine Chemicals, Sweden), although other suitable heparin-resins such as heparin-agarose (Sigma) and chelating-resins such as iminodiacetic acid-agarose (Sigma) may be used in lieu thereof.

The chromatographic media is preferably constituted so that the number of available heparin-binding sites is approximately equal to the number of available copper-binding sites. When heparin-Sepharose and copper-saturated chelating-Sepharose are employed as the heparin and copper components of the bi-affinity column, the ratio by weight percent of heparin-Sepharose to the copper-saturated

chelating-Sepharose is preferably from about 10:1 to about 1:10, and most preferably about 1:1.

As noted above, the eluates used in connection with this invention will vary depending on the compound of interest. In general, the eluate should not contain materials which react unfavorably with the chromatography medium involved, or with the compounds sought to be recovered. To that end, such eluates will normally be aqueous solutions containing physiological salt and suitable buffering materials to maintain the pH at around 7. The preferred buffering composition is Tris, in a concentration of about 10 mM.

Preferred eluates for eluting heparin-binding proteins from the column comprise salt solutions, e.g. halides of metals of group 1 or 2 of the periodic table of the elements. NaCl is most preferred, and it can be used in increasing concentration gradients, or in varying concentrations, depending upon the usage and the materials to be eluted. Other suitable eluates include sulfates such as  $(\text{NH}_4)_2\text{SO}_4$ .

Preferred eluates for eluting copper-binding proteins from the column comprise solutions containing materials, generally organic materials, which have a greater affinity for the metal component in the chromatographic media than the proteins to be eluted so that the materials to be eluted are displaced from their association with the metal without interfering with the bond between the metal and the supporting resin. Imidazole is preferred, and can be used in increasing concentration gradients, or in varying concentrations, depending upon the usage and the materials to be eluted.

For most applications, including application of the present invention to FGFs, the bi-affinity column is

pre-equilibrated with an appropriate solution in which the compound of interest is stable, for example for FGF, a 1 to 3M NaCl, 10 mM Tris, pH 7.

Pre-equilibration helps to reduce the undesirable sticking of other components of the sample to the ligands used in the bi-affinity column. Eluates, reactants and other solutions which come in contact with the compound of interest should also contain suitable buffers and other ingredients in order to avoid undesirable changes in the structure or function of the compound.

Samples which contain the compound of interest to be separated, identified or purified are preferably partially purified prior to contact with the bi-affinity column of the present invention by, for example, batchwise adsorption to a conventional affinity chromatography column containing a ligand known to bind to that compound. This partial purification helps to prevent overloading of the bi-affinity column with contaminants or other undesirable components of the sample.

The partially purified sample is applied directly to the pre-equilibrated bi-affinity column. The column is thereafter consecutively rinsed with, depending on the size of the column, from about 100 to about 200 ml volumes of one or more eluates to remove materials from the column other than those which bind to both ligands. When the compound of interest is FGF, the consecutive rinses comprise alternating rinses in 10 mM Tris, pH 7 of: a 2.0 to 2.5 M NaCl solution and a 10.0 to 20.0 mM solution of imidazole with intervening rinses of a 0.1 to 0.6 M NaCl solution. Most preferably, when the compound of interest is FGF the column is consecutively rinsed with (i) a 2 M NaCl

solution, (ii) a 0.6 M NaCl solution, (iii) a 0.6 M NaCl plus a 10 mM imidazole solution, and (iv) a 0.6 M NaCl solution. Rinse (i) is to remove those components of the sample which bind to heparin, but not to copper. Rinse (ii) is to re-equilibrate the column to allow the protein of interest to bind back to the other ligand, i.e. heparin. Rinse (iii) is to remove those components of the sample which bind to copper, but not to heparin. Rinse (iv) is to again re-equilibrate the column prior to the ultimate elution of the protein of interest. Of course, all of rinses (i) through (iv) would tend to remove materials or contaminants which were not bound to heparin, copper or some other component of the chromatography media.

