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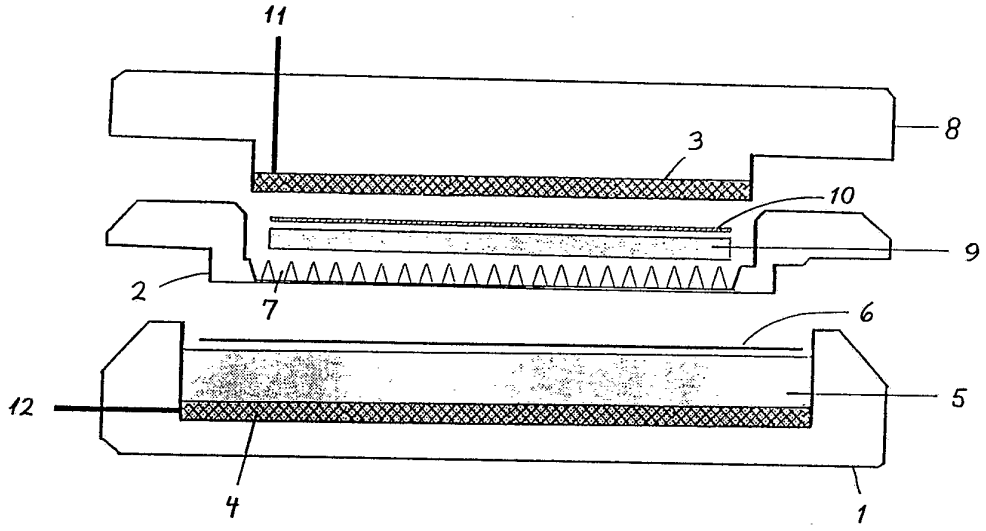
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(54) Title: PROCESS FOR ELECTROELUTION OF A GEL CONTAINING SEPARATED CHARGED MACROMOLECULES, SUCH AS PROTEINS OR DNA/RNA, AND AN APPARATUS AND MEANS FOR USE IN THE PROCESS



(57) Abstract

In a process for electroelution of a gel containing separated charged macromolecules, such as proteins or DNA/RNA, a preparatory separation after pre-equilibration of the gel is performed by collective elution of the entire gel in an electroelution apparatus in a suitable specific buffer, whereby the macromolecules are divided into narrow fractions each containing a specific macromolecule or closely related compounds. The electroelution apparatus comprises a base (1) containing a graphite anode (4) and a buffer containing sponge (5) covered by a dialysis membrane (6), a frame (2) containing multiple parallel triangular chambers (7), side vents in the frame to recover the isolated compounds, a graphite cathode (3) built into a safety cover (8) and a template for the precise excision of the gel before electroelution.

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Process for electroelution of a gel containing separated charged macromolecules, such as proteins or DNA/RNA, and an apparatus and means for use in the process

The present invention concerns a process of the type stated in the opening paragraph of claim 1 for electroelution of a gel containing separated charged macromolecules, such as proteins or DNA/RNA and the process according to the invention is novel according to the characterizing portion stated in claim 1. Further, the invention concerns an apparatus and various means for use in the process.

Electrophoresis of proteins or DNA/RNA in gels is widely used to separate compounds which differ in size and/or charge. Such compounds exhibit different migration distances and can be investigated by staining the gel or by transfer to a membrane suited for the study of antibody-antigen or other affinity interactions. Recovery of individual protein bands, however, is a laborious and time consuming procedure which generally has been based on cutting the gel into segments containing a single band, followed by homogenizing and elution, either by electroelution or by simple diffusion. Such a process is often ineffective and yields very high dilutions.

US Patent Specification No. 3 956 099 discloses a complex system suited for continuous operations, which system separates components into separate compartments during the electrophoretic run. The procedure is not based on the elution of macromolecules already separated in a conventional gel, but includes instead a separating unit suited for large scale purification of proteins.

The processes and components described in US Patent

Specifications No. 4 049 534, No. 4 725 348 and No. 4 747 918 are all suited for electroelution of small gel fragments containing single components. The procedure includes a preceeding localization and excision of the desired molecule, a technique which is time consuming and involves problems with respect to accuracy. The method is therefore not suited for the simultaneous investigation of all components separated in a gel.

EP Published Application No. 0 380 313 discloses a field inversion electroblotting and elution device specially suited for transfer of high molecular mass compounds. The procedure includes a preceeding identification of the band of interest and therefore has the same limitations as mentioned in relation to the methods described in the above-mentioned US Patent Specifications No. 4 049 534, No. 4 725 348 and No. 4 747 918.

