(CONVENTION. By one or more persons and/or a Company.) Form 4

603764

COMMONWEALTH OF AUSTRALIA

Patents Act 1952-1969

CONVENTION APPLICATION FOR A PATENT

(1) Here insert (in full) Name or Names of Applicant or Applicants, followed by Address (es).

I/We (1) BEHRINGWERKE AKTIENGESELLSCHAFT,

of D-3550 Marburg 1, Federal Republic of Germany

(2) Here insert Title of Invention.

hereby apply for the grant of a Patent for an invention entitled: (2)

PROCESS FOR THE PREPARATION OF A MATERIAL FOR AFFINITY CHROMATOGRAPHY

PATENT OFFICE
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(3) Here insert number(s) of basic application(s)

which is described in the accompanying complete specification. This application is a Convention application and is based on the application numbered (3)

P35 19 011.6

(4) Here insert Name of basic Country or Countries, and basic date or dates

for a patent or similar protection made in (4) Federal Republic of Germany on 25th May 1985

APPLICATION ACCEPTED AND AMENDMENTS
Accepted 31 · 8 · 90

~~My~~ Our address for service is Messrs. Edwd. Waters & Sons, Patent Attorneys, 50 Queen Street, Melbourne, Victoria, Australia.

DATED this 22nd day of May 19 86.

(5) Signature (s) of Applicant (s) or Seal of Company and Signatures of its Officers as prescribed by its Articles of Association.

(5) BEHRINGWERKE AKTIENGESELLSCHAFT

by James Murray

Reg'd. Patent Attorney

To:

THE COMMISSIONER OF PATENTS.

Edwd. Waters & Sons, Melbourne.

COMMONWEALTH OF AUSTRALIA

Form 7

Patents Act 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION UNDER PART XVI. FOR A PATENT.

In support of the Convention application made under Part XVI. of the Patents Act 1952 by BEHRINGWERKE AKTIENGESELLSCHAFT of Marburg/Lahn, Federal Republic of Germany

for a patent for an invention entitled:

PROCESS FOR THE PREPARATION OF A MATERIAL FOR AFFINITY CHROMATOGRAPHY

We, of

Dr. Dietmar O. Schütze 25 Vogelsberg, D-3550 Marburg-Cappel

Dr. Philipp Stein 28 Höhenweg, D-3550 Marburg

do solemnly and sincerely declare as follows:

1. We are authorized by BEHRINGWERKE AKTIENGESELLSCHAFT the applicant for the patent to make this declaration on its behalf.

2. The basic application as defined by Section 141 of the Act was made at München in the Federal Republic of Germany under P 35 19 011.6

on the 25th day of May 19 85 by BEHRINGWERKE AKTIENGESELLSCHAFT

3. a) Werner Stüber, 12 Cölber Weg, D-3551 Lahntal
b) Eric-Paul Pâques, 18 Schmiedeacker, D-3550 Marburg
a) - b) Federal Republic of Germany

~~X~~/are the actual inventor(s) of the invention and the facts upon which BEHRINGWERKE AKTIENGESELLSCHAFT is entitled to make the application are as follows:

The said BEHRINGWERKE AKTIENGESELLSCHAFT is the assignee of the said Werner Stüber, Eric-Paul Pâques

4. The basic application referred to in paragraph 2 of this Declaration was the first application made in a Convention country in respect of the invention the subject of the application.

DECLARED at Marburg/Lahn, Federal Republic of Germany

this 20th day of March 1986

To the Commissioner of Patents

BEHRINGWERKE AKTIENGESELLSCHAFT

(Schütze) Director

(ppa. Stein) Prokurist

(12) PATENT ABRIDGMENT (11) Document No. AU-B-57851/86
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(56) Prior Art Documents
EP 110409
GB 2006642
US 4308254

(57) Claim

1. A process for the preparation of a sulfated polysaccharide which is bonded to an insoluble polymer carrier which contains hydroxyl groups and amino groups which comprises treating a sulfated polysaccharide with an alkali metal periodate which oxidizes glycols to aldehydes, thereby cleaving the carbon chain, and reacting the modified sulfated polysaccharide with the insoluble polymer.