After rinsing, the FGFs are eluted with an appropriate eluting gradient such as an NaCl/imidazole gradient. The NaCl/imidazole gradient preferably comprises a 0 to 0.6 M NaCl without imidazole to a 2 to 3 M NaCl plus a 10 to 20 mM imidazole at a pH of about 7. The volume of the gradient depends on the size of the column employed. For example, for a 1 cm x 9 cm bi-affinity column containing about 7 ml of the heparin and copper components, the NaCl/imidazole gradient comprises about 100 ml of 0.6 M NaCl without imidazole to about 100 ml of 2 M NaCl plus 10 mM imidazole at a pH of about 7. This means that imidazole is increasing in concentration from 0 to 10 mM, and that NaCl is increasing in concentration from 0.6 to 2 M. As will be understood by those skilled in the art, depending on the source of the sample (e.g. brain vs. pituitary tissue), this gradient can be optimized by changing the imidazole and NaCl concentrations to improve separation of the various species of FGF present in the particular sample.

The flow rate of the eluents will vary depending on a number of factors, including the size of the column. In general, for a 1 cm x 9 cm column containing 7.0 ml of the heparin and copper components, the flow rate is between about 10 ml/hr and about 40 ml/hr, preferably between about 15 ml/hr and 30 ml/hr, and most preferably about 20 ml/hr.

The volume of fractions collected from the column will also vary depending on a number of factors including the resolving power of the column. That is, the greater the resolving power, the greater the fraction that can be collected. In general, for the above described column, the volume of fractions collected is between about 2 and 10 ml, preferably between about 3 and 7 ml, and most preferably about 5 ml.

As the skilled artisan will appreciate, fractions eluted from the bi-affinity column in accordance with the present invention may be analyzed by a number of methods to confirm resolution of the compound of interest from the original sample. For FGFs, for example, such analytical techniques include analysis of mitogenic activity, e.g., by measuring uptake of tritiated thymidine by quiescent confluent monolayers of BALB/c mouse 3T3 cells, as described in Klagsbrun et al., Proc. Natl. Acad. Sci. U.S.A. 82, 805-809 (1985)), the disclosure of which is incorporated herein by reference. Other techniques may also be used, such as SDS polyacrylamide gel electrophoresis followed by silver staining, electrophoretic immunoblots using site-specific polyclonal antibodies raised against either bFGF or aFGF. Other techniques such as radioimmunoassay may also be used, as will be readily appreciated by those skilled in the art.

The invention will be further illustrated with reference to the following examples which will aid in the understanding of the present invention, but which are not to be construed as a limitation thereof.

#### EXAMPLE I

##### Isolation of FGFs from Hypothalamus

A crude extract of bovine hypothalmi FGF was prepared as follows: Bovine hypothalami (100 g) obtained from Pel-Freez (Rogers, Arkansas) were homogenized in 300 ml of 0.15 M  $(\text{NH}_4)_2\text{SO}_4$  at pH 6 and extracted by stirring at 4°C for 2 hours. The crude extract was centrifuged at 15,000 X g for 1 hour and the supernatant solution was loaded directly onto a heparin-Sepharose column (1.5 cm X 12 cm) pre-equilibrated with 0.6 M NaCl in 10 mM Tris, pH 7. The column was rinsed with 300 ml of 0.6 M NaCl in 10 mM Tris, pH 7. A sample of crude extract containing FGFs were substantially eluted with 40 ml of 2 M NaCl in the same buffer.

About  $7 \times 10^5$  units of bovine fibroblast growth factor activity were extracted from bovine hypothalmi. More than 95% of this activity bound to heparin-sepharose in 0.6 M NaCl and could be eluted with 2 M NaCl as described below.