US Patent Specification No. 4 181 594 discloses a matrix recovery apparatus wherein compounds contained in a slap gel are electroeluted directly upwards into multiple wells. Each compound is eluted into several wells separated by walls of a considerable thickness. This thickness presumably leads to an uneven and inefficient transfer. The wells are filled with different protein indicators. The apparatus does not involve a dialysis membrane and the products obtained therefore contain salts and detergents from the gel.

The Blotelutor® apparatus (manufactured by Biometra, Göttingen, Germany) provides a unit which is exclusively suited for the elution of proteins from two-dimensional gels. The proteins are eluted into wells separated by walls of a considerable thickness, which may lead to an uneven transfer. Furthermore, the method underlying this apparatus is not based on a physiological buffer and does

not include a pre-equilibration step to ensure a fixed positioning of the gel during elution.

None of these prior systems meet the need for conventional, simultaneous elution of all compounds separated in a one-dimensional gel. A system with these features would greatly facilitate the biological and chemical screening procedure of complex protein/DNA mixtures.

It is thus the purpose of the present invention to render it possible to perform a preparatory separation of macromolecules, such as proteins or DNA/RNA. According to the process of the invention a simultaneous elution of the separated charged macromolecules, such as proteins, in an entire gel, such as a polyacrylamide or agarose gel, is performed. Thus the mixture is divided into narrow fractions each containing single or closely located compounds, and the greater part of the present sodium dodecyl sulphate (SDS) is removed.

The novelty according to the process of the invention is the collective elution of an entire gel. As it appears from the afore-mentioned, the hitherto known methods have generally been based on a direct cutting of the gel matrix upon the termination of the electrophoresis. After homogenizing the material has been transferred to an electroelution apparatus. As mentioned such a method is very laborious and time consuming, especially in cases where a collective screening of the entire protein content of the gel is desirable (typically 20-30 individual fractions).

The present invention additionally provides an electroelution unit suited for the simultaneous elution of all compounds separated in a whole gel by the process of the invention. The unit very efficiently elutes ordinary gels (0.5-1 mm thick), but is also suitable for the elution of

very thick gels (3-4 mm).

The process and the apparatus according to the inventions is explained more fully with reference to the figures wherein:

fig. 1 is a schematically view of a multichamber electroelution apparatus according to the invention;

fig. 2 is a photography of a gel eluted in an apparatus as shown in fig. 1 by means of the process according to the invention;

fig. 3 is a photograph showing the fractionation of a complex mixture of proteins by means of the electroelution apparatus shown in fig. 1.

Fig. 4 is a graphic representation showing the cellular responses to fractions from the multichamber electroelutor, and

fig. 5 is a graphic representation demonstrating that fractions from the multichamber electroelution apparatus are non-toxic.

Referring now to fig. 1, a cut through the apparatus is shown. The entire instrument for carrying out the electro-elution is constructed in a detergent resistant plastic material, except for the graphite electrodes and their terminals.

The apparatus is composed of a base (1) containing a graphite anode (4) and a sponge (5) filled with buffer. In the base is located a reservoir which receives excess buffer generated as the sponge is compressed during mounting of the frame (2). A sheet of a semipermeable membrane,

such as a dialysis membrane, is placed on top of the sponge, and the frame is tightly connected to the base by means of finger screws. Optionally the dialysis membrane may be fixed to the sponge, e.g. by glueing. The graphite cathode (3) is built into a safety cover (8).

The frame contains multiple parallel chambers (7) of a triangular shape and closed downwards by the close surface to surface contact with the dialysis membrane (6).

The volume of each chamber is less than 15 ml, preferably about 3 ml.

The frame contains an indentation in which the gel (9) is placed. A sheet of filter paper (10) wetted with buffer is preferably placed between the gel (9) and the graphite cathode (3). A template which ensures a precise trimming of the gel according to the size of the indentation is a part of the unit.

The template is made of clear acrylic plastic and each well is indicated. The template is furthermore equipped with adjustment lines which are used to orientate the template before excision.

A voltage potential of about 40 volts is provided, and the power supply should be adjustable to provide such a voltage. The power supply is connected to the cathode (3) and the anode (4) by means of cables (11 and 12, respectively). Optionally the power supply can be built into the safety cover (8).

The necessary period for complete elution is generally between 8 and 15 minutes for a 0.75 mm gel. After elution the current is reversed for 10 seconds to loosen material sticking to the membrane. The product obtained is harvest-

ed by a plastic pipette through the harvesting side vents on the apparatus.

