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(54) Title: OLIGOSACCHARIDES HAVING GROWTH FACTOR BINDING AFFINITY

(57) Abstract

Oligosaccharides having a high specific binding affinity for FGF growth factors and made up of less than ten disaccharide units in all are disclosed which include sulphated disaccharide units composed of an N-sulphated glucosamine residue and a 2-0-sulphated iduronic acid residue. A method is also disclosed for preparing these oligosaccharides in a purified and relatively homogeneous state from glycosaminoglycans such as heparan sulphate. For the best FGF-binding affinity there are preferably at least four of the sulphated disaccharide units arranged as an internal contiguous sequence. The most favoured structures contain fourteen monosaccharide residues in all, but structures having twelve monosaccharide residues can also have quite high FGF-binding affinity, at least for bFGF. These oligosaccharides can either activate and stimulate FGF activity or inhibit FGF activity, and uses thereof as drugs for therapeutic purposes in medicine are also disclosed.

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OLIGOSACCHARIDES HAVING GROWTH FACTOR BINDING AFFINITY

The present invention relates to the field of biochemistry and medicine. More particularly, it concerns certain novel oligosaccharide products and preparations thereof which have particular binding affinity for certain bioactive proteins or polypeptides present in biological systems, especially certain growth factors or cytokines such as fibroblast growth factors (FGF's). It also concerns uses of such oligosaccharide products, especially in medicine.

BACKGROUND

In complex multicellular living organisms, for example humans and other mammals, it is well known that various aspects of cell development, migration, growth and/or proliferation, involving in some cases cell-cell interactions, are often under the control of or are regulated by various extracellular mediators or cytokines, commonly referred to as growth factors, which are generally specialised soluble proteins or polypeptides secreted by cells of the tissues concerned.

These growth factors, of which many have already been isolated and subsequently synthesised using recombinant DNA technology, are believed to act through a variety of mechanisms, but in general their effect appears to result from an initial interaction with specific receptors or binding sites on the surface of target cells which are thereby activated to bring about a chain or sequence of intracellular biochemical events.

Certain of these protein growth factors, characterised *inter alia* by a high binding affinity for heparin, are designated by the general term Fibroblast Growth Factor (FGF) of which two main forms having partial amino acid sequence identity but differing isoelectric

points are recognised, acidic Fibroblast Growth Factor (aFGF) and basic Fibroblast Growth Factor (bFGF). FGF's are present in a wide variety of mammalian tissues; they appear to function in both normal and in diseased physiological states as important signalling molecules involved in regulation of cell growth and differentiation and they act as potent mitogens stimulating proliferation for a range of cell types. A review by D Gospodarowicz of some of the characteristics and properties in FGF's is to be found in *Cell Biology Reviews* (1991) 25 (4), 305-314.

In particular, basic fibroblast growth factor (bFGF) appears to have an important role in processes such as embryonic development, wound repair and tumour growth, and it has been specifically implicated as being directly concerned in various disorders or degenerative conditions involving cell proliferation, including for example diabetic retinopathy, capsular opacification following cataract operations, restenosis after angioplasty, tumour angiogenesis, and various forms of chronic inflammation. It delivers its signal to cells by binding with specific cell surface tyrosine kinase receptors (K_d 10-500 pM), such as receptors which are the expression products of the gene *flg*, that generate intracellular signals. However, the mode of action of bFGF and similar growth factors or cytokines is complex and appears also to involve an interaction with the heparan sulphate component of heparan sulphate proteoglycans (HSPGs) (K_d 5-50 nM) on the cell surface or in the extracellular matrix of mammalian cells. Recent work has shown, for example, that in cells which are deficient in heparan sulphate (HS) synthesis the *flg* receptor will not respond to bFGF, but that addition of heparin or heparan sulphate (HS) can restore responsiveness. It seems clear that in at least many cases such growth factors need to be activated before they can exert their biological effect. It has been suggested that polysaccharides such as HS and heparin induce a conformational

change in growth factors such as bFGF with which they interact and that this is a prerequisite for binding to the signal transducing receptor. Thus, on this basis a model invoking a dual-receptor mechanism, at least for the action of bFGF, has been proposed. Hitherto, however, the nature of a supposed bFGF binding site in HS has not been fully elucidated. HS is probably the most complex mammalian glycosaminoglycan (GAG), consisting of a linear polysaccharide chain having an ordered arrangement of domains rich in N- and O- sulphate groups, in which the basic disaccharide repeat unit consists of glucuronic acid or iduronic acid linked to an N-sulphated glucosamine (i.e. GlcA/IdoA-GlcNSO$_3$), spaced apart by regions of low sulphation in which N-acetylated disaccharides (GlcA-GlcNAc) predominate. Since bFGF is a heparin-binding growth factor the sulphated domains which contain some "heparin-like" regions might be expected to provide the most likely location of the bFGF binding site. On the other hand, the size of these domains, their sulphation pattern and their iduronic acid content are highly variable, and a possibility arises that the strong interaction with bFGF may require a strictly defined sequence of sulphated monosaccharide isomers providing a specialised binding domain in a manner similar to the specific pentasaccharide sequence in heparin which has been found to have high affinity for antithrombin III. Endothelial cell derived HS has already been demonstrated by affinity chromatography to bind strongly to bFGF, and a weaker interaction with HS from the Engelbreth Holm Swarm (EHS) tumour has also been reported, but the full structural requirements for such interactions have not previously been known.

As compared to bFGF, acidic fibroblast growth factor (aFGF) seems generally to be less potent, but nonetheless it is known as an active mitogen and differentiation factor for a wide variety of cells, especially mesodermal derived cell types, it is present in a variety of tissues,

it binds to the same cell surface receptors as bFGF with substantially the same affinity, it likewise binds strongly to heparin and to the heparan sulphate of cell surface or extracellular matrix heparan sulphate proteoglycans, and the mechanism of interaction would appear to be the same as with bFGF. A number of other growth factors or cytokines also bind to heparan sulphate or similar sulphated glycosaminoglycans of extracellular matrix or cell surface proteoglycans, and again this may be a necessary prerequisite for their biological activity under physiological conditions.

Since these growth factors or cytokines such as FGF appear to have such an important and wide-ranging role in controlling or regulating cellular processes that are responsible both for maintaining or restoring a normal physiological state or for promoting certain disease states, the possibility of controlling or modulating their activity for the purpose of therapeutic treatment is an attractive proposition. Thus, some consideration has already been given for example to the development and use of agents which would block the cell surface signal transducing binding receptors in order to inhibit growth factor activity, and in other cases, such as wound healing for example, where increased growth factor activity may be beneficial the use and administration of exogenous growth factors as therapeutic drugs has been considered. Another possibility for blocking or reducing activity would be to employ agents that would act as antagonists or agonists to interfere with the preliminary binding interaction between such growth factors and the proteoglycan or glycosaminoglycan, such as heparan sulphate, which appears to be necessary before binding to the cell surface signal inducing receptors can take place, and for this purpose the possible use and administration of heparin for acting as a competitive inhibitor could be considered, at least in principle. However, heparin (or heparan sulphate itself) is not particularly suitable for use as a drug in

this context, not least because of its complexity and heterogeneity with a large number of different disaccharide sequences in its molecular composition such that it is likely to have multiple activities giving other undesirable effects and it would lack specificity. What is needed for use as a drug is a high purity or substantially homogeneous preparation of a relatively small molecular compound of known composition which can be conveniently administered and which would have a very high degree of specificity for binding to the particular glycosaminoglycan binding sites of the growth factors in question with a low risk of promoting unpredictable or unwelcome side effects. In other words, it would be most desirable to have a molecule of minimal size consistent with high specific binding affinity, or a high value for the ratio of binding affinity or biological activity to size. Such a drug could then provide a valuable regulatory therapeutic agent for blocking or inhibiting subsequent binding to the cell surface signal inducing receptors and thus reducing growth factor activity, or in other cases it might act to stimulate growth factor activity by promoting subsequent growth factor binding to the cell surface signal inducing receptors. Also, if it is desired to administer exogeneous growth factors for therapeutic purposes, as perhaps in wound healing or various other tissue repair applications, it may be advantageous for such growth factors at the time of administration to be complexed with a protective or activating agent in the form of a relatively small molecular compound as referred to above which could be co-administered with the growth factor and which would bind with a high degree of specificity to the glycosaminoglycan binding sites of the growth factor.

Although it may be expected that, like the parent molecule, at least certain fragments of heparan sulphate would also have some specific binding affinity for FGF growth factors, and it is known that heparan sulphate can

be partially depolymerised by selective scission reagents (e.g. enzymes such as heparinase and heparitinase) to yield preparations of relatively short length oligosaccharides, such oligosaccharide preparations generally comprise a complex mixture of various molecular species having a wide range of different compositions and sizes. These preparations would therefore be no more suitable for use as drugs than would be heparan sulphate itself or heparin, and whilst various fractionations and partial purifications of such oligosaccharide preparations or mixtures have been carried out in the course of experimental work, the lack of more detailed knowledge about the particular structural characteristics that provide high specific binding affinity for FGF growth factors has been a problem that has hindered development of more well defined oligosaccharide products or preparations having optimum efficiency and better suited for possible medical use as drugs or therapeutic agents.

The present invention has originated in the course of work which was undertaken to investigate human skin fibroblast heparan sulphate and which has led to the isolation and characterisation of distinct oligosaccharide structures having particular specific binding affinity for FGF's and similar heparin or heparan sulphate binding growth factors. As a consequence, the invention enables oligosaccharide products to be prepared which, for medical use, especially as FGF growth factor modulating agents in connection with the treatment of various conditions herein referred to, are more suitable than any oligosaccharide preparations hitherto known.

ABBREVIATIONS

Throughout the present specification, including the claims, the following abbreviations are used:

GAG - glycosaminoglycan;
HS - heparan sulphate;
HSPG - heparan sulphate proteoglycan;
bFGF - basic fibroblast growth factor;
aFGF - acidic fibroblast growth factor;
dp - degree of polymerisation (e.g. for a disaccharide, dp=2, etc);
GlcA - D-glucuronic acid;
IdoA - L-iduronic acid;
IdoA(2S) - L-iduronic acid 2-sulphate;
GlcNAc - N-acetyl D-glucosamine;
$GlcNSO_3$ - N-sulphated D-glucosamine;
$GlcNSO_3(6S)$ - N-sulphated D-glucosamine 6-sulphate;
GlcA(2S) - D-glucuronic acid 2-sulphate;
ΔHexA - unsaturated uronic acid residue;
ΔGlcA - unsaturated hexuronate residue designated GlcA on the basis that it is believed to be derived from the saturated residue GlcA in an original polymer chain, e.g. based on the known specificity of heparitinase scission (see later);
$aMan_R$ - 2,5-anhydro-D-mannitol formed by reduction of terminal 2,5-anhydromannose residues with $NaBH_4$.

The symbol (Δ) is used to indicate that the monosaccharide residue concerned may or may not be unsaturated, and the symbol (±6S) denotes that a residue may or may not be sulphated at the C6 position.

SUMMARY OF THE INVENTION

As indicated, the invention broadly provides novel oligosaccharide products having a high specific protein or polypeptide binding affinity, especially in respect of HS-binding proteins or polypeptides exemplified by growth factors such as FGF's.

More particularly, in one aspect the invention

provides an oligosaccharide product having a specific binding affinity for fibroblast growth factors (FGF's), characterised in that it consists essentially of oligosaccharide chains which are substantially homogeneous with respect to FGF binding affinity and which contain at least four, preferably at least six, disaccharide units including sulphated disaccharide units, preferably arranged as a contiguous sequence, that are each composed of an N-sulphated glucosamine residue (±6S) and a 2-O-sulphated iduronic acid residue.

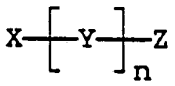
Also, it is preferred that each of said sulphated disaccharide units is IdoA(2S)-α1,4-GlcNSO$_3$, and that the oligosaccharide chains consist of a sequence of less than ten disaccharide units in all. In preferred embodiments, the oligosaccharide chains may consist of a sequence of six disaccharide units in all of which at least four are included in the aforesaid contiguous sequence of sulphated disaccharide units, although in the most preferred embodiments there are a total of seven disaccharide units of which at least five are included in said contiguous sequence of sulphated disaccharide units.

It is also preferred that the predominating majority of the oligosaccharide chains should all be of the same length and that the content (if any) of glucosamine residues O-sulphated at C6 should be less than 20%, or more preferably less than 5%. Oligosaccharides in accordance with the invention will generally be substantially completely resistant to depolymerisation by heparitinase but not by heparinase, and may be obtainable from heparan sulphate (HS) of human fibroblast heparan sulphate proteoglycan (HSPG) by enzymic partial depolymerisation to the fullest extent with heparitinase followed by size fractionation, using for example gel filtration size exclusion chromatography, followed by, in respect of a selected fraction or fractions recovered from the size fractionating stage, affinity chromatography

using an FGF growth factor as the immobilised ligand in order to separate out the FGF-binding fragments, and then eluting selectively over a range of salt concentrations under a salt gradient, advantageously a serially stepped gradient, to fractionate said fragments in respect of FGF binding affinity, followed by recovering the most strongly bound fragments and, optionally, further purifying the recovered product by carrying out at least one additional step of size fractionation and selection of recovered product using the methods herein referred to.

Alternatively, an oligosaccharide product having a specific binding affinity for fibroblast growth factors (FGF's) in accordance with the invention may be defined as being characterised in that

(a) it is composed predominantly of a molecular species:



in which

X is ΔHexA-GlcNSO$_3$(±6S),
Y is IdoA(2S)-GlcNSO$_3$(±6S),
Z is IdoA-GlcR(±6S) or
IdoA(2S)-GlcR(±6S)
where R is NSO$_3$ or NAc, and
n is in the range 4 to 7

(b) the content, if any, of monosaccharide residues having a 6-O-sulphate group is less than 20%;

(c) it is obtainable by a process comprising the steps of digesting a heparan sulphate with heparitinase so as to bring about partial depolymerisation thereof to the fullest extent, followed by size fractionating the oligosaccharide mixture produced using for example gel filtration size exclusion chromatography, collecting a fraction or fractions containing oligosaccharide chains having a particular size

selected within the range of 12 to 18 monosaccharide residues, then subjecting said selected fraction or fractions to affinity chromatography using an immobilised FGF ligand and recovering the more strongly FGF-binding constituents by eluting under a salt gradient over a range of salt concentrations and collecting a selected fraction or fractions containing the bound material which desorbs only at the highest salt concentrations,

and preferably being further characterised in that:

Y is exclusively IdoA(2S)-GlcNSO$_3$,

n is 5 or 6 with there being a total of seven disaccharide units in all, or is 4 with there being a total of six disaccharide units in all, and

the content, if any, of residues having a 6-O-sulphate group is less than 5%.