9. The use of a sulfated polysaccharide which is bonded to a carrier and produced according to the process of claim 1 in a process in which a protein which binds to sulfated polysaccharides is adsorbed into this polysaccharide and, where appropriate, is desorbed therefrom.

603764

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Form 10

PATENTS ACT 1952-69

COMPLETE SPECIFICATION

(ORIGINAL)

Application Number: 57851/86 Class Int. Class

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Complete Specification Lodged:

Accepted:

Published:

Priority :

Related Art :

This document contains the amendments made under Section 49 and is correct for printing.

Name of Applicant : BEHRINGWERKE AKTIENGESELLSCHAFT

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Actual Inventor: WERNER STUBER and ERIC-PAUL PAQUES

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50 QUEEN STREET, MELBOURNE, AUSTRALIA, 3000.

Complete Specification for the invention entitled:

PROCESS FOR THE PREPARATION OF A MATERIAL FOR AFFINITY CHROMATOGRAPHY

The following statement is a full description of this invention, including the best method of performing it known to us

BEHRINGWERKE AKTIENGESELLSCHAFT.

85/B 011 - Ma 527
Dr. Ha/Sd.

Process for the preparation of a material for affinity chromatography

The invention relates to a process for the preparation of a material for affinity chromatography, in which a sulfated polysaccharide is bonded to a carrier material which has amino groups.

This material can be used for the isolation and purification of substances which interact with sulfated polysaccharides.

Background of the Invention

The isolation of enzymes has been considerably improved in recent years by the technique of affinity chromatography. This method makes use of specific interactions between substances. For this purpose, a substance is covalently bonded as ligand onto an insoluble carrier (matrix). The ligand must be able to undergo an interaction, of the nature of a complex, with the substance which is to be isolated. The ligand retains only those substances which specifically react with it. Other substances are washed out. The retained substances can be eluted from the carrier material using a solution of unbonded ligand or, for example, using a salt gradient.

The success of an affinity chromatography depends on how well the interactions which naturally takes place between the substance which is to be isolated and the ligand are simulated. Thus, the choice of the matrix and the manner of immobilization of the ligand are important. The matrix ought to be hydrophilic and have good mechanical and chemical stability. Steric effects impeding the interaction can be favorably affected by spacers. Neither the matrix nor the spacer ought to give rise to non-specific adsorption.

For the isolation of a protein, it is of course most favorable to use a ligand which interacts only with one protein or with few proteins. The capacity of the adsorbent depends on the ligand loading of the matrix being sufficiently high. The chemical bonding ought to be as uniform and stable as possible, that is to say as difficult to hydrolyze as possible.

Affinity chromatography can be used for the isolation of proteins, for example from plasma, especially of antithrombin III. Immobilized sulfated polysaccharides have proved useful as affinity material for this purpose, especially carrier-bound heparin. However, it is known that, on immobilization of heparin and other sulfated polysaccharides, modifications of the ligand, with a reduction in biological activity, may occur.

The present invention had the object of preparing a material for affinity chromatography, by covalently bonding a sulfated polysaccharide to a carrier so that a material with high biological activity, ligand density and stability is obtained.

Prior Art

Affinity chromatography, especially using heparin, is utilized for the isolation of proteins which form complexes with sulfated polysaccharides.

For this purpose, a sulfated polysaccharide is bonded to a suitable carrier, the polysaccharide frequently being the mucopolysaccharide heparin. Sepharose^{R} 4B (an agarose gel in bead form; Pharmacia, Sweden) has proved especially useful as carrier matrix.

The covalent bonding of a sulfated polysaccharide to the carrier is mainly accomplished by activation of the carrier material or of the sulfated polysaccharide with

cyanogen bromide (Thromb.Res. 5, 439-452 (1974), German Offenlegungsschrift 2,243,688). However, this method has associated deficiencies. The isourea links which are formed between amino groups of ligands and hydroxyl groups of customary carrier materials are to a certain extent unstable toward nucleophilic reagents. This means that detachment of ligands must be expected under the conditions of elution, especially at elevated pH (Enzyme Microb. Technol. 4, 161-163, 1981).