To start the electroelution process the gel containing the separated molecules is pre-equilibrated in a buffer, preferably for about 30-40 minutes. A preferred buffer, if the products are to be used in cellular assays, is a physiological buffer of low ionic strength, preferably a 2mM phosphate buffer (pH=6.5). During equilibration the gel will swell and obtain its final size, thereby ensuring a fixed positioning of the gel during elution. The buffer is changed three times to ensure the removal of excess salt and ionic detergents from the gel. The gel is removed from the buffer and placed on a clean glass plate, and then the part which is to be eluted is excised by the aid of the template. In the preferred use, gels are run with pre-stained molecular weight markers in the peripheral lanes. By aiming the template adjustment lines towards the markers a precise orientation and reproducible excision of the gel can be obtained. This method guarantees the important parallel course of gel bands and elution chambers.

The sponge in the base of the apparatus is filled with buffer. A dialysis membrane soaked in the buffer is placed on top of the sponge and the frame is mounted by means of the finger screws. The sponge functions as a support for the membrane and its compression ensures a tight closing of each chamber. The chambers are filled with buffer and the template is used to assist transfer of gels to the indentation of the frame. Two sheets of thick filter paper adjusted to the size of the gel are soaked in buffer and placed on top of the gel. The graphite cathode is mounted and a voltage potential of preferentially about 40 volts is provided. During the elution charged molecules will migrate from the gel into the buffer contained in the

chambers. The upper part of the walls separating the triangular chambers is sharply pointed to minimize any area of suboptimal transfer. Ionic detergents (SDS) will migrate towards the anode through the dialysis membrane, and then they can be effectively removed in the buffer. The period necessary for complete elution depends on the compounds under investigation and the gel matrix used, but is preferably between 8 and 16 minutes. The elution is followed by reversal of the current for a short period of time, preferentially about 10 seconds, to loosen molecules sticking to the membrane. After elution the product is recovered by aspirating with a pipette through the vents on the side of the frame. After harvesting of the product the apparatus is disassembled and the gel is stained for protein/DNA to check whether the transfer was complete. Before the product is used in cellular assays all fractions are made isotonic by addition of a sufficient volume of PBS x 10.

A different aspect of the present invention is the use of this apparatus for the simultaneous electroelution and electroblotting. In accordance with this aspect a sheet of suitable membrane is inserted in one side of the apparatus, thereby covering a variable part of all chambers. In this way molecules can be transferred into solution and simultaneously blotted onto a membrane. In a preferred form T cell reactivity and antibody response towards separated protein mixtures can be investigated simultaneoulsy in a simple and accurate way.

In accordance with another aspect of the present invention there is provided a method for purification of single compounds in a simple and quick way. This method comprises the step of removing a narrow strip of gel or electroblotted membrane containing the separated material and localizing the compounds of interest by staining or by

reaction with specific probes, which in a preferred embodiment could be monoclonal antibodies. Each elution chamber is indicated on the membrane by impressions from the pointed chamber walls. If a gel strip is stained, a number of 2-3 mm holes are punched to indicate the localization of the elution chambers. A precise punching is easily done by the aid of the template which carry a precise marking for each elution chamber. These reference strips enable a precise identification of the chamber which contains the relevant compound.

As it appears from fig. 2, the process and the apparatus according to the invention provides effective elution of all parts of the gel. The gel is divided into two areas, A and B:

Part A of the gel was removed before elution and stained for protein.

The eluted gel (part B) was stained for protein after termination of the elution process to evaluate if the transfer was complete.

In the gel are seen narrow faint lines indicating the location of chamber wall edges, and two blurred areas in the bottom of the gel. These findings emphasize two important findings:

1: the sharply pointed chamber walls minimize the areas of suboptimal transfer.

2: Air bubbles underneath the gel will interfere with the protein transfer.

The photograph in fig. 3 shows the fractionation of a complex mixture of proteins.

Mycobacterium tuberculosis culture filtrate was separated in 10-20% SDS-PAGE and fractionated with the multichamber electroelution apparatus of the invention. The filtrate and the obtained fractions were analyzed by SDS-PAGE and silver staining.

Lane F is the Mycobacterium tuberculosis filtrate. Lanes 1-18 are the fractions 1 through 18.

Fig. 4 shows the proliferative responses of spleen lymphocytes obtained from mice infected with M. tuberculosis. The cells were grown in cultures stimulated with fractions of M. tuberculosis culture filtrate in a dilution of 1:5. The proliferative response obtained with intact filtrate was 18600 cpm and the response of unstimulated cells was 500 cpm, the proliferation being measured by liquid scintillation counting of ^{3}H thymidine incorporation in the DNA of proliferative cells.