The invention may also be defined as providing an oligosaccharide product having a specific binding affinity for fibroblast growth factors (FGF's) that, at least in preferred embodiments, is substantially all composed of oligosaccharide chains which are either fourteen monosaccharide residues in length and which contain an internal contiguous sequence of 5 or 6 disaccharide units each consisting of an IdoA(2S) residue linked to a GlcNSO$_3$(±6S) residue, with less than 20% of the glucosamine residues (terminal or internal) being 6-O-sulphated, or which are twelve monosaccharide residues in length and which contain an internal contiguous sequence of 4 disaccharide units each consisting of an IdoA(2S) residue linked to a GlcNSO$_3$(±6S) residue, again with less than 20% of the glucosamine residues (terminal or

internal) being 6-O-sulphated, the predominant oligosaccharide chain sequence, accounting for substantially more than 50% of the component oligosaccharide chains and preferably more than at least 70% of the component oligosaccharide chains, being preferably selected from the following:

$(\Delta)GlcA\text{-}GlcNSO_3\text{-}[IdoA(2S)\text{-}GlcNSO_3]_5\text{-}IdoA\text{-}GlcR$,
$(\Delta)GlcA\text{-}[GlcNSO_3\text{-}IdoA(2S)]_6\text{-}GlcR$, and
$(\Delta)GlcA\text{-}GlcNSO_3(\pm 6S)\text{-}[IdoA(2S)\text{-}GlcNSO_3]_4\text{-}IdoA\text{-}GlcR(\pm 6S)$,
where R is NSO_3 or NAc.

Oligosaccharides in accordance with the invention include in particular the main constituent of the oligosaccharide product or preparation hereinafter designated oligo-H having a disaccharide sequence:

$\Delta GlcA\text{-}\beta 1,4\text{-}GlcNSO_3\text{-}\alpha 1,4\text{-}[IdoA(2S)\text{-}\alpha 1,4\text{-}GlcNSO_3]_5\text{-}\alpha 1,4\text{-}IdoA\text{-}\alpha 1,4\text{-}GlcR$,

or

$\Delta GlcA\text{-}\beta 1,4\text{-}[GlcNSO_3\text{-}\alpha 1,4\text{-}IdoA(2S)]_6\text{-}\alpha 1,4\text{-}GlcR$,
where R is NAc or NSO_3,

and minor variants thereof having at least the same relatively high specific binding affinity to bFGF.

Oligosaccharides in accordance with the invention also include, however, related highly sulphated oligosaccharides such as those comprising the main constituent of oligosaccharide preparations hereinafter designated oligo-M and oligo-L which have a weaker, but still significant, binding affinity to bFGF. These include, at least for oligo-M, oligosaccharide chains having the sequence

$\Delta GlcA\text{-}GlcNSO_3(\pm 6S)\text{-}[IdoA(2S)\text{-}GlcNSO_3]_4\text{-}IdoA\text{-}GlcR(\pm 6S)$
where R is generally NAc but may be NSO_3

The main components of oligo-L appear to comprise

the sequences

$\cap$GlcA-GlcNSO$_3$-IdoA-GlcNAc(6S)-GlcA-GlcNSO$_3$(6S)-[IdoA(2S)-GlcNSO$_3$]$_2$-IdoA-GlcR(±6S)

and

$\cap$GlcA-GlcNSO$_3$-IdoA(2S)-GlcNSO$_3$-IdoA-GlcNAc(6S)-GlcA-GlcNSO$_3$(6S)- IdoA(2S)-GlcNSO$_3$-IdoA-GlcR(±6S)

where R is generally NAc but may be NSO_3

Oligosaccharide products in accordance with the invention may either be isolated from natural sources or may be made synthetically.

In respect of isolation from natural sources, in broad terms the invention further provides a method of isolating from a glycosaminoglycan such as heparan sulphate small oligosaccharides in a purified and relatively homogeneous state which have a specific binding affinity for a selected bioactive protein or polypeptide that itself binds to said glycosaminoglycan or to the corresponding proteoglycan in multicellular biological systems, said method comprising the steps of:

(a) preparing an affinity chromatographic matrix or substrate incorporating a sample of said protein or polypeptide as the affinity ligand immobilised thereon;

(b) treating said glycosaminoglycan with a selective scission reagent so as to cleave the polysaccharide chains thereof selectively in regions of relatively low sulphation;

(c) subjecting the product of step (b) to size fractionation,, for example by gel filtration size exclusion chromatography, and collecting selectively therefrom fractions that appear to contain oligosaccharides composed of less than ten disaccharide units,

(d) contacting the affinity chromatographic matrix or substrate from step (a) with a selected fraction, or set of fractions, from step (c)

containing a specific number of disaccharide units in the range of four to nine in order to extract from the latter and retain on said matrix or substrate size selected oligosaccharide fragments of the glycosaminoglycan that have at least some binding affinity for the immobilised said protein or polypeptide;

(e) eluting the affinity chromatographic matrix or substrate using a progressively increasing salt concentration or gradient in the eluant;

(f) collecting the fraction or set of fractions containing oligosaccharide fragments eluting in selected highest ranges of eluant salt concentration; and optionally,

(g) further purifying the product of the selected fraction, or set of fractions, from step (f) by selectively repeating step (c) using said selected fraction or set of fractions collected in step (f) instead of the reaction mixture obtained from step (b), and optionally also repeating steps (d), (e) and (f).

In carrying out the above method, the partial depolymerisation of the glycosaminoglycan may be carried out by a chemical method in which the polysaccharide is first N-deacetylated, e.g. by hydrazinolysis and is then treated with nitrous acid at about pH 4, this being used as the selective scission reagent, to bring about deaminitive cleavage at the free amino groups of the glucosamine residues resulting from the N-deacetylation.

However, at least in the case of heparan sulphate the preferred selective scission reagent is the polysaccharide lyase enzyme heparitinase which is commercially available from Seikagaku Corporation of Tokyo, Japan under the designation "Heparitinase I", or from Sigma Chemical Co. under the designation "Heparinase III", and which has the classification EC 4.2.2.8. This enzyme will select-

ively cleave glycosidic linkages on the non-reducing side of GlcA-containing disaccharides, such as in GlcNAc-α1,4-GlcA present in regions of low sulphation, but in general it will not cleave bonds of sulphated disaccharides containing L-iduronic acid or 2-sulphated L-iduronic acid, i.e. IdoA or IdoA(2S). This is in contrast to the enzyme known as heparinase (EC 4.2.2.7) which cleaves glycosidic linkages between disaccharides containing 2-sulphated L-iduronic acid (for a review of these enzymes see R J Linhardt *et al* (1990) *Biochemistry* 29, 2611-2617). There are several known varieties of the heparitinase enzyme which have substantially the same linkage specificity but which vary for example in depolymerisation efficiency according to the size of the substrate molecules. However, in general throughout the present specification, including the claims, unless otherwise stated the term "heparitinase" is used to denote the enzyme supplied by Seikagaku Corporation as "Heparitinase I", or any other equivalent enzyme having the same glycosidic linkage specificity.

In connection with the cleavage of polysaccharide or oligosaccharide glycosidic linkages, e.g. 1,4 linkages, by enzymes such as heparitinase and heparinase, it should incidentally be appreciated that in the one fragment produced the monosaccharide residue at the non-reducing end which is immediately adjacent the cleaved bond will generally become unsaturated with a double-bond formed between C4 and C5. This unsaturation, however, is not likely to affect significantly the growth factor binding affinity of the fragment concerned, although it may perhaps affect stability of the molecule.

Oligosaccharides or oligosaccharide products in accordance with the invention generally have a well defined composition, readily capable of further purification if necessary, and considering also their size and specific growth factor binding affinity they can be very well suited for

pharmaceutical use to exploit a considerable potential in the field of medicine, e.g. as growth factor inhibitors or activators and mobilising agents. Accordingly, they are expected to have valuable applications as therapeutic drugs, particularly for controlling or regulating the activity of FGF's, especially bFGF. This may arise for example where there is a need to control or reduce FGF-activity dependent cell growth or proliferation in clinical treatment of conditions such as diabetic retinopathy, restenosis after angioplasty, capsular opacification, proliferation vitreoretinopathy, arthritis and other chronic inflammatory conditions, cancer cell growth and tumour angiogensis, mild muscular dystrophy, Alzheimer disease and various viral infections (e.g. Herpes Simplex type 1). This may also arise where there is a need to stimulate endogeneous FGF's or to administer and activate exogeneous FGF's for promoting healing or tissue repair, for example in clinical treatment of conditions such as wound healing, bone healing, nerve regeneration, duodenal or venous ulcers, various ocular and retinal disorders, atherosclerosis, degenerative muscle disorders, ischaemia, or for protecting tissues against serious damage during radiation treatment. For these purposes, the oligosaccharide products (or pharmaceutically-acceptable salts thereof) may be made up into pharmaceutical formulations as required, and such uses are also within the scope of the invention.

By way of further explanation so that the skilled person in the art will more readily be able to appreciate the nature of the invention and will more readily be able to put it into practical effect, there now follows a fuller description of the invention, including some of the background experimental work carried out by the inventors and various practical details thereof. In connection with this description, reference should also be had to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1: This shows the fractionation of native and partially depolymerised HS on a bFGF-affinity column in experiments in which ^{3}H-labelled samples of HS chains (control - panel A), or HS treated with heparinase (panel B), or heparitinase (panel C), were fractionated on a bFGF affinity column as hereinafter described. Bound material was eluted with a step gradient of sodium chloride as shown in panel A (dotted line). Heparitinase-resistant oligosaccharides retaining high affinity for bFGF (see panel C, material eluting at 1 to 1.5M NaCl, fractions 22-35) were pooled, then dialysed (Spectrapor 7 1000-Mr cut-off, Spectrum UK) and freeze dried. Their size was then established by gel filtration on a Bio-Gel P6 column (1 x 120cm) at a flow rate of 4ml/hour in 0.5M NH_4HCO_3 (panel D).

FIGURE 2: This shows the effect of heparinase on the affinity of HS oligosaccharides for bFGF in experiments in which ^{3}H-labelled HS chains were first treated with heparitinase and size fractionated by Bio-Gel P6 chromatography. Fractions of the heparitinase-resistant oligosaccharides of size dp12 and dp14 were pooled and then fractionated by bFGF affinity chromatography. Three major fractions eluting at 0.75M, 1.0M and $\geq$ 1.25M NaCl were obtained, designated oligo-L (low), oligo-M (medium) and oligo-H (high) affinity oligosaccharides respectively. The affinity of these oligosaccharides for bFGF was tested by re-application to the affinity column either intact (solid line), or after heparinase treatment (dashed line), and elution with a step gradient of sodium chloride (panel A, dotted line).

FIGURE 3: This shows the results of Bio-Gel P6 chromatography of HS oligosaccharides having differing affinities for bFGF in experiments in which HS oligo-saccharides (dp12-14) with relatively low (oligo-L),

medium (oligo-M) and high (oligo-H) affinity for bFGF were prepared as in connection with Fig. 2. Their size distribution was established by Bio-Gel P6 chromatography either intact (solid line) or after heparinase treatment (dashed line).

FIGURE 4: This shows the results of Bio-Gel P6 chromatography of bFGF-binding HS oligosaccharides subjected to deaminitive scission in experiments in which HS oligosaccharides (dp 12-14) with low (oligo-L), medium (oligo-M) and high (oligo-H) affinity for bFGF, prepared as described in connection with Fig. 2, were treated with nitrous acid and fractionated by Bio-Gel P6 chromatography. The disaccharides (dp2) were partially resolved into mono-sulphated (main peak) and non-sulphated species.

FIGURE 5: Shows the results of Bio-Gel P6 chromatography of bFGF-binding oligosaccharides (Oligo-H and Oligo-M) after being subjected to heparitinase IV depolymerisation;

FIGURE 6: Shows the results of bFGF affinity chromatography of heparitinase-resistant oligosaccharides for each different size from dp=2 to dp=14 after preparation by Bio-Gel P6 gel filtration;

FIGURE 7: Shows graphs "A" and "B", for bFGF and aFGF respectively, illustrating the effect of size of HS-binding oligosaccharides and binding affinity in relation to growth factor activation;

FIGURE 8: Shows a typical result of Bio-Gel P6 gel filtration of a heparitinase digest of ^{3}H-labelled fibroblast HS prior to bFGF-affinity chromatography, as referred to in the Example described herein.

DETAILED DESCRIPTION and EXAMPLES

In the initial background experimental work that led to the present invention, investigations were conducted using as source materials heparan sulphate derived from human skin fibroblasts and human recombinant bFGF.

The human recombinant bFGF was prepared in a manner similar to that described previously for acidic FGF by Ke, Y. *et al*, (1990) *Biochem Biophys. Res. Comm.* 171, 963-971. Briefly, the recombinant bFGF was purified by heparin-Sepharose chromatography and reverse phase or cation-exchange HPLC from lysates of bacterial cells, harbouring a PKK 233-2-bFGF construct (see Amann, E *et al*, (1985) *Gene*, 40, 183-190) encoding amino acids 1-155 of human bFGF (see Abraham, J.A. *et al*, (1986) *EMBO J.* 5, 2523-2528), to yield a single compound of MW 17kDa on SDS-PAGE. The amino acid sequence was consistent with that of human bFGF and the recombinant protein possessed full biological activity.

HSPG and HS chains biosynthetically radiolabelled with ^{3}H-glucosamine were prepared from confluent cultures of adult human skin fibroblasts as described in a paper by Turnbull and Gallagher (see Turnbull, J.E. *et al*, (1991) *Biochem. J.* 273, 553-559), the content of which is incorporated herein by reference.