#### EXAMPLE II

##### Heparin-copper Bi-affinity Chromatography

Copper-saturated chelating-Sepharose component was prepared by adding 100 ml of 2 mg/ml copper (II) chloride to 50 ml of chelating-Sepharose (Pharmacia), followed by several gentle mixings over a one-hour

period and decanting. A heparin-copper bi-affinity column was prepared by thoroughly mixing 3.5 ml each of heparin-Sepharose (Pharmacia) and copper-Sepharose, and placing the mixture in a 7.0 ml column (1 cm x 9 cm). The mixture was blue in appearance. The sample (40 ml) of FGF, which was partially purified by batchwise adsorption to heparin-Sepharose as described above, was applied directly to this blue color bi-affinity column which had been pre-equilibrated with 2 M NaCl, 10 mM Tris, pH 7. The column was rinsed consecutively with 40 ml each of the following reagents, all in 10 mM Tris, pH 7: (i) 2 M NaCl, (ii) 0.6 M NaCl, (iii) 0.6 M NaCl plus 10 mM imidazole, (iv) 0.6 M NaCl. Finally, FGFs were eluted at a flow rate of 20 ml/hr with a linear NaCl/imidazole gradient from 100 ml of 0.6 M NaCl without imidazole to 100 ml of 2 M NaCl plus 10 mM imidazole in 10 mM Tris, pH 7. Fractions (10 ml) were collected and assayed for growth factor activity.

### EXAMPLE III

#### Growth Factor Assay

As can be seen from Fig. 2A, growth factor activity was detected starting at about fraction No. 9, which corresponded to eluate containing about 1.3 M NaCl and 5 mM imidazole. About 30% of the initial growth factor activity was recovered. FGF activity was assessed by measuring the incorporation of [<sup>3</sup>H]thymidine into the DNA of quiescent, confluent monolayers of BALB/c mouse 3T3 cells in 96-well plates as previously described (Klagsbrun et al. Proc. Natl. Acad. Sci. U.S.A. 82, 805-809 (1985)). One unit of activity was defined as the amount of growth factor required to stimulate half-maximal DNA synthesis in 3T3 cells (about 10,000

cells per 0.25 ml of growth medium per well). For determination of specific activity and amount of recovery, protein concentration was estimated by the method of Lowry et al. (Lowry et al. J. Biol. Chem. 193, 265-275 (1951)).

#### SDS Polyacrylamide Gel Electrophoresis

As illustrated in Fig. 2B, analysis of eluates from fraction 9 to fraction 20 by SDS polyacrylamide gel electrophoresis followed by silver staining revealed the existence of multiple protein bands corresponding to the various FGF species present in bovine hypothalmi. The FGF-containing samples were analyzed by electrophoresis on 15% polyacrylamide gels as described by Laemmli (Laemmli et al. Nature (London) 227, 680-685 (1970)). The polypeptide bands were visualized by a silver stain described by Oakley (Oakley et al. Anal. Biochem. 105, 361-363 (1980)).

#### Preparation of Anti-FGF Polyclonal Antibodies

Peptide fragments corresponding to positions 1-12 (Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe) of basic FGF (Esch et al. Proc. Natl. Acad. Sci. U.S.A. 82, 6507-6511 (1985)) and position 59-90 (Thr-Glu-Thr-Gly-Gln-Phe-Leu-Ala-Met-Asp-Thr-Asp-Gly-Leu-Leu-Tyr-Gly-Ser-Gln-Thr-Pro-Asn-Glu-Glu-Cys-Leu-Phe-Leu-Glu-Arg-Leu-Glu) of acidic FGF (Gimenez-Gallego et al. Science 230, 1385-1388 (1985)) were synthesized by solid phase methods using an automated Applied Biosystems 430 A peptide synthesizer (See Merrifield et al. J. Am. Chem. Soc. 85, 2149-2154 (1963)), Sakakibara et al. Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, 1:51-85 (Weinstein, B., ed, Marcel Dekker, New York 1971)). The synthetic peptide fragments were

conjugated to keyhole limpet hemocyanin (KLH) using maleimidohexanoyl-N-hydroxy-succinimide ester as a cross-linking agent (Ishikawa et al. Immunoassay 4, 209-327, (1983)). Rabbits were injected at multiple dorsal intradermal sites with 500  $\mu$ g each of KLH-peptide conjugate emulsified with complete Freund's adjuvant. Animals were boosted regularly at 3-6 weeks intervals with 200  $\mu$ g of KLH peptide conjugated emulsified in incomplete Freund's adjuvant. The titer of the antisera after the second booster injection was about 1:15,000 to 1:50,000 as determined in an ELISA using unconjugated peptide as the antigen.