This disadvantage can be eliminated by replacement of the cyanogen bromide by activators which contain oxirane groups. It is possible using epichlorohydrin (J.Chromatogr. 51, 479, 1970) or using bis-oxiranes, such as 1,4-butanediol bis(epoxypropyl) ether (J.Chromatogr. 90, 87, 1974), to introduce oxirane groups into a matrix which contains hydroxyl groups. The oxirane groups which have been introduced can be converted into amino groups with ammonia. It is possible using a carbodiimide (Anal. Biochem. 126, 414-421, 1982) to bond a sulfated polysaccharide which contains carboxyl groups to these amino groups.

The resulting amide bonds are distinguished by high chemical stability. The disadvantage of this method is that a number of carboxyl groups is converted into N-acylurea derivatives during the activation with carbodiimides. This means that although this process accomplishes extensive loading of the carrier with sulfated polysaccharides, nevertheless, as a consequence of their chemical modification, relatively low binding capacities are available for the substances which are to be isolated by affinity chromatography.

However, the bonding of a sulfated polysaccharide to a carrier material into which amino groups have been introduced can be accomplished in a chemically unambiguous manner by reductive coupling (Analyt.Biochem. 126, 414-

421, 1982). This entails the reducing end (aldehyde group) of the saccharide chain being coupled to an amino group of a polymer, with the formation of a Schiff's base. To stabilize the C=N double bonds they are reduced, for example with sodium cyanoborohydride, to the secondary amine. This method allows adequate loading of Sepharose^{R} 4B, into which amino groups have been introduced, with, for example, heparin, but it cannot be straightforwardly applied to other polymers.

In place of Sepharose^{R} 4B, which is widely used, it is in principle possible to use other polymers which contain hydroxyl groups as the carrier material, for example Fractogel^{R} HW-65 (F) (a synthetic hydrophilic polymer containing hydroxyl groups; J.Chromatogr. 239, 747-754, 1982). This polymer is, by reason of its chemical and physical properties, frequently more favorable than Sepharose^{R} 4B for industrial use.

However, there is no known process for bonding Fractogel^{R} HW-65 (F), after introduction of amino groups, to the reducing end of a sulfated polysaccharide in satisfactory yield.

The process described in this application does not have the disadvantages of the prior art and it makes it possible to couple, with good yields, a suitably derivatized sulfated polysaccharide to a carrier material into which amino groups have been introduced.

Summary of the Invention

In the process described, sulfated polysaccharides are modified with a diol-cleaving oxidizing agent, additional aldehyde groups thus being produced on the polysaccharide. Derivatives of this type can be bonded in good yields to carriers into which amino groups have been introduced. The resulting Schiff's bases can be reduced to the amine

with reducing agents, for example sodium cyanoborohydride.

Thus the invention relates to a process for the preparation of a sulfated polysaccharide bonded to a carrier, which comprises treatment of a sulfated polysaccharide with an oxidizing agent which oxidizes glycols to aldehydes, with cleavage of the carbon chain, and reaction of this modified sulfated polysaccharide with a polymer carrier which has amino groups.

It is possible to use as starting material one of the known sulfated polysaccharides, preferably heparin or its sodium salt. Aldehyde groups have been generated in aqueous solution by treatment with an oxidizing agent known to react with diols to form aldehyde groups, preferably an alkali metal periodate. The pH of the reaction solution was maintained preferably in the range 5-9, more preferably 6-8, with a base, preferably an alkali metal hydroxide, especially lithium hydroxide. 5-100 mg of alkali metal periodate, preferably 30-40 mg of sodium periodate, per gram of heparin have proved particularly favorable. The reaction times are preferably in the range from 10 min to 5 hours, the reaction temperature being maintained preferably at between 0 and 30°C. The oxidation reaction is preferably carried out for one hour at 4°C.

For coupling the polyaldehyde-derivatized sulfated polysaccharide it is possible to add the oxidation mixture directly to a carrier into which amino groups have been introduced. Carriers into which amino groups have been introduced are described in Analyt.Biochem. 126, 414-421 (1982).