Amongst the experimental techniques used, depolymerisation of HS chains with heparitinases, heparinase or low pH nitrous acid, and gel chromatography of oligosaccharides on Bio-Gel P6 or P2 were carried out as also previously described in the above-mentioned paper of Turnbull, J.E. *et al*, and in another paper of Turnbull, J.E. *et al*, (1991) *Biochem. J.* 277, 297-303. Chemical N-desulphation/re-N-acetylation was carried out as described by Inoue and Nagasawa (see Inoue, Y. *et al* (1976) *Carbohydrate Res.* 46, 87-95).

The work also involved the use of affinity chromatography and strong-anion exchange HPLC of disaccharides, the affinity chromatography involving a bFGF-Affi-Gel 10 affinity matrix. To prepare the latter, 1ml of packed Affi-Gel 10^{RTM} activated affinity gel from Bio-Rad Laboratories was washed four times with five volumes of double distilled water using centrifugation at 800g for 1 minute. Heparin (500µg) was added to bFGF (500µg in 3ml 0.6M NaCl, 25 mM Na_2HPO_4, pH 6.6) and mixed with 1ml of washed and packed Affi-Gel 10 overnight at 4°C. 2ml of 4M Tris-HCl, pH 8.0 was added to block unreacted groups on the gel. 5mg of heparin was added to stabilise bound bFGF and 4.5µl of 20% (w/v) NaN_3 as preservative. The gel was washed with 10 volumes of 2M NaCl in 10mM Tris-HCl, pH 6.5. No bFGF was detected in the wash by reverse-phase HPLC, indicating a high coupling efficiency.

The Affinity chromatography was generally carried out as follows:

Approximately 1ml of the bFGF-Affi-Gel 10 affinity matrix was packed into a glass column (bed dimensions 6mm x 35mm)). Samples were loaded onto the column in 10mM Tris-HCl, pH 6.5, at a flow rate of 0.25ml per minute. Unbound material was eluted by collecting five 1ml fractions. Bound material was eluted with a step gradient of sodium chloride (0 - 2M NaCl in column buffer in 0.25M steps) at a flow rate of 0.5ml per minute. Five 1ml fractions were collected at each concentration. The column was stored at 4°C in running buffer containing 10µg/ml heparin (Sigma), 0.01% (w/v) sodium azide and 0.2M NaCl.

To analyse the disaccharide composition of HS oligosaccharides, the latter were completely depolymerised enzymically with a mixture of heparitinase, heparitinase II and heparinase (obtained from Seikagaku Kogyo Co., Tokyo, Japan). Disaccharides were recovered by Bio-Gel

P2^{RTM} chromatography and separated by HPLC on a ProPac PA1 analytical column (4 x 250mm; Dionex, UK). After equilibration in mobile phase (double distilled water adjusted to pH 3.5 with HCl) at 1ml/minute samples were injected and disaccharides eluted with a linear gradient of sodium chloride (0 - 1M over 45 minutes) in the same mobile phase. The eluant was monitored in-line for UV absorbance (A_{232} for unlabelled disaccharides) and for radioactivity (Radiomatic Flo-one/Beta A-200 detector). Disaccharides released by nitrous acid treatment were separated by HPLC as has been described previously (see Pejler, G. *et al*, (1987) *Biochem. J.* 248, 67-69 and Bienkowski, M.J. *et al*, *Biochem. J.* 260, 356-365).

In initial experiments, HSPG, metabolically-labelled with ^{3}H-glucosamine, was purified from the medium of cultures of human skin fibroblasts. HS chains were prepared by Pronase treatment of the HSPG and applied to an affinity column prepared with human recombinant bFGF as hereinbefore described. Bound material was eluted stepwise with NaCl concentrations ranging from 0.25M - 2.0M in 0.25M steps. The majority of the HS bound strongly to bFGF, the major peak eluting at 1.25M NaCl (see Fig 1A). A similar elution profile was obtained for the intact HSPG (results not shown), indicating that the heparan sulphate (HS) chains are the principal determinant of proteoglycan binding to bFGF. Hyaluronic acid did not bind to the bFGF column and fibroblast-derived chondroitin and dermatan sulphate eluted in the range 0 - 0.5M NaCl; however commercial heparin eluted mainly at 1.25M and 1.5M NaCl (data not shown). These results indicated a specific interaction of bFGF with N-sulphated polysaccharides. The importance of N-sulphate groups was confirmed by the findings that either deaminitive scission with nitrous acid, or N-desulphation/re-N-acetylation of HS, abolished the high affinity interaction (results not shown).

The problem of identifying the bFGF binding domains

in HS was addressed using the enzymes heparinase and heparitinase which selectively cleave the polysaccharide in different structural domains. As already mentioned, heparinase acts in the N-sulphated regions and specifically cleaves disaccharides that contain 2-O-sulphated iduronate i.e. $GlcNSO_3(\pm 6S)$-α1,4-IdoA(2S), the major products being oligosaccharides of 9-10 kDa, while in contrast heparitinase cleaves GlcA-containing disaccharides, principally GlcNAc-α1,4-GlcA present in regions of low sulphation, but does not attack N-sulphated sequences of the type $[GlcNSO_3(\pm 6S)$-α1,4-IdoA$(\pm 2S)]_n$.

Heparinase scission of HS resulted in products with significantly reduced affinities for bFGF, elution occurring in the range 0.25 - 0.75M NaCl (see Fig. 1B). The effects of heparitinase digestion were even more marked with the majority of the material either failing to bind to the column or eluting at 0.25 - 0.75M NaCl (see Fig.1C). However, a minor population of oligosaccharides in the heparitinase digest displayed an affinity for bFGF that was comparable to the intact HS (eluting in the range 1.0 to 1.5M NaCl). Gel filtration size exclusion chromatography on Bio-Gel P-6 showed that these high affinity products comprised two oligosaccharide fractions predominantly dp12 and dp14 in size (see Fig. 1D), equivalent to six and seven disaccharide units. These are the largest fragments present in significant quantities in heparitinase digests of human skin fibroblast HS. The foregoing data indicated that extended N-sulphated sequences in HS contain the highest affinity binding site for bFGF, and that IdoA(2S) residues make an important contribution to the interaction.

Specificity of binding of oligosaccharides

To investigate the specificity of oligosaccharide interaction in more detail and the structural features involved, quantities of heparitinase-resistant oligosaccharides were prepared directly from heparitinase

digests of HS using Bio-Gel P-6 gel filtration size exclusion chromatography. Selected components of size dp12 and dp14 (12.5% of total product) were pooled and then fractionated again by bFGF affinity chromatography. Three major fractions were identified which eluted at 0.75M, 1.0M and 1.25M NaCl and were designated oligo-L (low), oligo-M (medium) and oligo-H (high) affinity oligosaccharides. Re-application of the fractions to the column confirmed their different affinities for bFGF (see Fig. 2). Oligosaccharides of size dp14 were mainly present in the oligo-H fraction whereas the oligo-M and oligo-L fractions were predominantly dp12 (see Fig 3).

Heparinase treatment caused a marked reduction in binding of these oligosaccharides to bFGF (see Fig. 2), and the extent of depolymerisation (Fig. 3) correlated closely with loss of affinity. The presence of major products dp4 and dp6 in size (Fig. 3) was indicative of cleavage of internal linkages.

Disaccharide composition of oligosaccharides

The disaccharide composition of the H, M and L oligosaccharides (see Table 1) was assessed by polysaccharide lyase depolymerisation and strong anion exchange HPLC as hereinbefore described. The calculated molar ratios are shown in Table 2. The most striking aspect of the analyses was the high content of disulphated disaccharides of the type ΔHexA(2S)-α1,4-GlcNSO$_3$, particularly in oligo-H and oligo-M (approximately 74% and 60% respectively of disaccharide units). The heparinase sensitivity of these fractions (Figs. 2 and 3) indicated that the majority of the 2-sulphated HexA residues were originally IdoA(2S) before cleavage of their glycosidic bonds and becoming unsaturated. Since the content of IdoA(2S)-disaccharides in the native HS was approximately 10-12%, the results indicated an enrichment of these residues of approximately seven-fold in oligo-H and six-fold in oligo M. Overall the concentration of ΔHexA(2S)-

α1,4-GlcNSO$_3$ strongly correlated with the differing bFGF affinities of the H, M and L oligosaccharides (see Table 1 and Fig. 2). In contrast there was a marked inverse correlation of binding strength with the content of the 6-O-sulphated derivatives ΩHexA-α1,4-GlcNAc(6S) and ΩHexA-α1,4-GlcNSO$_3$(6S). These accounted for 26% of disaccharides in oligo-L but were minor components in oligo-H (Tables 1 and 2). The amount of N-acetylated disaccharide ΩHexA-α1,4-GlcNAc was similar in each of the oligosaccharide preparations and corresponded to approximately one per fragment (Tables 1 and 2).

Deaminitive scission with low pH nitrous acid resulted almost exclusively in disaccharide products with the oligo-H and oligo-M fractions (see Fig. 4A and 4B; 99% and 95% respectively), whereas both disaccharides (76%) and tetrasaccharides (24%) were major products from the oligo-L fraction (Fig. 4C). Thus, virtually all the internal hexosaminidic linkages within the oligosaccharides in the H and M fractions involved GlcNSO$_3$ residues and the N-acetylated unit would clearly be at the reducing end of the fragment (see below). This is in contrast to oligo-L for which the results indicated the presence of an internal N-acetylglucosamine in a large proportion (60-70%) of the oligosaccharides. The disaccharides released from the H, M and L fractions by nitrous acid were also examined by strong anion exchange in order to establish the identity of the constituent uronic acid residues. Oligo-H yielded 71% of labelled product eluting in the position of the standard IdoA(2S)-aMan$_R$; the remaining labelled product eluted as an unsulphated peak, corresponding to ΩGlcA-aMan$_R$ and IdoA-GlcNAc (results not shown). Oligo-M and oligo-L yielded 61% and 37% respectively as IdoA(2S)-aMan$_R$; thus, the content of the disaccharide IdoA(2S)-aMan$_R$ in each of the fractions correlated well with that of ΩHexA(2S)-GlcNSO$_3$ established by lyase depolymerisation (Table 1).

Table 1

Disaccharide composition of HS oligosaccharides with differing affinities for bFGF

HS oligosaccharides (dp12-14) with low (oligo-L), medium (oligo-M) and high (oligo-H) affinity for bFGF were prepared as described in connection with Fig. 2. Disaccharide composition was analysed by strong anion exchange HPLC as described.

Disaccharide	Oligo-L	Oligo-M	Oligo-H
ΔHexA-GlcNAc	13.6	16.0	11.3
ΔHexA-GlcNAc(6S)	12.4	2.6	0.9
ΔHexA-GlcNSO$_3$	27.3	11.3	7.5
ΔHexA-GlcNSO$_3$(6S)	14.0	4.5	1.4
ΔHexA(2S)-GlcNSO$_3$	31.0	59.8	74.2
ΔHexA(2S)-GlcNSO$_3$(6S)	0.6	2.2	1.0
Disaccharide yield %	98.9	96.4	96.3

Table 2

Stoichiometry of constituent disaccharides of HS oligosaccharides with differing affinities for bFGF

This table shows the average relative molar ratios of the constituent disaccharides of the HS oligosaccharides (based on the disaccharide composition data of Table 1) and the predominant average size of these oligosaccharides.

Disaccharide	Oligo-L	Oligo-M	Oligo-H
ΔHexA-GlcNAc	0.82	1.00	0.82
ΔHexA-GlcNAc(6S)	0.75	0.16	0.07
ΔHexA-$GlcNSO_3$	1.65	0.70	0.55
ΔHexA-$GlcNSO_3$(6S)	0.85	0.28	0.10
ΔHexA(2S)-$GlcNSO_3$	1.88	3.72	5.39
ΔHexA(2S)-$GlcNSO_3$(6S)	0.04	0.14	0.07
Total Disaccharide (moles/mole oligosaccharide)	6	6	7

A novel enzyme, heparitinase IV (from Seikagaku Kogyo Co.), was also used to characterise the sequence of these oligosaccharides. This enzyme has a similar linkage specificity to heparinase [i.e. $GlcNSO_3$(±6S)-α(1-4)-IdoA(2S)], but is much more efficient at cleaving small substrates (such as tetrasaccharides and hexasaccharides) which contain susceptible linkages.

Treatment of Oligo-H with the enzyme heparitinase IV resulted in a high degree of depolymerisation to give disaccharide and tetrasaccharide products (69% and 31% of ^3H label respectively, as shown in Figure 5). These results indicated a ratio of 4.5:1 for the number of disaccharides to tetrasaccharides, in good agreement with the expected ratio (5:1) based on the predominant sequence proposed for Oligo-H.

Treatment of oligo-M with heparitinase IV also resulted in a high degree of depolymerisation giving mainly disaccharide and tetrasaccharide products, but also some hexasaccharides (51%, 36% and 13% of ^3H label respectively - see also Figure 5). These results indicate a molar ratio of 4:1.4:0.3 for di:tetra:hexasaccharides, in reasonable agreement with the expected ration (4:1:0) based on the predominant sequence proposed for Oligo-M.

The experimental work described above showed that a sulphated oligosaccharide fraction (oligo-H) in fibroblast HS composed of a sequence of seven disaccharides bound particularly strongly to bFGF. The dominant structural unit in the oligosaccharide was IdoA(2S)-α1,4-GlcNSO$_3$ (74% of disaccharides; Table 1) and both the 2-O-sulphate and the N-sulphate groups appeared to be essential for binding activity. Analysis of the disaccharide composition following deaminitive scission confirmed that the identity of the uronic acid moiety of this disaccharide was IdoA(2S) and not GlcA(2S). The only other disaccharides present in approximately stoichiometric amounts were ΔHexA-α1,4-GlcNSO$_3$ and ΔHexA-α1,4-GlcNAc. Because oligo-H was a product of heparitinase digestion it was deduced that the sequence of the principal or most predominant oligosaccharide component or components is:

ΔGlcA-β1,4-GlcNSO$_3$-α1,4-[IdoA(2S)-α1,4-GlcNSO$_3$]$_5$-α1,4-IdoA-α1,4-GlcR

or, alternatively,

ΠGlcA-β1,4-[GlcNSO$_3$-α1,4-IdoA(2S)]$_6$-α1,4-GlcR
where R is generally NAc but may be NSO$_3$

These structures have subsequently been confirmed, although further minor variations can occur by the occasional presence of 6-O-sulphate groups on any of the amino sugars (e.g. 1% of disaccharides contain GlcNAc(6S); see Table 1) without significantly affecting the specific binding affinity. Polysaccharide lyase depolymerisation of these sequences would produce a disaccharide composition for oligo-H which closely matches that shown in Table 1, and the fact that the GlcNAc residue is placed at the reducing end of the sequence (position 14) correlates with the fact that essentially all the internal linkages were sensitive to deaminitive scission (Fig. 4A), indicating the presence of a single contiguous sequence of N-sulphated disaccharides. If the GlcNAc was located elsewhere in the sequence, even at position 2, deaminitive scission would also produce a significant fraction of tetrasaccharide products in addition to disaccharides (e.g. as observed with oligo-L, Fig. 4C).