#### Immunoblotting (Western blot)

As illustrated in Figs. 2C and 2D, further analysis of these protein bands by electrophoretic immunoblot (Western blot) using site-specific polyclonal antibodies raised against either bFGF (Figure 2C), or aFGF (Figure 2D) demonstrated that it was possible to resolve from hypothalamus at least two bFGF species with Mr values of 19,000 and 18,000 and three aFGF species with Mr values of 18,000, 16,400 and 15,600. Proteins were separated by electrophoresis on SDS 15%-polyacrylamide gels and transferred electrophoretically to a nitrocellulose sheet as previously described (Towbin et al. Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354 (1979)). The nitrocellulose sheet was incubated with antiserum against either bFGF or aFGF and visualized by successive incubations with biotinylated goat anti-rabbit IgG, streptavidin-biotinylated peroxidase complex, and enzyme substrate (4-chloro-1-naphthol) until color develops (Nakane et al. J. Histochem. Cytochem. 16, 557-560 (1968)).

## WHAT IS CLAIMED IS:

1. A method for separating a compound from a sample, the compound being one which has affinity for at least two ligands, comprising the steps of:

(a) contacting the sample with a bi-affinity column comprising two ligands for which the compound has affinity;

(b) contacting the column with an eluate which elutes compounds which do not bind to at least one of the two ligands; and

(c) contacting the column with an eluate which elutes compounds which has affinity for both of the ligands.

2. A method for separating a compound from a sample, the compound being one which releasably binds to at least two ligands, comprising the steps of:

(a) contacting the sample with a bi-affinity column comprising two ligands to which the compound releasably binds;

(b) contacting the column with an eluate which elutes compounds which do not bind to at least one of the two ligands; and

(c) contacting the column with an eluate which elutes compounds which releasably bind to both of the ligands.

3. A method for separating a compound from a sample, the compound being one which releasably binds to at least two ligands, comprising the steps of:

(a) contacting the sample with a bi-affinity column comprising two ligands to which the compound releasably binds;

(b) contacting the column with an eluate which elutes compounds which releasably bind to one of the two ligands;

(c) contacting the column with an eluate which elutes compounds which releasably bind to the other of the ligands; and

(d) contacting the column with an eluate which elutes compounds which releasably bind to both of the ligands.

4. The method of claim 3, in which one of the ligands in the column is heparin.

5. The method of claim 4, in which one of the ligands in the column is copper.

6. The method of claim 3, 4 or 5, in which the compound is a protein.

7. The method of claim 6, in which the compound is a growth factor.

8. The method of claim 6, in which the compound is a fibroblast growth factor.

9. The method of claim 6, in which the compound is a human fibroblast growth factor.

10. The method of claim 5, wherein step (b) comprises contacting the column with an eluate which elutes compounds which releasably bind to heparin, step (c) comprises contacting the column with an eluate which elutes compounds which releasably bind to copper; and step (d) comprises contacting the column with an

eluate which elutes compounds which releasably bind to both heparin and copper.

11. The method of claim 10, wherein the eluate which elutes compounds which releasably bind to heparin comprises a salt solution.

12. The method of claim 10, wherein the eluate which elutes compounds which releasably bind to copper comprises a salt solution.

13. The method of claim 10, wherein the eluate which elutes compounds which releasably bind to both heparin and copper comprises a salt solution containing imidazole.

14. The method of claim 10, wherein the eluate which elutes compounds which releasably bind to both heparin and copper comprises a salt solution containing imidazole, the amounts of salt and imidazole being linearly increased over time.

15. A method for separating at least one growth factor from a sample, comprising:

- (a) contacting the sample with a bi-affinity column comprising heparin and copper;
- (b) eluting the fibroblast growth factor with an eluting gradient; and
- (c) recovering the fibroblast growth factor.