The following are suitable as carrier materials for functionalization:
insoluble polymers which contain hydroxyl groups and into which amino groups can be introduced in a suitable manner, such as polymers based on carbohydrates. These include dextran and agarose resins as well as copolymers of

DBM/KJS:EK(13:21)

methacrylic acid derivatives, pentaerythritol, polyethylene glycol and divinylbenzene, which are marketed under the name Fractogel^{R}.

A carrier into which amino groups have been introduced in this manner is reacted with a polyaldehyde-derivatized polysaccharide, preferably 30-40 g of polysaccharide with 1,000 ml of carrier. The reaction is carried out preferably at pH 6-9, preferably at room temperature and preferably in a buffered aqueous solution, in particular at pH 6-7 in a phosphate buffer. After the reactants have been mixed, sodium cyanoborohydride is preferably added. The reaction at room temperature preferably takes 1 to 30 days. The preferred reaction time is 12 to 16 days. The product is preferably but not essentially washed with water and treated with acetic anhydride, by which means amino groups which are still free are acetylated.

It is know that, using suitable oxidizing agents, diols having hydroxyl groups on adjacent carbon atoms (glycols), such as, for example, sugars, provide aldehyde groups with cleavage of the carbon-carbon bond. Surprisingly, the action of the necessary "strong" oxidizing agents did not result in a loss of biological activity of the sulfated polysaccharide, and heparin which has been polyaldehyde-derivatized by the described process exhibited high binding affinity for an activity toward proteins which form complexes with heparin. This biological activity is also retained in the derivatives bonded to the carrier. A favourable coupling behavior is achieved owing to the large number of aldehyde groups in the ligands, since the coupling yields depend not only on the number of amino groups on the carrier but also on the number of aldehyde groups on the ligand. Carriers with few amino groups require a large number of reactive aldehyde groups.

DBM/KJS:EK (13:21)

The binding capacity of the adsorbent for the protein which is to be adsorbed depends directly on the ligand loading.

It is possible using the affinity materials obtained in the described manner to adsorb proteins which bind to sulfated polysaccharides from solutions of these proteins and, where appropriate, then to desorb them, for example antithrombin III from blood plasma. The effect of the number of amino groups in the resins on the coupling yields for heparin and polyaldehyde-derivatized heparin has been demonstrated with the polymers which are preferably used, Sepharose^{R} 4B and Fractogel^{R} HW-65 (F).

When a heparin which had been polyaldehyde-derivatized by this method was bonded to amino-Sepharose 4B it emerged that the binding capacity for antithrombin III was up to 2.5-fold that of a material which had been prepared from heparin and amino-Sepharose by a known method. The differences were even more drastic with carriers having lower numbers of amino groups, for example amino-Fractogel HW-65(F). Owing to the additional aldehyde groups, the binding capacity increased to 2 to 5 times that for the unmodified immobilized ligand.

By reason of the chemical nature of the ligand linkage, there was a very low tendency to lose ligands shown by the adsorbents which had been prepared using the derivatized (oxidized) sulfated polysaccharides. Compared with a ligand immobilized via isourea links, as are produced by activation with cyanogen bromide, the loss of ligands under comparable conditions was reduced by the described process to 0.1 to 1 %.

Example 1

Introduction of amino groups into Sepharose^{R} 4B

1,500 ml of water and 650 ml of 2N sodium hydroxide solution were added to 1,000 ml of thoroughly washed Sepharose^{R} 4B. This suspension was heated to 40°C, 150 ml of epichlorohydrin were added, and the mixture was shaken at 40°C for two hours. The product was washed to neutrality with water, and was treated with 750 ml of ammonia solution (density 0.91 g/ml) at 40°C for 90 min. Washing with water to a neutral reaction was carried out to remove excess reagents.

Oxidation of the sodium salt of heparin

30 g of the sodium salt of heparin (about 160 IU/g) were dissolved in 500 ml of water, and the pH was adjusted to 7 with a 20 g/l lithium hydroxide solution. This solution was cooled to 4°C, and 1.2 g of sodium periodate were added. The oxidation was continued for one hour, the pH being maintained at 7 by dropwise addition of a 20 g/l lithium hydroxide solution. This solution was used immediately for the coupling to the carrier into which amino groups had been introduced.