In the case of oligo-M, it has been established that the principal or predominant oligosaccharide chains have a sequence

ΠGlcA-GlcNSO$_3$(±6S)-[IdoA(2S)-GlcNSO$_3$]$_4$-IdoA-GlcR(±6S)
where R is generally NAc but may be NSO$_3$

It is believed that this is the first time an extended contiguous sequence of IdoA(2S)-α1,4-GlcNSO$_3$ units has been identified in HS. The surprisingly low content of 6-O-sulphate groups clearly distinguishes this oligosaccharide from typical N-sulphated sequences in heparin in which the GlcNSO$_3$ residues are frequently sulphated at C-6 i.e. [IdoA(2S)-α1,4-GlcNSO$_3$(6S)]$_n$. The oligo-H sequence identified here may not necessarily represent the minimal sequence for optimal binding to bFGF, but it seems noteworthy that full activation of bFGF

(measured by its ability to bind to the *flg* receptor) requires heparin fragments of about the same size as oligo-H, i.e. dp14-dp16. The related cytokine acidic FGF is also strongly activated by heparin oligosaccharides in this size range and has also been found to bind to oligosaccharides of the kind herein identified.

An indication of the structural requirements for optimum high affinity interactions with bFGF can be obtained by comparing the composition of oligo-H with the oligosaccharides of medium and low affinity for bFGF which, as oligosaccharide products resistant to heparitinase digestion, contain the same basic disaccharide repeat of IdoA-$GlcNSO_3$. Oligo-H and oligo-M have similar degrees of sulphation (1.6 and 1.5 sulphates/disaccharide respectively) but oligo-M contains approximately 60% of disaccharides in the form of IdoA(2S)-α1,4-$GlcNSO_3$ compared to 74% in oligo-H. About 10% of amino sugars in oligo-M are 6-O-sulphated (Table 1) which in terms of overall sulphation largely offsets the lower concentrations of IdoA(2S). The only other detectable difference between the two fractions is in size, oligo-M containing predominantly six disaccharides compared to seven in oligo-H (Fig. 3). It is believed that the combined effects of fragment size and enrichment of IdoA(2S) are the key properties that facilitate a stronger interaction of oligo-H with bFGF. The importance of IdoA(2S) is emphasised by the analytical data on the low affinity fragment oligo-L (size dp12) in which only 31% of disaccharides contain this component and there appear to be no more than two of the basic IdoA-$GlcNSO_3$ disaccharide repeat units which may or may not be contiguous within the sequence. However, oligo-L is still quite highly sulphated (1.3 sulphates/disaccharide) because of the higher content of $GlcNSO_3$(6S) and GlcNAc(6S) (Table 1), and has some specific bFGF-binding activity so that the main oligosaccharide components thereof, having the sequences hereinbefore specified, may have some utility.

The data obtained provide some revealing insights into the structural heterogeneity of HS. The differential O-sulphation of the large N-sulphated oligosaccharides probably reflects a complex mechanism of HS biosynthesis in which the 2- and 6- sulphotransferases may be regulated independently. Although a specific sequence consisting of $GlcNSO_3$ and IdoA(2S) appears to be designed for strong binding to bFGF, it is believed that sequences with different sulphation patterns, especially those with mixed 2- and 6-sulphate isomers, may interact with other HS-binding proteins and other members of the FGF family may bind preferentially with HS sequences which are slightly different to those preferentially recognized by bFGF. Thus, it will be appreciated that while retaining the same basic characteristics, there is scope for some structural variations. In particular, even for bFGF (or aFGF) a higher proportion of 6-sulphated glucosamine residues is probably unlikely to be seriously detrimental to binding affinity, and even sequences similar to oligo-M and oligo-L can provide oligosaccharides having a useful degree of specific binding affinity although not as high as for oligo-H.

The identification of specific binding sequences in glycosaminoglycans (GAGs) is central to an understanding of their biological functions. The sequences of the antithrombin-III binding region in heparin was a major advance in this field (Lindahl, 1984). The interaction is specific, requiring a distinct sugar sequence and sulphation pattern, rather than being determined mainly by relatively unspecific electrostatic forces. Antithrombin-III is activated by heparin in a manner analogous to HS/heparin activation of bFGF. Thus, specific interactions with GAGs can convert proteins from latent to active forms, and the inventors hereof have obtained evidence indicating that the oligosaccharides of the present invention can also be effective in activating FGF's such as bFGF. This activation ability, however,

does not appear to be present, at least to a significant extent, in oligosaccharides composed of less that six disaccharide units. When a protein ligand for HS or a similar GAG is known, the method herein disclosed in accordance with the present invention, wherein specific polysaccharide scission and size fractionation is followed by affinity chromatography, should also prove useful for isolating and characterising the protein-binding domains in these other cases.

Biological Activity

In connection with the biological activity of heparitinase-resistant oligosaccharides, further studies have been carried out on the relationship between oligosaccharide affinity and activation of aFGF and bFGF, using a HS-dependent bioassay of FGF-stimulated mitogenesis. This assay depended on the fact that 3T3 fibroblasts grown in the presence of the chemical sodium chlorate (which supresses polysaccharide sulphation) do not respond to aFGF or bFGF, but responsiveness (measured by incorporation of ^{3}H-thymidine) is restored by addition of HS or heparin (as little as 1-10ng/ml) to the culture medium, thus allowing testing of the ability of exogenous HS oligosaccharides to activate FGFs. A number of oligosaccharides with a range of structures and affinities for FGFs have been studied using this assay, in particular heparitinase-resistant oligos of size dp6, 8, 10, 12, 14 and 16 and larger (from porcine mucosal HS). Preliminary results for both bFGF and aFGF are shown in Figure 7, and indicate that oligosaccharides dp12 or larger are active, whereas those dp10 or smaller are inactive. This suggests that only the larger oligosaccharides of a particular structure excised from HS by heparitinase (and presumably of similar structure to those herein described) are capable of full biological activation of FGFs, and that smaller structures, even if they have some degree of binding affinity, do not activate these growth factors.

Preparation

In practice, the oligosaccharides of this invention may be conveniently prepared from purified heparan sulphate, native or recombinant, using the gel filtration chromatography and FGF-affinity chromatography techniques herein described in relation to the investigative experimental work, although generally the heparan sulphate may not need to be radiolabelled for purely preparative purposes. Oligosaccharides derived by heparitinase scission can readily be monitored by virtue of the unsaturated terminal uronic acid residue which absorbs strongly in the ultraviolet range (maximum at 232nm).

A specific example of the preferred method of carrying out of the invention and of preparing oligosaccharide products having a relatively high affinity for bFGF in accordance therewith will now be described in more detail, starting with the preliminary purification of the heparan sulphate source material.

EXAMPLE

Preliminary Purification of HS

Generally applicable procedures for the extraction and purification of PGs and GACs have been described in two detailed reviews which cover methods for both connective tissue and cultured cells (Heinegard & Sommarin, 1987, *Methods in Enzymology*, 144, 319-372; Yanagashita *et al*, 1987, *Methods in Enzymology*, 138, 279-289), but a preferred procedure for the purpose of this example is now described for the purification of HS from skin fibroblast cells grown in culture.

Confluent cultures of fibroblasts are maintained at 37°C (CO_2/air, 1:19) in Eagle's minimal essential medium supplemented with 15% (v/v) donor-calf serum, 2mM-glutamine, 1mM-sodium pyruvate, non-essential amino acids,

penicillin (100 units/ml) and streptomycin (100µg/ml).

Cells can be harvested at confluence, after biosynthetic radiolabelling if necessary [by incubating for 72 hours with $Na^{35}SO_4$ (e.g. at 10-50µCi/ml) and/or [^{3}H]glucosamine (e.g. at 10-20µCi/ml)]. HS can be extracted from both the medium and the cell layer. The medium is removed and the cell layers washed twice with warm (37°C) phosphate-buffered saline (PBS). These combined solutions are centrifuged (200xg, 10 min) to pellet cells and other debris and the resulting supernatant constitutes the medium extract. HS is efficiently extracted from the cell layers by treatment with 0.05% (w/v) trypsin in PBS at 37°C for 30 min. The resulting cell suspension is centrifuged as above, and the supernatant removed carefully. After washing the pellet twice with PBS the combined supernatants constitute the cell layer trypsin extract.

The crude soluble extracts are subjected to initial purification by anion exchange chromatography. Samples in PBS are loaded onto a DEAE-Sephacel column (1cm x 5cm) and washed with 0.3M NaCl in 20mM phosphate buffer, pH 6.8, to elute contaminating proteins and hyaluronic acid. PGs and GAGs which remain bound are eluted with a gradient of 0.3-1.0M NaCl in 20mM phosphate bufffer. Fractions corresponding to HS (typically eluting at approximately 0.53M NaCl) are collected, pooled, desalted on a Sephadex G-25 column (2.5cm x 40cm) with distilled water as the eluant, and freeze dried. Traces of contaminating GAGs (e.g. chondroitin and dermatan sulphate) can be removed by treatment with 1 unit of chrondroitinase ABC for 3-4 at 37°C. Protein cores of HSPGs can then be removed by adding Pronase (5mg/ml final concentration) and calcium acetate (5mM final concentration) to the Chondroitinase ABC digest and digesting for 24 hours at 37°C. HS chains are recovered by step elution from DEAE-Sephacel with 1M NaCl after eluting contaminants with 0.3 NaCl. The

fractions containing HS are then heated at 100°C for 10 minutes, followed by either dialysis against distilled water (using Spectrapor 7 high purity dialysis membrane) or by desalting on a Sephadex G-25 column as above, followed by freeze drying.

Depolymerisation of HS to selectively produce sulphated oligosaccharides:

Biosynthetically ^{3}H and/or $^{35}SO_4$ labelled HS chains purified as above are treated with heparitinase, i.e. heparitinase I (EC 4.2.2.8) from Seikagaku Kogyo Co, Tokyo, Japan, to provide cleavage within regions of relatively low sulphation while leaving intact the more highly sulphated domains rich in N- and O-sulphate groups and iduronate residues. More specifically, a sample of freeze dried HS ($7x10^6$ dpm ^{3}H) is treated with heparitinase I (5 milli-units) in 200µl of 100mM Na acetate, pH 7.0, containing 0.2mM Ca acetate, at 37°C, for 16 hours, followed by addition of a further aliquot of 5 milli-units of the heparitinase I and incubation for 1 hour at 37°C. Digestion is typically complete in 3-4 hours, but should normally be continued for 16 hours in order to ensure complete cleavage of all heparitinase-susceptible linkages. Progress of the reaction can be monitored in the case of unlabelled HS by measuring the increase in absorbance at 282nm due to formation of 4,5-unsaturated hexuronate residues at the non-reducing ends of digestion products. Digestion is terminated by heating at 100°C for 2min.

An alternative chemical method for selective preparation of sulphated domains from HS is to specifically de-N-acetylate the polysaccharide, followed by specific cleavage at the resulting N-unsubstituted glucosamine residues. The methodological details have been described in detail previously (Shaklee and Conrad (1984), *Biochem. J.* 217, 187-197; Guo and Conrad (1989),

Analytical Biochemistry, 176, 96-104). Briefly, de-N-acetylation is carried out by hydrazinolysis by heating the sample at 96°C in 70% (w/v) aqueous hydrazine containing 1% (w/v) hydrazine sulphate, for approximately 4 hours. After drying in a stream of air the mixture is neutralized by addition of 500mM sulphuric acid. The sample is then subjected to deaminitive cleavage at pH 4.0 to specifically cleave at the resulting N-unsubstituted glucosamine residues. This method generates oligosaccharides which differ from those prepared by heparitinase treatment in that they terminate in intact hexuronate residues at their non-reducing ends and in 2,5-anhydromannose residues at their reducing ends. The latter residues are normally converted to their 2,5-annhydro-D-mannitol derivatives by reduction with $NaBH_4$ Radiolabel can be introduced into the oligosaccharides at this stage, if required, by using NaB^3H_4 as the reducing agent.

Fractionation of oligosaccharides by gel filtration:

The oligosaccharide products of the heparitinase (or chemical) treatment method are partially resolved on the basis of size by gel filtration chromatography, the result being individual peaks consisting of complex mixtures of oligosaccharides composed of defined numbers of disaccharide units, ranging in size from disaccharides upwards, each differing from the next by an increase in size of one complete disaccharide unit. For analytical purposes (e.g. sample loads up to approximately 10mg) columns (1 x 120cm or 1 x 240cm) packed with Bio-Gel P6 or Bio-Gel P10 (commercially available from Biorad Ltd.) are suitable, but for the separation of larger quantities the column diameter can be increased appropriately to allow scaling-up. Bio-Gel P10 is particularly suitable for separation of oligosaccharides larger than dp10 in size. The sample is loaded on to the top of the gel and eluted with 500mM NH_4HCO_3 at a flow rate of 4ml/hour. Fractions of 1ml are collected and a small aliquot taken from each for liquid scintillation counting if the HS has been

labelled. Alternatively, unlabelled HS oligosaccharides can be detected by measuring the absorbance at 232 nm, either of the individual fractions or continuously with a UV monitor. Fractions corresponding to oligosaccharide peaks of defined sizes (determined by previous calibration with standards) are pooled and freeze dried. Figure 8 shows a typical result for gel filtration of Bio-Gel P6 of the heparitinase digest of ^{3}H-labelled fibroblast HS described above.

Fractionation of oligosaccharides by bFGF affinity chromatography:

Individual peaks containing mixtures of oligosaccharides composed of defined numbers of disaccharide units are further fractionated on the basis of their affinity for bFGF. A description of the preparation of an analytical scale affinity matrix containing bFGF immobilized on Affi-Gel 10 has been described earlier. For use on a preparative scale, a similar procedure will be followed but the quantity of bFGF coupled and the amount of gel will be determined by the sample capacity required. The following description is of the use of bFGF-Affi-Gel 10 matrix for the separation of 3H-labelled HS oligosaccharides from the fibroblast HS of this particular example.