16. A method for analyzing a sample for at least one fibroblast growth factor, comprising:

- (a) contacting the sample with a bi-affinity column comprising heparin and copper;

(b) eluting the fibroblast growth factor with an eluting gradient; and

(c) recovering the fibroblast growth factor.

17. The method of claim 15 or 16, further comprising the steps of:

(i) contacting the column with an eluate which elutes compounds which releasably bind to heparin; and

(ii) contacting the column with an eluate which elutes compounds which releasably bind to copper;

wherein steps (i) and (ii) are conducted before eluting the fibroblast growth factor.

18. The method of claim 17, wherein the growth factor is eluted by contacting the column with an eluate which elutes compounds which releasably bind to both heparin and copper.

19. The method of claim 15 or 16, wherein the heparin and copper components comprise a mixture of heparin-Sepharose and copper-saturated chelating-Sepharose.

20. The method of claim 19, wherein the eluate which elutes compounds which releasably bind to heparin comprises sodium chloride.

21. The method of claim 19, wherein the eluate which elutes compounds which releasably bind to copper comprises imidazole.

22. The method of claim 18, wherein the eluate

which elutes compounds which releasably bind to heparin and to copper comprises an eluate having a NaCl/imidazole concentration gradient.

23. The method of claim 19, wherein the ratio by weight percent of heparin-Sepharose to copper-saturated chelating-Sepharose is from about 1:10 to about 10:1.

24. The method of claim 19, wherein the ratio by weight percent of heparin-Sepharose to copper-saturated chelating-Sepharose is about 1:1.

25. The method of claim 19, wherein the eluate which elutes compounds which releasably bind to heparin comprises from about 100 to 200 ml of a 2 to 3 M NaCl.

26. The method of claim 19, wherein the eluate which elutes compounds which releasably bind to copper comprises from about 100 to 200 ml of 2 to 3 M of NaCl and 10 to 20 mM of imidazole.

27. The method of claim 22, wherein the NaCl/imidazole gradient comprises from about 100 to 200 ml of a 0.1 to 0.6 M NaCl without imidazole to about 100 to 200 ml of a 2 to 3 M NaCl plus a 10 to 20 mM imidazole at a pH of about 7.

28. The method of claim 27, wherein the NaCl/imidazole gradient comprises from about 0.6 M to about 2 M NaCl, and from about 0 mM to about 10 mM imidazole.

29. The method of claim 17, wherein the column is contacted with consecutive rinses of: (i) a 2 M NaCl

solution; (ii) a 0.6 M NaCl solution; (iii) a 0.6 M NaCl plus a 10 mM imidazole solution; and a (iv) a 0.6 M NaCl solution.