Coupling of oxidized heparin to amino-Sepharose 4B

1,000 ml of Sepharose^{R} 4B into which amino groups had been introduced were suspended in 1,000 ml of 0.2 mol/l dipotassium hydrogen phosphate buffer, pH 9. 30 g of oxidized heparin and 11.5 g of sodium cyanoborohydride were added to this suspension. The mixture was stirred at room temperature for 16 days, and the solid was filtered off and thoroughly washed with water. The product was suspended in 1,000 ml of 0.2 mol/l of sodium acetate solution and, at 4°C, 500 ml of acetic anhydride were added. Washing with water to neutrality was carried out after 30 min.

Example 2

Introduction of amino groups into Fractogel^{R} HW-65 (F)

325 ml of water and 275 ml of 5 normal sodium hydroxide solution were added to 1,000 ml of Fractogel^{R} HW-65 (F). 200 ml of epichlorohydrin were added to this mixture, which was shaken at 45°C for two hours. The product was filtered off and washed several times with water. The introduction of the amino groups was carried out with 500 ml of ammonia solution (density 0.91 g/ml) at 45°C for 90 min. The resin was then filtered off and washed with water to neutrality.

Coupling of oxidized heparin to Fractogel^{R} HW-65 (F) into which amino groups have been introduced

1,000 ml of amino-Fractogel HW-65 (F) were suspended in 500 ml of 0.5 mol/l sodium phosphate buffer, pH 6.5, and 30 g of heparin oxidized as in Example 1 were added. The pH was maintained at 6.5 with ortho-phosphoric acid (850 g/l solution). 11.5 g of sodium cyanoborohydride dissolved in 30 ml of water were added to this suspension. It was stirred at room temperature for 16 days, and the solid was filtered off and washed with water. The product was suspended in 100 ml of 0.2 mol/l sodium acetate solution, and 500 ml of acetic anhydride were added, and the mixture was stirred at 4°c for 30 min. The solid was filtered off with suction and washed with water.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for the preparation of a sulfated polysaccharide which is bonded to an insoluble polymer carrier which contains hydroxyl groups and amino groups which comprises treating a sulfated polysaccharide with an alkali metal periodate which oxidizes glycols to aldehydes, thereby cleaving the carbon chain, and ~~reaching~~ reacting the modified sulfated polysaccharide with the insoluble polymer.

2. The process as claimed in claim 1, wherein the sulfated polysaccharide is heparin.

3. The process as claimed in claim 1, wherein the oxidizing agent is an alkali metal periodate.

4. The process as claimed in claim 1, wherein the polymer which has amino groups is insoluble.

5. The process as claimed in claim 1, wherein the polymer which has amino groups is an agarose.

6. The process as claimed in claim 1, wherein the polymer which has amino groups is Fractogel^{R} into which amino groups have been introduced.

7. The process as claimed in claim 1, wherein heparin in aqueous solution is treated with 5-100 mg of alkali metal periodate per gram of heparin at a pH of 6-8 and at a temperature of 0 to 30°C for 10 minutes to 5 hours, the reaction product is allowed to react with a polymer which has amino groups, and the reaction product is, where appropriate, reduced with an alkali metal borohydride.

8. A sulfated polysaccharide bonded to a carrier, prepared as claimed in claim 1.

9. The use of a sulfated polysaccharide which is bonded to a carrier and ~~can be~~ produced according to the process of claim 1 in a process in which a protein which binds to sulfated polysaccharides is adsorbed into this polysaccharide and, where appropriate, is desorbed therefrom.

10. The use of a sulfated polysaccharide which is bonded to a carrier and produced according to the process of claim 1 in a process in which antithrombin III is adsorbed onto this polysaccharide and, where appropriate, desorbed therefrom.

DATED this 20th day of July, 1990.

BEHRINGWERKE AKTIENGESELLSCHAFT

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