Approximately 1ml of bFGF-Affi-Gel 10 affinity matrix is packed into a glass column (bed dimensions 6mm x 35mm). Samples are loaded onto the column in 10mM Tris-HCl, pH 6.5, at a flow rate of 0.25 ml/min. Unbound material is eluted by collecting five 1ml fractions. Bound material is eluted with a gradient of sodium chloride (0-2M NaCl in column buffer) at a flow rate of 0.5 ml/min. This can be conveniently achieved by a discontinuous step gradient (e.g. increasing concentration of NaCl by steps of 250mM NaCl or other suitable increment). Five 1ml fractions are collected at each concentration. Alternatively, a linear continuous

gradient (e.g. with a total volume of 50ml) may be used to elute bound fragments, and 1ml fractions collected. A small aliquot is taken from each fraction for liquid scintillation counting (or detection by UV absorbance).

Figure 6 shows a typical result of the bFGF affinity chromatography of heparitinase-resistant oligosaccharides peaks of different sizes (dp2-dp14) prepared by Bio-Gel P6 gel filtration. Selected fractions containing oligosaccharides having the same affinities for bFGF are pooled, desalted either by dialysis against distilled water using Spectrapor 7 1000-Mr cut-off membrane (Pierce Ltd) and/or by again using gel filtration on a Bio-Gel P2 column (1.5cm x 30cm) eluted with 500mM NH_4HCO_3 at a flow rate of 10ml/hour, and freeze dried. The fractions of particular interest are those for dp=12 and dp=14.

Additional Purification:

Additional purification of the oligosaccharides with selected specific affinities for bFGF can be achieved by further steps of gel filtration chromatography and bFGF-affinity chromatography. This was carried out for example in the preliminary experimental work in respect of which Figure 2 illustrates the bFGF-affinity profiles of oligosaccharides (dp12 and dp14) selected for low (750mM fraction), medium (1000mM fraction) and high ($\geq$1250mM fraction) affinity for bFGF by an bFGF-affinity step following a second application to the affinity column. In addition, it is also possible if desired to further purify the oligosaccharides to apparent homogeneity by application of two additional techniques: strong anion exchange (SAX) HPLC which separates mainly according to anionic charge properties, and gradient polyacrylamide gel electrophoresis (PAGE) which separates predominantly according to molecular size.

Thus, to obtain increased purity of binding oligosaccharides by SAX HPLC chromatography, separations are

made on ProPac PA1 columns (from Dionex Ltd), either an analytical column (4 x 250mm) or alternatively a semi-preparative column (9 x 250mm). After equilibration in mobile phase (double distilled water adjusted to pH 3.5 with HCl) at 1ml/min samples are injected and oligosaccharides eluted with a linear gradient of sodium chloride (0-2M over 180 minutes) in the same mobile phase. The eluant is monitored in-line for UV absorbance (A_{282}) for detection of unlabelled oligosaccharides, and/or for radioactivity (e.g. using an in-line monitor such as a Radiomatic Flo-one/Beta A-200 detector, Canberra Packard Ltd).

Gradient PAGE methods have been described in detail in previous publications (e.g. Turnbull, J.E. & Gallagher, J.T. (1988) *Biochem. J.* 251, 597-608; Turnbull, J.E. & Gallagher, J.T. (1990) *Biochem. J.* 265, 715-724; Turnbull *et al* (1993), "Approaches to the structural analysis of GAGS" in *Extracellular Matrix Macromolecules: A Practical Approach*, Oxford University Press; Turnbull (1993), "Oligosaccharide mapping and sequence analysis of GAGs", in *Methods in Molecular Biology: Membrane Methods*, Humans Press, Chapter 24). This methodology provides a very powerful technique for resolving complex mixtures of large oligosaccharides into single apparently homogeneous species, and it can be adapted to preparative scale for the separation of large quantities of oligosaccharides, either by eluting directly from the gel using appropriate apparatus or by electrotransfer from the gel onto a positively-charged nylon membrane, followed by recovery from the membrane by elution with salt as described in the above references.

Various combinations of these techniques described can thus enable a very high degree of final purification of homogeneous preparations of well defined oligosaccharide products in accordance with the invention which have specific sequences and defined affinities for growth

factors such as bFGF.

Insofar as the basic features of oligosaccharide sequences have been identified and characterised that give rise to a specific FGF binding affinity, which in the case of oligo-H can be of the same order as in heparan sulphate, it will be appreciated that this knowledge can also enable such oligosaccharides and analogues thereof having like binding affinities now to be made or constructed synthetically and they may be "tailor made" to suit requirements using conventional synthetic methods. For example, insofar as these compounds can be regarded as being built up of three types of main disaccharide units, precursors of these units designated A, B and C, which are to be arranged as A—[B]$_n$—C, may be separately synthesised with O-acetyl or O-methylchloroacetonyl (OMCA) protected terminal groups, e.g. MCAO—B—OAC, A—OAC and MCAO—C. The MCAO and OAC groups can then be converted selectively to —OH groups, e.g. by pyridine and hydrazine respectively, to enable firstly the required number of B units to be coupled together followed by the selective coupling of the required terminal units to build up the chain, and the structure produced can then be subjected to deacylation, O-sulphation, hydrogenolysis and N-sulphation as necessary to give the final product. For a general review to synthetic methods, reference may be made to "Heparin", edited by D.A. Lane and V. Lindahl, page 51 onwards.

THERAPEUTIC USES

In general, for therapeutic use of the oligosaccharide products of the present invention and administration to mammals in need of treatment, an effective growth factor binding amount of the active oligosaccharide, which may be in the form of a pharmaceutically acceptable salt, will be made up as a pharmaceutical formulation ready for administration in any suitable manner, for example orally, parenterally (including subcutaneously, intramuscularly and intravenously), or topically, or in a slow-release dispensing device for implantation. Such formulations may be presented in unit dosage form and may comprise a pharmaceutical composition, prepared by any of the methods well known in the art of pharmacy, in which the active oligosaccharide component or components is in intimate association or admixture with at least one other ingredient providing a compatible pharmaceutically acceptable carrier, diluent or excipient. Alternatively, such formulations may comprise a protective envelope of compatible or relatively inert pharmaceutically acceptable material within which is contained the active oligosaccharide component or components with or without association or admixture with any other ingredients.

It may be noted that for pharmaceutical use, it may be preferable for the oligosaccharides of the present invention to be in the form in which their non-reducing ends are unsaturated, as obtained by heparitinase scission, since there is some evidence that this form may be more resistant to bio-transformation which could reduce efficiency. However, this may not be essential for all applications.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the active component,

with capsules being a preferred type of formulation for providing the most effective means of oral delivery. For parenteral administration the formulations may comprise sterile liquid preparations of a predetermined amount of the active oligosaccharide component contained in sealed ampoules ready for use.

The amount of the oligosaccharide products of the invention, and dosing regimen required for effective therapeutic use will of course vary and will be ultimately at the discretion of the medical or veterinary practitioner treating the mammal in each particular case. The factors to be considered by such a practitioner, e.g. a physician, include not only the particular disorder being treated (and whether growth factor stimulation or growth factor inhibition is required) but also the route of administration and type of pharmaceutical formulation; the mammal's body weight; surface area, age and general condition. However, a suitable effective bFGF inhibitory dose, e.g. for antitumour treatment, might perhaps be in the range of about 1.0 to about 75 mg/kg bodyweight, preferably in the range of about 5 to 40 mg/kg with most suitable doses being for example in the range of 10 to 30 mg/kg. In daily treatment for example, the total daily dose may be given as a single dose, multiple doses, e.g. two to six times per day, or by intravenous infusion for any selected duration. For example, for a 75 kg mammal, the dose range could perhaps be about 75 to 500 mg per day, and a typical dose would commonly be about 100 mg per day. If discrete multiple doses are indicated, treatment might typically be 50mg of an oligosaccharide product, as hereinbefore defined, given 4 times per day in the form of a tablet, capsule, liquid (e.g. syrup) or injection.

As previously indicated, in some cases where the treatment required consists in administering a growth factor such as bFGF, for example to promote tissue repair as in wound healing applications, the active oligo-

saccharide component may be co-administered with the growth factor.

Apart from their use in conjunction with the administration of growth factors in wound healing applications and in other medical applications where it is desired to increase or stimulate growth factor activity, e.g. bone healing, nerve regeneration, duodenal or venous ulcers, various ocular and retinal disorders, atherosclerosis, degenerative muscle disorders, ischaemia, or for protecting tissues against serious damage during radiation treatment, the medical uses of the oligosaccharide compounds or products of the present invention will probably be most frequently targetted to the inhibition of growth factor activity, and pharmaceutical formulations or compositions containing these oligosaccharides are expected to be especially useful, as previously indicated, for treating conditions that arise, or are aggravated, as a result of activity of growth factors promoting harmful growth or cell proliferation, e.g. conditions, such as diabetic retinopathy, capsular opacification, proliferative vitreoretinopathy, tumour angiogenesis, cancer cell growth and metastasis, rheumatoid arthritis, mild muscular dystrophy, Alzheimer disease, various viral infections (e.g. Herpes Simplex type 1), or restenosis following angioplasty and other forms of chronic inflammation.

As will be seen, the invention provides a number of different aspects and, in general, it embraces all novel and inventive features and aspects, including novel compounds, herein disclosed either explicitly or implicitly and either singly or in combination with one another. Moreover, the scope of the invention is not to be construed as being limited by the illustrative examples or by the terms and expressions used herein merely in a descriptive or explanatory sense.

CLAIMS

1. An oligosaccharide product having a specific binding affinity for fibroblast growth factors (FGF's), characterised in that it consists essentially of oligosaccharide chains which are substantially homogeneous with respect to FGF binding affinity and which contain at least six disaccharide units including a contiguous sequence of sulphated disaccharide units that are each composed of an N-sulphated glucosamine residue (±6S) and a 2-O-sulphated iduronic acid residue.

2. An oligosaccharide product as claimed in Claim 1 in which each of said sulphated disaccharide units is IdoA(2S)-α1,4-GlcNSO$_3$.

3. An oligosaccharide product as claimed in Claim 1 or 2 in which said oligosaccharide chains consist of a sequence of less than ten disaccharide units.

4. An oligosaccharide product as claimed in any of Claims 1 to 3, further characterised in that it is substantially completely resistant to depolymerisation by heparitinase but not by heparinase.

5. An oligosaccharide product as claimed in any of Claims 1 to 4 in which at least the predominating majority of said oligosaccharide chains are all of the same length.

6. An oligosaccharide product as claimed in any of the preceding claims in which substantiaaly all said oligosaccharide chains consist of a sequence of six disaccharide units in all.

7. An oligosaccharide product as claimed in any of the preceding claims in which said oligosaccharide chains include a contiguous sequence of at least four said sulphated disaccharide units.

8. An oligosaccharide product as claimed in any of Claims 1 to 4 in which substantially all the oligosaccharide chains consist of a sequence of seven disaccharide units of which at least five are included in said contiguous sequence of sulphated disaccharide units.

9. An oligosaccharide product as claimed in any of the preceding claims in which the content (if any) of glucosamine residues in the oligosaccharide chains which are O-sulphated at C6 is less than 20%.

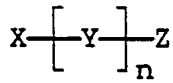
10. An oligosaccharide product as claimed in Claim 9 in which the content (if any) of glucosamine residues in the oligosaccharide chains which are O-sulphated at C6 is less than 5%.

11. An oligosaccharide product as claimed in any of Claims 1 to 10 further characterised in that it is obtainable from heparan sulphate (HS) of human fibroblast heparan sulphate proteoglycan (HSPG) by enzymic partial depolymerisation to the fullest extent with heparitinase followed by size fractionation, using for example gel filtration size exclusion chromatography, followed by, in respect of a selected fraction or fractions recovered from the size fractionating stage, affinity chromatography using an FGF growth factor as the immobilised ligand in order to separate out the FGF-binding fragments, and then eluting selectively over a range of salt concentrations under a salt gradient, advantageously a serially stepped gradient, to fractionate said fragments in respect of FGF binding affinity, followed by recovering the most strongly bound fragments and, optionally, further purifying the recovered product by carrying out at least one additional step of size fractionation and selection of recovered product.

12. An oligosaccharide product having a specific binding affinity for fibroblast growth factors (FGF's),

characterised in that

(a) it is composed predominantly of a molecular species:



in which

X is ΔHexA-GlcNSO$_3$(±6S),
Y is IdoA(2S)-GlcNSO$_3$(±6S),
Z is IdoA-GlcR(±6S) or
IdoA(2S)-GlcR(±6S)
where R is NSO$_3$ or NAc, and
n is in the range 4 to 7

(b) the content, if any, of monosaccharide residues having a 6-O-sulphate group is less than 20%;

(c) it is obtainable by a process comprising the steps of digesting a heparan sulphate with heparitinase so as to bring about partial depolymerisation thereof to the fullest extent, followed by size fractionating the oligosaccharide mixture produced using for example gel filtration size exclusion chromatography, collecting a fraction or fractions containing oligosaccharide chains having a particular size selected within the range of 12 to 18 monosaccharide residues, then subjecting said selected fraction or fractions to affinity chromatography using an immobilised FGF ligand and recovering the more strongly FGF-binding constituents by eluting under a salt gradient over a range of salt concentrations and collecting a selected fraction or fractions containing the bound material which desorbs only at the highest salt concentrations.

13. An oligosaccharide product as claimed in Claim 12, wherein Y is exclusively IdoA(2S)-GlcNSO$_3$.

14. An oligosaccharide product as claimed in Claim 12 or 13, wherein n is 5 or 6.

15. An oligosaccharide product as claimed in Claim 14 wherein said molecular species consists of a total of seven disaccharide units in all.

16. An oligosaccharide product as claimed in Claim 12 or 13 wherein n is 4 and the total number of disaccharide units in said molecular species is 6.

17. An oligosaccharide product as claimed in any of Claims 12 to 16 in which the content, if any, of residues having a 6-O-sulphate group is less than 5%.