30. A composition of matter comprising a physiologically acceptable chromatography media comprising heparin and copper.

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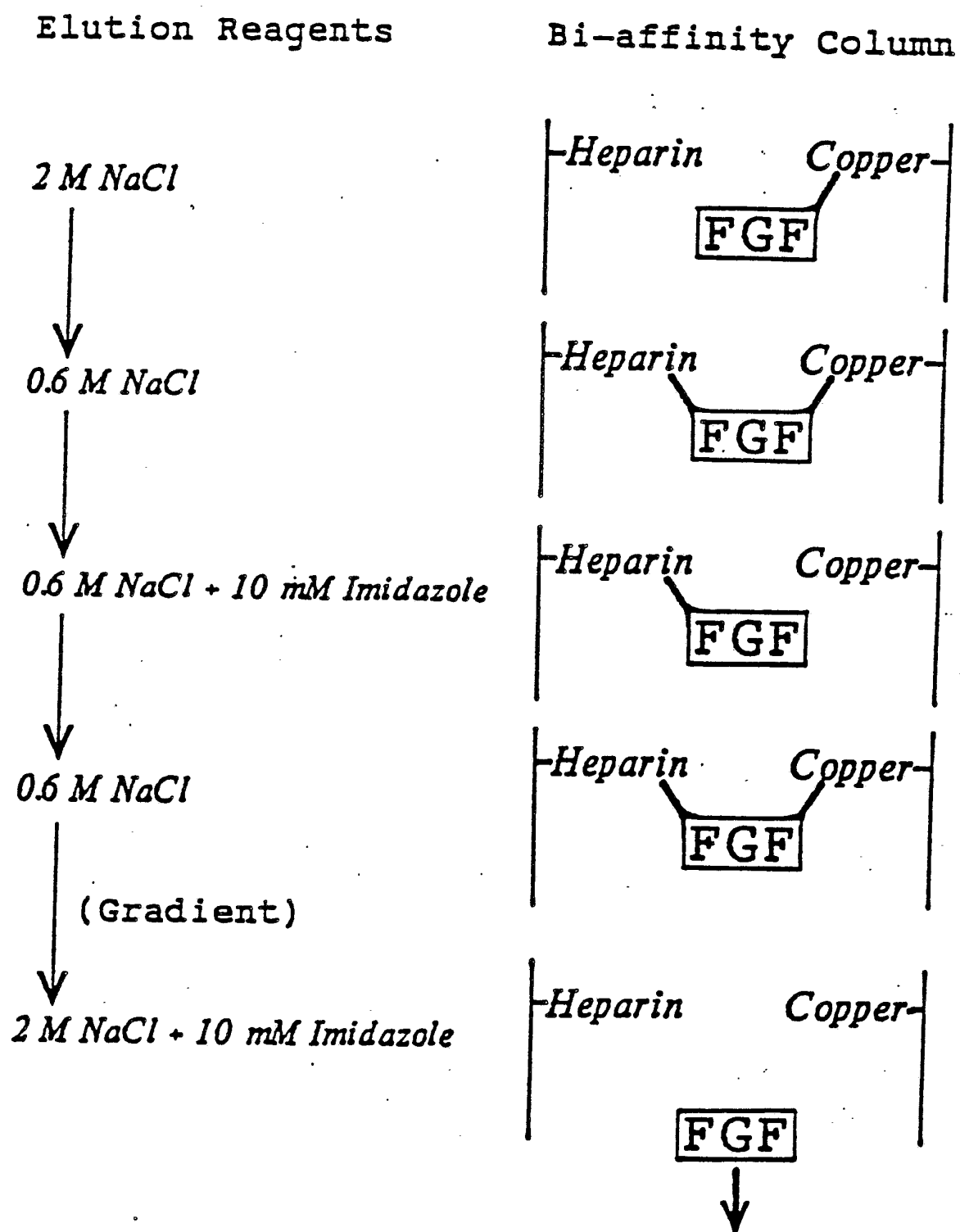
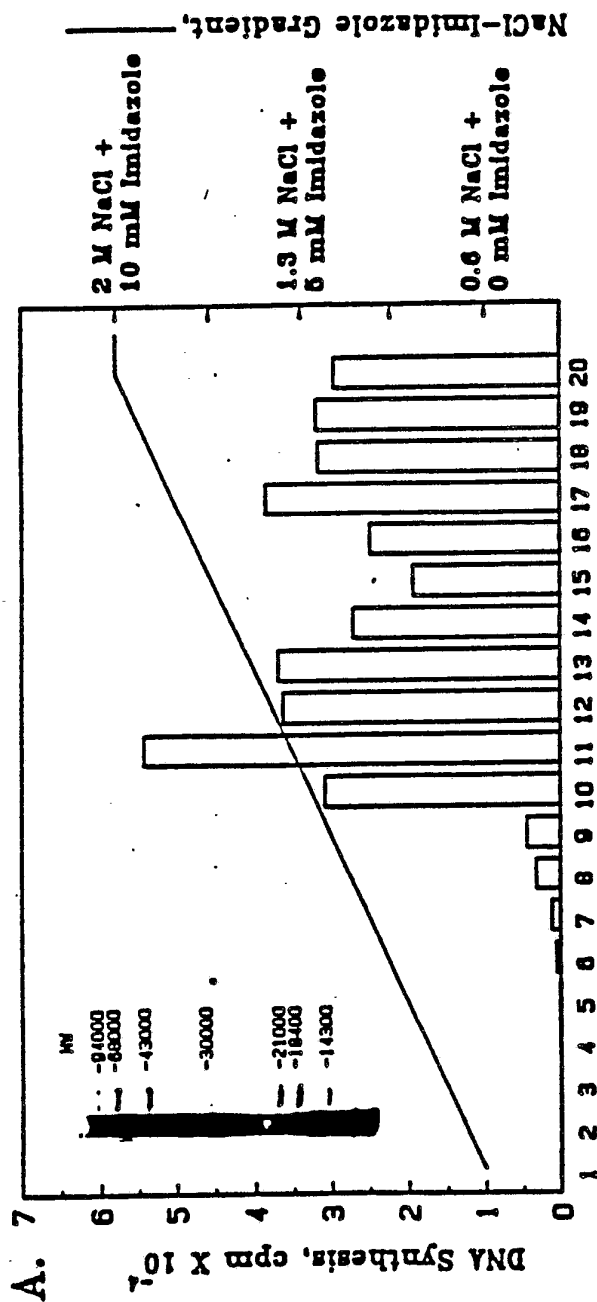