18. An oligosaccharide product having a specific binding affinity for fibroblast growth factors (FGF's) that is substantially all composed of oligosaccharide chains which are fourteen monosaccharide residues in length and which contain an internal contiguous sequence of 5 or 6 disaccharide units each consisting of an IdoA(2S) residue linked to a $GlcNSO_3(\pm 6S)$ residue, with less than 20% of the glucosamine residues (terminal or internal) being 6-O-sulphated.

19. An oligosaccharide product as claimed in Claim 18 wherein the oligosaccharide chains have sequences selected from

$(\Omega)GlcA\text{-}GlcNSO_3\text{-}[IdoA(2S)\text{-}GlcNSO_3]_5\text{-}IdoA\text{-}GlcR$

and

$(\Omega)GlcA\text{-}[GlcNSO_3\text{-}IdoA(2S)]_6\text{-}GlcR$

where R is NSO_3 or NAc.

20. An oligosaccharide product having a specific binding affinity for fibroblast growth factors (FGF's) that is substantially all composed of oligosaccharide chains which are twelve monosaccharide residues in length and which contain an internal contiguous sequence of 4 disaccharide

units each consisting of an IdoA(2S) residue linked to $GlcNSO_3$(±6S) residue, with less than 20% of the glucosamine residues (terminal or internal) being 6-O-sulphated.

21. An oligosaccharide product as claimed in Claim 20 wherein the predominant oligosaccharide chain sequence is
(∩)GlcA-$GlcNSO_3$(±6S)-[IdoA(2S)-$GlcNSO_3$]$_4$-IdoA-GlcR(±6S)
where R is NSO_3 or NAc.

22. An oligosaccharide product having a relatively high specific binding affinity for basic fibroblast growth factor (bFGF) and consisting essentially of oligosaccharide chains having a disaccharide sequence

∩GlcA-β1,4-$GlcNSO_3$-α1,4-[IdoA(2S)-α1,4-$GlcNSO_3$]$_5$-α1,4-IdoA-α1,4-GlcR

or ∩GlcA-[$GlcNSO_3$-IdoA(2S)]$_6$-GlcR

where R is NSO_3 or NAc

or minor variants thereof which have at least the same relatively high specific binding affinity for bFGF.

23. A method of isolating from a glycosaminoglycan such as heparan sulphate small oligosaccharides in a purified and relatively homogeneous state which have a specific binding affinity for a selected bioactive protein or polypeptide that itself binds to said glycosaminoglycan or to the corresponding proteoglycan in multicellular biological systems, said method comprising the steps of

(a) preparing an affinity chromatographic matrix or substrate incorporating a sample of said protein or polypeptide as the affinity ligand immobilised thereon;

(b) treating said glycosaminoglycan with a selective scission reagent so as to cleave the polysaccharide chains thereof selectively in regions of relatively low sulphation;

(c) subjecting the product of step (b) to size

fractionation,, for example by gel filtration size exclusion chromatography, and collecting selectively therefrom fractions that appear to contain oligosaccharides composed of less than ten disaccharide units,

(d) contacting the affinity chromatographic matrix or substrate from step (a) with a selected fraction, or set of fractions, from step (c) containing a specific number of disaccharide units in the range of four to nine in order to extract from the latter and retain on said matrix or substrate size selected oligosaccharide fragments of the glycosaminoglycan that have at least some binding affinity for the immobilised said protein or polypeptide;

(e) eluting the affinity chromatographic matrix or substrate using a progressively increasing salt concentration or gradient in the eluant;

(f) collecting the fraction or set of fractions containing oligosaccharide fragments eluting in selected highest ranges of eluant salt concentration; and optionally,

(g) further purifying the product of the selected fraction, or set of fractions, from step (f) by selectively repeating step (c) using said selected fraction or set of fractions collected in step (f) instead of the reaction mixture obtained from step (b), and optionally also repeating steps (d), (e) and (f).

24. A method as claimed in Claim 23, wherein the glycosaminoglycan is heparan sulphate derived from heparan sulphate proteoglycan of mammalian cells and the selected bioactive protein or polypeptide is a cytokine or growth factor that when activated under physiological conditions stimulates mammalian cells through binding interaction with signal transducing receptors on the surface of said cells.

25. A method as claimed in Claim 24 in which the selective scission reagent is heparitinase and the heparan sulphate is partially depolymerised to the fullest extent by digesting therewith until cleavage of the heparitinase sensitive linkages is complete.

26. A method as claimed in Claim 24 wherein the selective scission reagent is nitrous acid which, after prior treatment of the heparan sulphate with an N-deacetylating agent, is reacted at about pH 4 with the polysaccharide to cleave it at the free amino groups therein.

27. A method as claimed in any of Claims 23 to 26 wherein the bioactive protein or polypeptide selected is a fibroblast growth factor (FGF).

28. A method as claimed in any of Claims 23 to 26 wherein the selected bioactive protein or polypeptide is basic fibroblast growth factor (bFGF).

29. A method as claimed in any of Claims 23 to 28, wherein the fractions collected from the size fractionation stage are those that appear to contain oligosaccharides composed of seven disaccharide units.

30. A method as claimed in any of Claims 23 to 28 wherein the fractions collected from the size fractionation stage are those that appear to contain oligosaccharides composed of six disaccharide units.

31. An oligosaccharide product as claimed in any one of Claims 1 to 22 for therapeutic use as an active FGF-activity stimulating agent for promoting healing or tissue repair in treating mammals in need of such treatment, for example in conditions such as wound healing, bone healing, nerve regeneration, duodenal or venous ulcers, various ocular and retinal disorders, atherosclerosis,

disorders, ischaemia, or for protecting tissues against serious damage during radiation treatment.

32. A medical composition comprising the oligosaccharide product of Claim 31 in association with an exogenous FGF growth factor for co-administration therewith in carrying out the treatment therein referred to.

33. An oligosaccharide product as claimed in any one of Claims 1 to 22 for therapeutic use as an active FGF-activity inhibiting agent for controlling or reducing cell growth or proliferation in treating mammals in need of such treatment, for example in connection with conditions such as diabetic retinopathy, capsular opacification, proliferative vitreoretinopathy, tumour angiogenesis, cancer cell growth and metastasis, rheumatoid arthritis, mild muscular dystrophy, Alzheimer disease, various viral infections (e.g. Herpes Simplex type 1), or restenosis following angioplasty.

34. A pharmaceutical formulation or composition for medical use comprising a therapeutically effective non-toxic amount of an FGF-activity modulating agent comprising an oligosaccharide product as claimed in any of Claims 1 to 22 or pharmaceutically acceptable salts thereof, together with a pharmaceutically acceptable carrier or vehicle.

35. Use of an oligosaccharide product as claimed in any of Claims 1 to 22, for the manufacture of a medical preparation for use in the treatment of diabetic retinopathy, capsular opacification, proliferative vitreoretinopathy, tumour angiogenesis, cancer cell growth and metastasis, rheumatoid arthritis, mild muscular dystrophy, Alzheimer disease, various viral infections (e.g. Herpes Simplex type 1), or restenosis following angioplasty or for use in promoting repair of damaged tissues in conditions such as wound healing, bone healing,

tissues in conditions such as wound healing, bone healing, nerve regeneration, duodenal ulcers, various ocular and retinal disorders, atherosclerosis, degenerative muscle disorders, ischaemia, or for protecting tissues against serious damage during radiation treatment.

36. An oligosaccharide product having a specific binding affinity for fibroblast growth factors (FGF's), consisting essentially of oligosaccharide chains which are substantially homogeneous with respect to FGF binding affinity and which contain a sequence of less than ten disaccharide units including, intermediate its terminal residues, a plurality of sulphated disaccharide units that are each composed of an N-sulphated glucosamine residue (±6S) and a 2-O-sulphated iduronic acid residue.

37. A pharmaceutical composition or formulation for use in controlling the activity of fibroblast growth factors in mammals for promoting tissue repair or for inhibiting cell growth or proliferation in the treatment of disorders resulting therefrom, said composition or forumulation comprising a therapeutically useful and therapeutic amount of an essentially pure oligosaccharide product as claimed in Claim 36.

38. A method for treating a mammal to promote healing or tissue repair in the case of wounds, duodenal or venous ulcers, various ocular and retinal disorders, atherosclerosis, degenerative muscle disorders, or ischaemia, for promoting bone healing or nerve regeneration, or for protecting tissues against serious damage during radiation treatment, which comprises administering to a mammal in need of such treatment an effective amount of an essentially pure oligosaccharide product as claimed in Claim 36 or in any of Claims 1 to 22.

39. A method as claimed in Claim 38 in which the

oligosaccharide product is co-administered with a preparation of bFGF.

40. A method of treating diabetic retinopathy, capsular opacification, proliferative vitreoretinopathy, tumour angiogenesis, cancer cell growth and metastasis, rheumatoid arthritis, mild muscular dystrophy, Alzheimer disease, various viral infections (e.g. Herpes Simplex type 1), or restenosis following angioplasty in mammals by inhibiting FGF growth factor activity, which method comprises administering to a mammal in need of such treatment an effective amount of an essentially pure oligosaccharide product as claimed in Claim 36 or in any of Claims 1 to 22.

FIG.1.

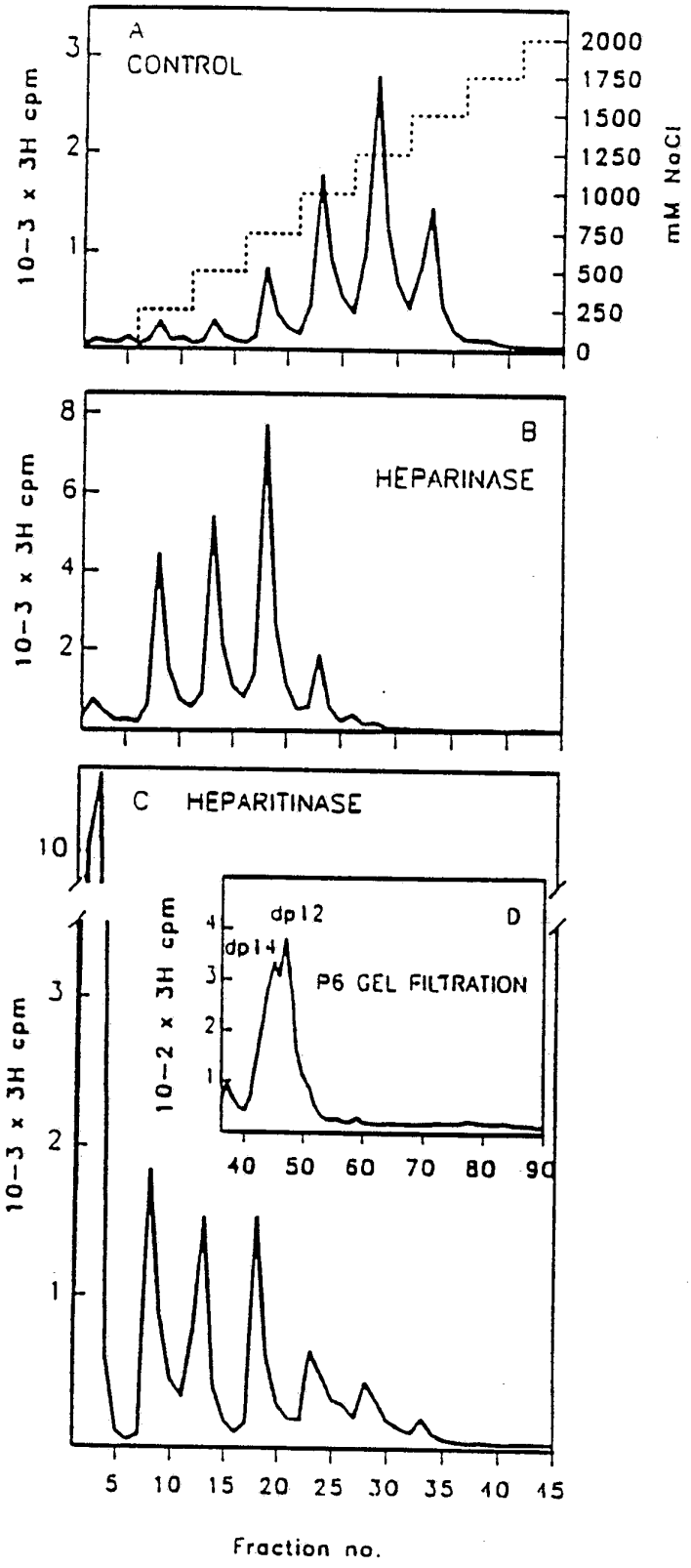

A
CONTROL
10-3 x 3H cpm
mM NaCl
B
HEPARINASE
C HEPARITINASE
D
dp12
dp14
P6 GEL FILTRATION
10-2 x 3H cpm
Fraction no.

FIG.2.

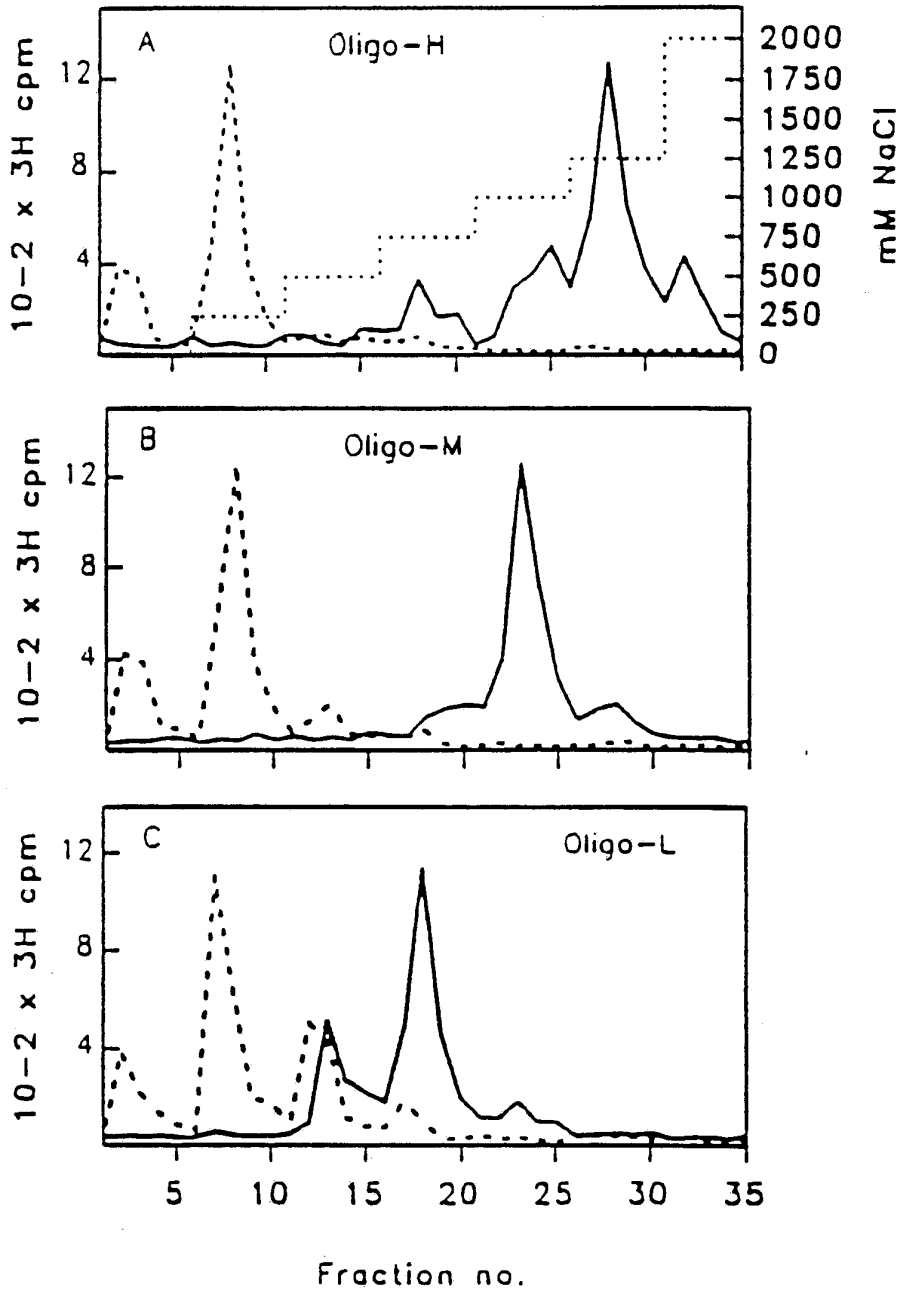

A
Oligo-H
B
Oligo-M
C
Oligo-L
10-2 x 3H cpm
12
8
4
2000
1750
1500
1250
1000
750
500
250
0
mM NaCl
5
10
15
20
25
30
35
Fraction no.

FIG.3.

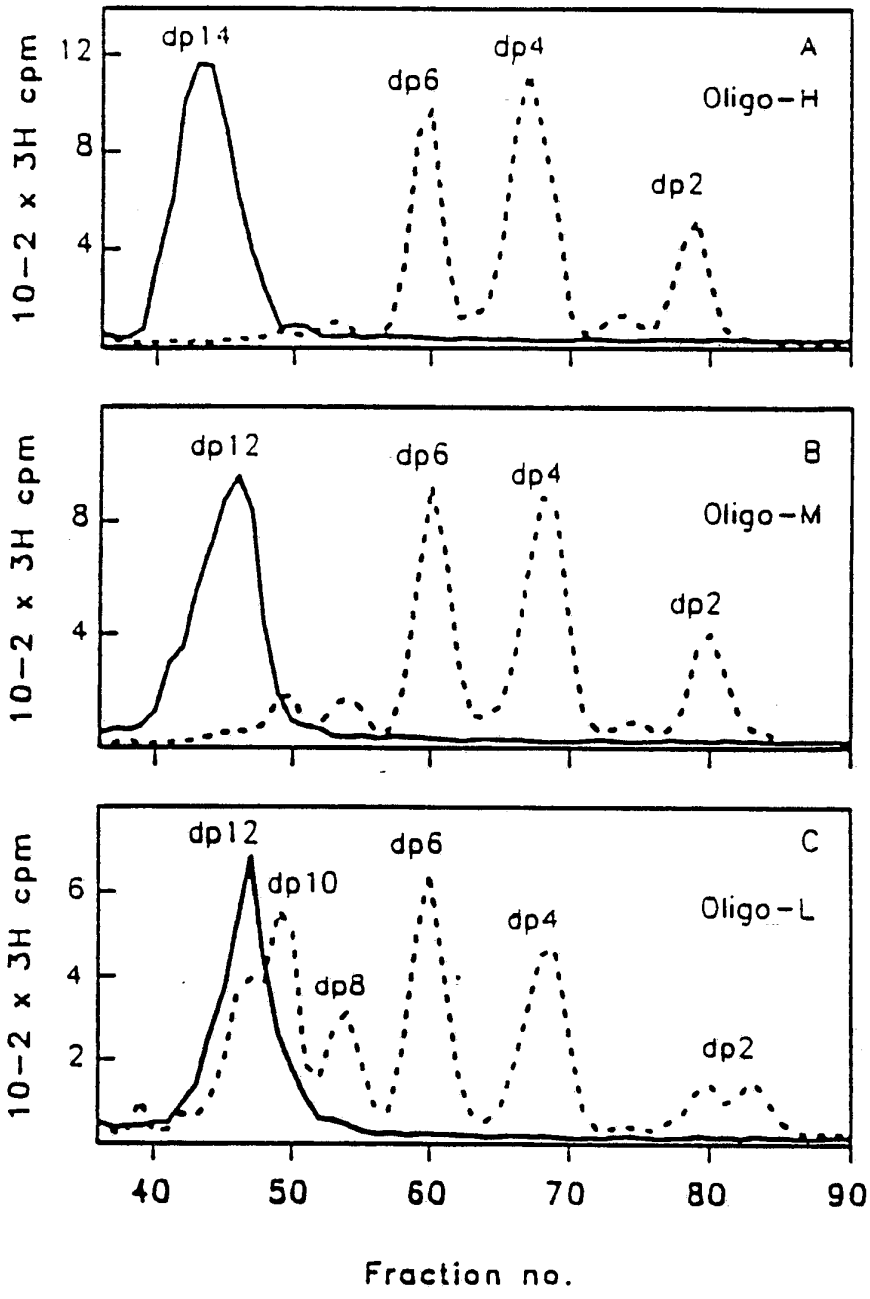

10−2 x 3H cpm
dp14
dp6
dp4
dp2
A
Oligo−H
dp12
dp6
dp4
dp2
B
Oligo−M
dp12
dp10
dp8
dp6
dp4
dp2
C
Oligo−L
40
50
60
70
80
90
Fraction no.

FIG.4.

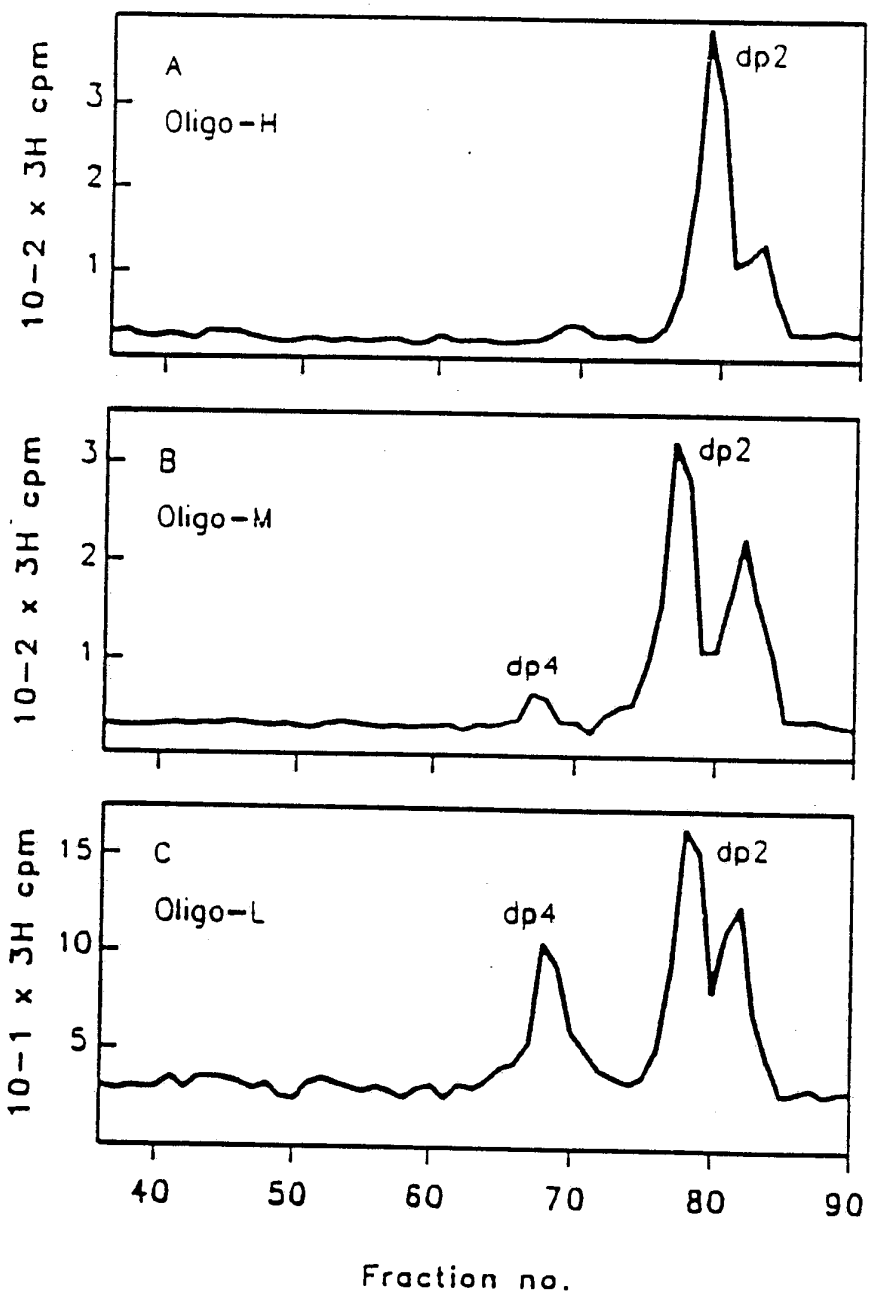

A
Oligo-H
dp2
10-2 x 3H cpm
3
2
1
B
Oligo-M
dp2
dp4
10-2 x 3H cpm
3
2
1
C
Oligo-L
dp2
dp4
10-1 x 3H cpm
15
10
5
40
50
60
70
80
90
Fraction no.

FIG. 5.

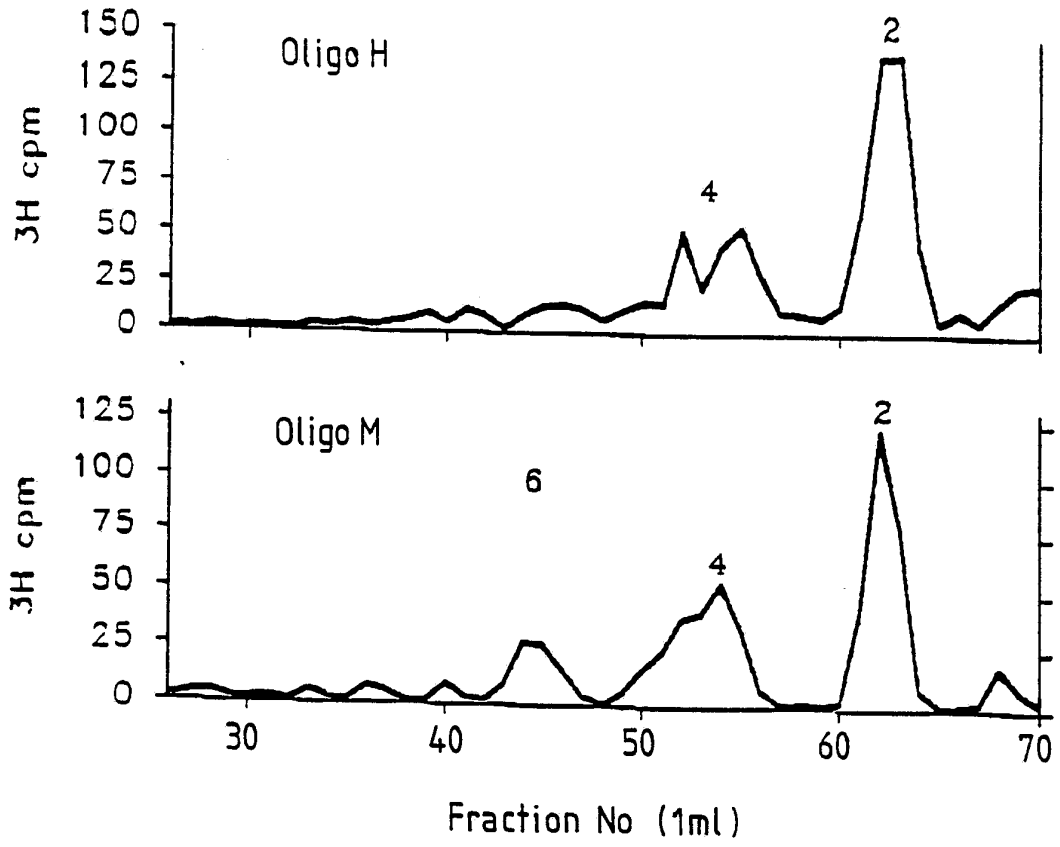

Oligo H
Oligo M
3H cpm
150
125
100
75
50
25
0
2
4
6
30
40
50
60
70
Fraction No (1ml)

FIG.6.