FIGURE 1

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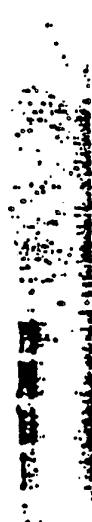
FIGURE 2



**B. Silver Stain**



**C. Western Blot**  
(Anti-basic FGF)



**D. Western Blot**  
(Anti-acidic FGF)



# INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/00587

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>3</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07K 3/18, 13/00

US. Cl.: 530/413, 415, 399

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>4</sup>

Classification System <sup>1</sup>

Classification Symbols

435/814,815

US

530/399,413,415

Documentation Searched other than Minimum Documentation  
to the extent that such Documents are Included in the Fields Searched <sup>5</sup>

COMPUTER SEARCH - CAS, BIOSIS

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

Category <sup>6</sup>	Citation of Document, <sup>15</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	J.M. ROWE, et al., "Purification and Characterization of a Human Pituitary Growth Factor", <u>Biochemistry</u> Vol. 25, No. 21, pages 6421-6425, published October 21, 1986 by American Chemical Society (Washington, D.C.). See pp. 6421-6422.	1-30
Y	M. IWANE, et al., "Expression of cDNA Encoding Human Basic Fibroblast Growth Factor in <u>E. Coli</u> ", <u>Biochem. Biophys. Res. Commun.</u> Vol. 146, No. 2, pages 470-477, published July 31, 1987 by Academic Press Inc. (New York, New York, USA). See pp. 470-477.	4-30

\* Special categories of cited documents: <sup>15</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Δ" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search <sup>2</sup>

10 MAY 1988

International Searching Authority <sup>1</sup>

ISA/US

Date of Mailing of this International Search Report <sup>3</sup>

09 JUN 1988

Signature of Authorized Officer <sup>20</sup>

CHARLES PATTERSON

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, <sup>1a</sup> with indication, where appropriate, of the relevant passages <sup>1b</sup>	Relevant to Claim No <sup>1c</sup>
Y	R. MIRA-Y-LOPEZ, et al., "Identification of a Pituitary Factor Responsible for Enhancement of Plasminogen Activator Activity in Breast Tumor Cells", <u>Proc. Natl. Acad. Sci, USA</u> Vol. 83, pages 7780-7784, published October, 1986 by National Academy of Sciences (Washington, D.C., USA) See pp. 7780-7782.	4-30
Y	A. BAIRD, et al., "Fibroblast Growth Factors are Present in the Extracellular Matrix produced by Endothelial Cells <u>In Vitro</u> : Implications for a Role of Heparinase like Enzymes in the Neovascular Response", <u>Biochem. Biophys. Res. Commun.</u> Vol. 142, No. 2, pages 428-435, published January 30, 1987 by Academic Press, Inc. (New York, New York, USA). See pp. 428-429	4-30