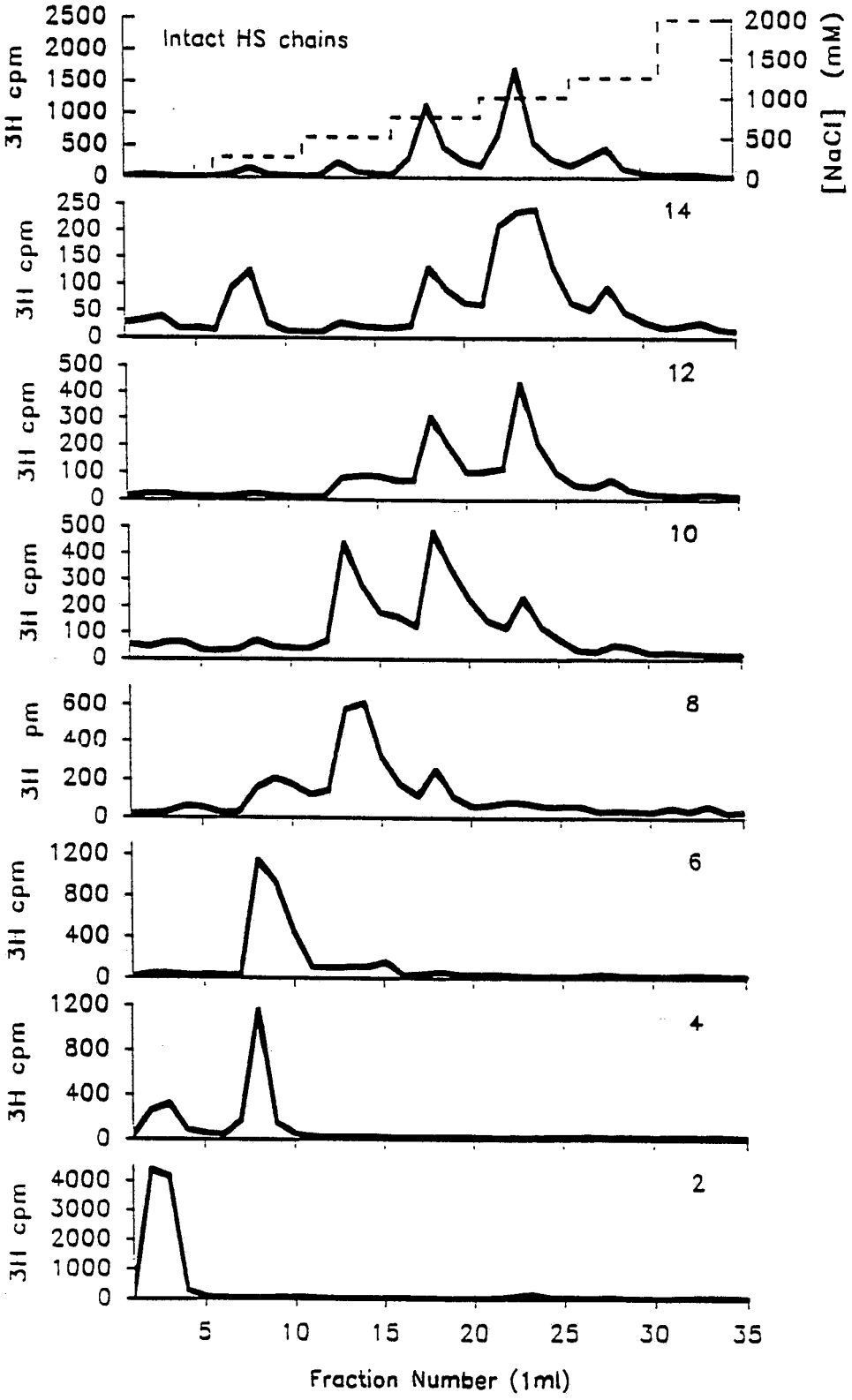

Intact HS chains
3H cpm
[NaCl] (mM)
14
12
10
8
6
4
2
3H pm
Fraction Number (1ml)

FIG.7A.

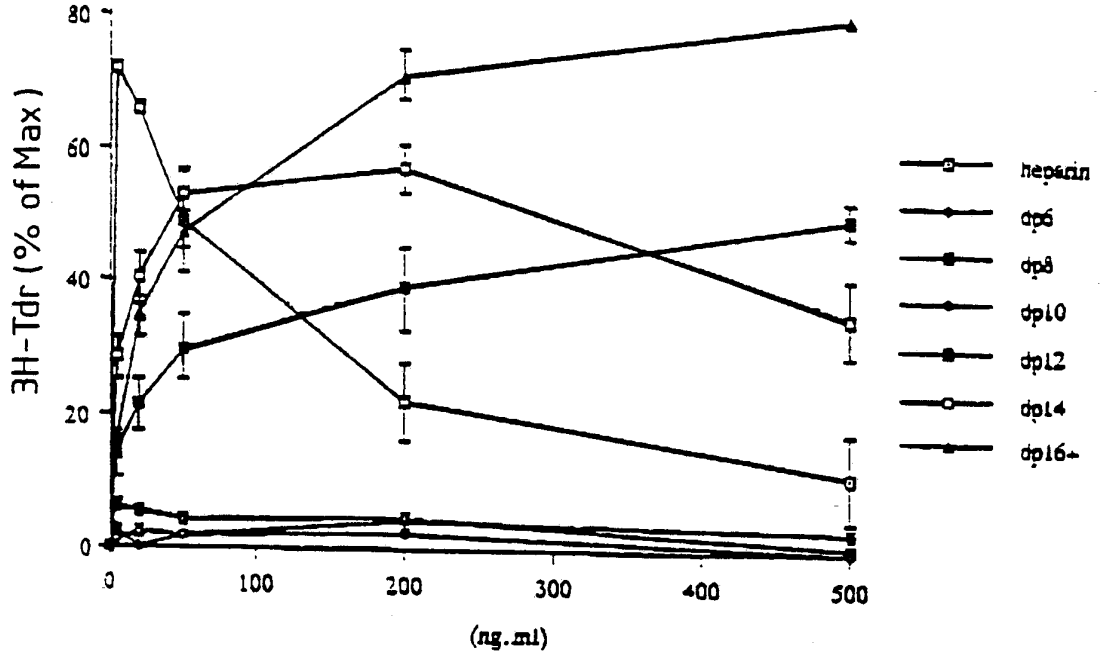

3H-Tdr (% of Max)
80
60
40
20
0
0
100
200
300
400
500
(ng.ml)
Heparin
dp6
dp8
dp10
dp12
dp14
dp16+

FIG.7B.

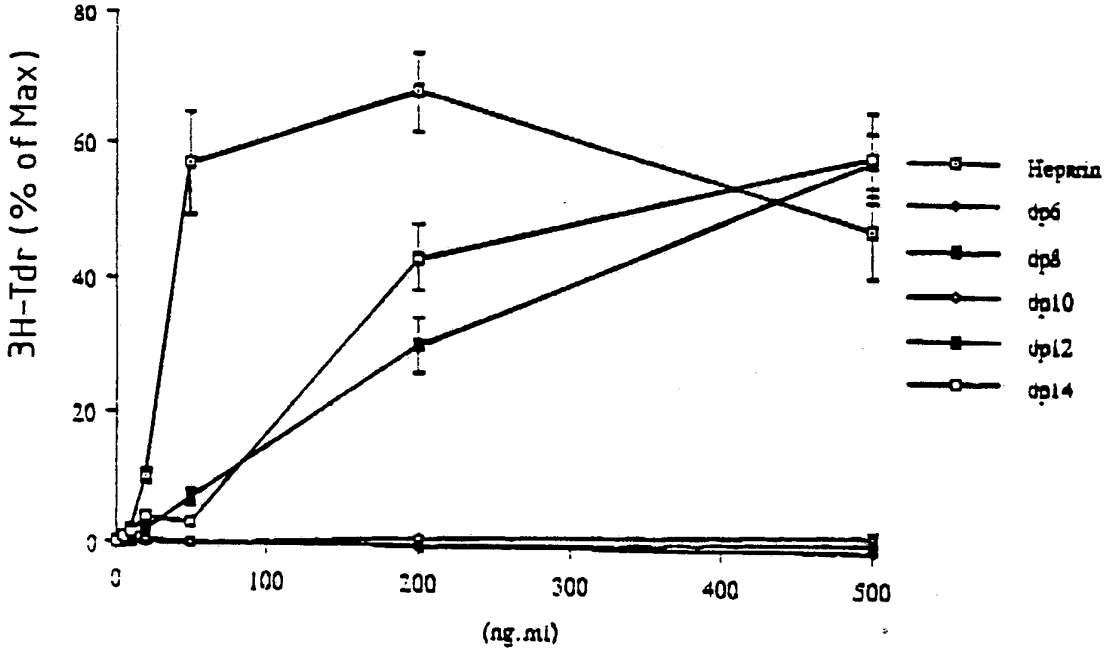
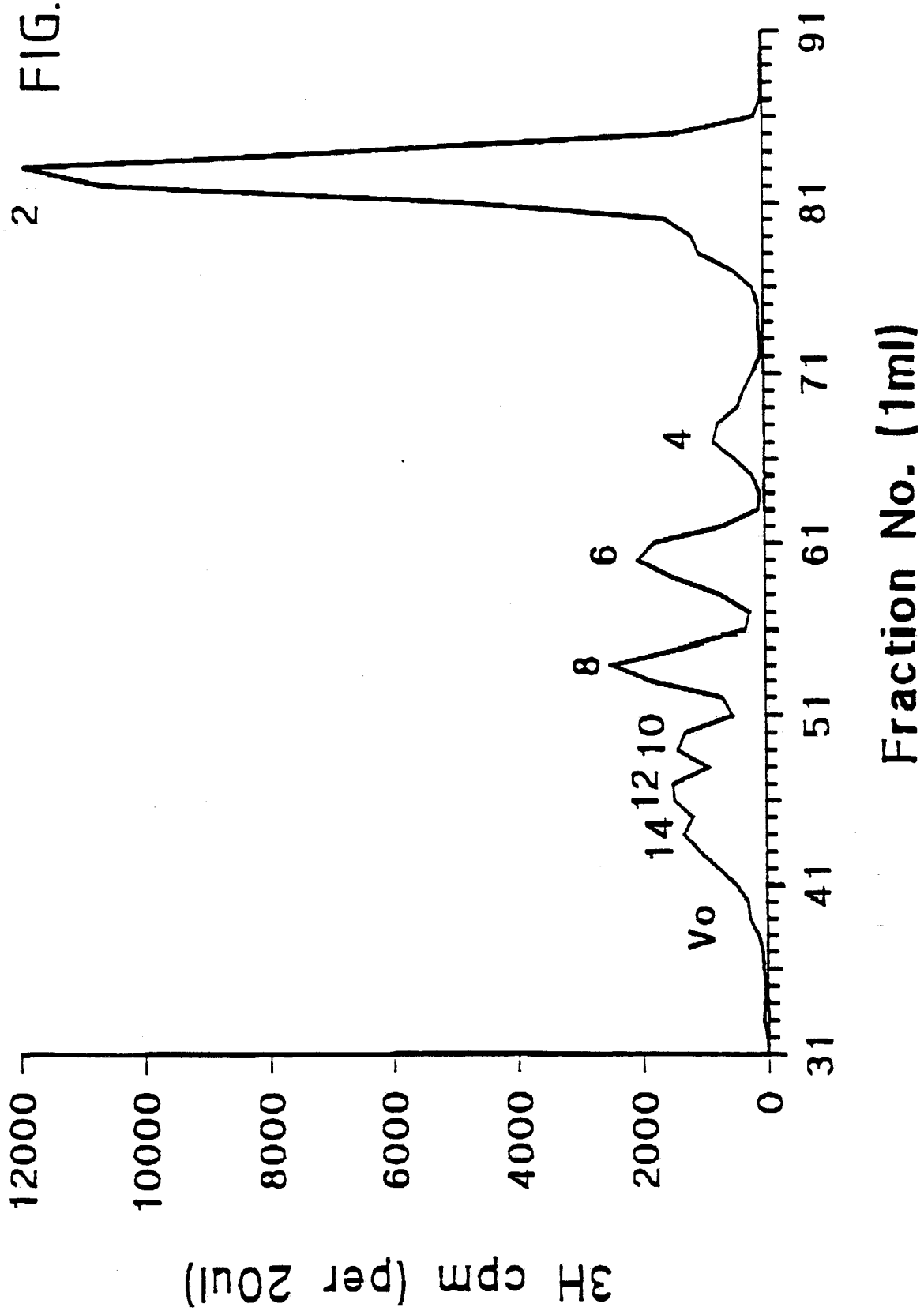

3H-Tdr (% of Max)
80
60
40
20
0
0
100
200
300
400
500
(ng.ml)
Heparin
dp6
dp8
dp10
dp12
dp14

FIG.8.


3H cpm (per 20ul)
12000
10000
8000
6000
4000
2000
0
Fraction No. (1ml)
31
41
51
61
71
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INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00597

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

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

Int.Cl. 5 C08B37/10; A61K31/725

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C08B ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
P,X	EP,A,0 509 517 (SEIKAGAKU KOGYO KABUSHIKI) 21 October 1992 see page 2, line 1 - line 6 see page 3, line 37 - line 39 see page 9, line 12 - line 13 see claims; example --- -/--	1,3, 31-37

° Special categories of cited documents : [10]

"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.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 JULY 1993	05. 08. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MAZET J.-F.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	EP,A,0 244 298 (SANOFI) 4 November 1987 see page 1, line 1 - line 7 see page 2, line 5 - line 23 see page 3, line 4 - line 15 see page 4, line 1 - line 18 see page 5, line 3 - line 17 see page 8, line 23 - line 33 see page 10, line 25 - page 11, line 5 see page 13, line 33 - page 14, line 9 see page 16, line 7 - line 10 see claims	1,11-13, 23-28, 31-37
A	EP,A,0 394 971 (KABI VITRUM AB) 31 October 1990 see page 2, line 1 - line 5 see page 3, line 1 - line 5 see page 3, line 47 - page 4, line 2 see claims; examples 1,7	1,11, 23-26, 31,33, 35,36
A	EP,A,0 014 184 (KABI AB) 6 August 1980 see claims	1,12,18, 20,22
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 267, no. 15, 25 May 1992, BALTIMORE, MD US pages 10337 - 10341 J.E TURNBULL ET AL. 'Identification of the basic fibroblast growth factor binding sequence fibroblast heparan sulfate'	1-30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB93/00597

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
38,39,40 Method for treatment of the human or animal body by therapy (E.P.C. article 52(4)).

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9300597 SA 72606

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/07/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0509517	21-10-92	AU-A- 1500392	11-03-93
		JP-A- 5148305	15-06-93
EP-A-0244298	04-11-87	FR-A- 2597484	23-10-87
		JP-A- 63066192	24-03-88
		US-A- 5034520	23-07-91
EP-A-0394971	31-10-90	AU-A- 5445290	16-11-90
		CA-A- 2053883	25-10-90
		WO-A- 9012580	01-11-90
EP-A-0014184	06-08-80	AT-T- 8998	15-09-84
		CA-A- 1136620	30-11-82
		WO-A- 8001383	10-07-80
		SU-A- 1209033	30-01-86
		US-A- 4303651	01-12-81