From fig. 5 it appears that the fractions from the multichamber electroelution apparatus according to the invention are non-toxic.

Low, medium and high stimulatory fractions from the multichamber electroelution apparatus were investigated in dilutions ranging from 1:5 to 1:500. All fractions exhibited a positive dose-response relationship, i.e. the higher dose gave a higher proliferative response. These results emphasize the non-toxic character of products from the electroelution apparatus.

As it appears from the afore-mentioned, the process and the apparatus according to the invention provide several great advantages:

First of all it is possible in a quick and simple way to obtain a simultaneous elution of all components separated in a one-dimensional gel which as a routine is commonly used. Moreover it is possible to use one-dimensional gels of varying thickness ensuring maximum flexibility of the invention. The electroelution apparatus can therefore form a part of an ordinary protein purification procedure and the yield can be quantitatively and qualitatively analyzed.

The triangular wells provide an even transfer of all protein bands.

Stretching of the separation frame against a sponge works as an efficient packing easy to handle.

The buffer is non-toxic and free of chloride ions which might otherwise cause problems during the elution. The same buffer is used in all parts of the electroelution apparatus, which is labor saving.

Upon completed elution it is possible to suck out the product without having to disassemble the machine. This secures a high degree of purity. The product is completely non-toxic and therefore suited for a number of different purposes.

Finally, it should be mentioned that the used template ensures an accurate and reproducible fractionation.

P a t e n t C l a i m s:

1. A process for electroelution of a gel, such as a polyacrylamide or agarose gel containing separated charged macromolecules, such as proteins or DNA/RNA, c h a r a c t e r i z e d in that a preparatory separation after pre-equilibration of the gel is performed by collective elution of the entire gel in an electroelution apparatus in a suitable specific buffer, whereby the macromolecules are divided into narrow fractions each containing a single or a few closely located compounds, while the greater part of the used sodium dodecyl sulphate (SDS) is removed, and that prior hereto the gel has been cut by means of a template securing an accurate and reproducible fractionation.

2. A process according to claim 1, c h a r a c t e r i z e d in that it comprises two or more of the following steps:

(a) pre-equilibration of the gel in a buffer, preferably for about 30-40 minutes to remove excessive salts and most of the used SDS;

(b) use of a specific buffer with low ionic strength in the elution step;

(c) reversal of the current for a short period of time, preferably about 10 seconds, to loosen molecules sticking to the membrane, and

(d) addition of a sufficient volume of PBS x 10 to all fractions in order to make them isotonic.

3. A process according to claim 2, c h a r a c t e r -

i z e d in that the buffer used in step (b) is a phosphate buffer, preferably 2mM.

4. An electroelution apparatus for simultaneous elution of all components separated in a gel into a number of separate cells by the process of claim 1, c h a r a c t e r i z e d in that it comprises:

a) a base (1) containing a graphite anode (4) and a buffer containing sponge (5) covered by a dialysis membrane (6), said dialysis membrane optionally being fixed to the sponge, e.g. by glueing;

b) a frame (2) containing multiple parallel chambers (7) each confined by said dialysis membrane at the bottom;

c) side vents in said frame to harvest the isolated compounds;

d) a graphite cathode (3) built into a safety cover (8), and

e) a template for the precise excision of the gel before electroelution.

5. The apparatus of claim 4, c h a r a c t e r i z e d in that said chambers (7) are triangular with sharply pointed edges ensuring an optimal transfer of all compounds separated in the gel.

6. The apparatus of claim 4 wherein the dialysis membrane (6) serves to concentrate the compounds of interest, while salts and ionic detergents pass through the membrane whereafter they can be removed.

7. The apparatus of claim 4, c h a r a c t e r i z e d

in that said sponge (5) covered with dialysis membrane (6) is being forced into intimate contact with said frame (2), thereby providing a number of sealed cells.

8. The apparatus of claim 4, c h a r a c t e r i z e d in that the power supply is built into the safety cover (8), said power supply being able to reverse the current automatically.

9. The apparatus of claim 4, c h a r a c t e r i z e d in that said side vents are accessible from the outside enabling a harvest of the obtained fractions without disassembling the apparatus, ensuring safe handling and products of very high purity.

10. The apparatus of claim 4, c h a r a c t e r i z e d in that said template is provided with adjustment lines which permit a precise and reproducible orientation of the gel according to the migration of the molecules.

11. The apparatus of claim 4, c h a r a c t e r i z e d in that a membrane is inserted for simultaneous elution and blotting.

12. A template for use in the apparatus of claim 4, c h a r a c t e r i z e d in that it carries an indication of each chamber in order to identify a chamber containing a single molecule of interest.

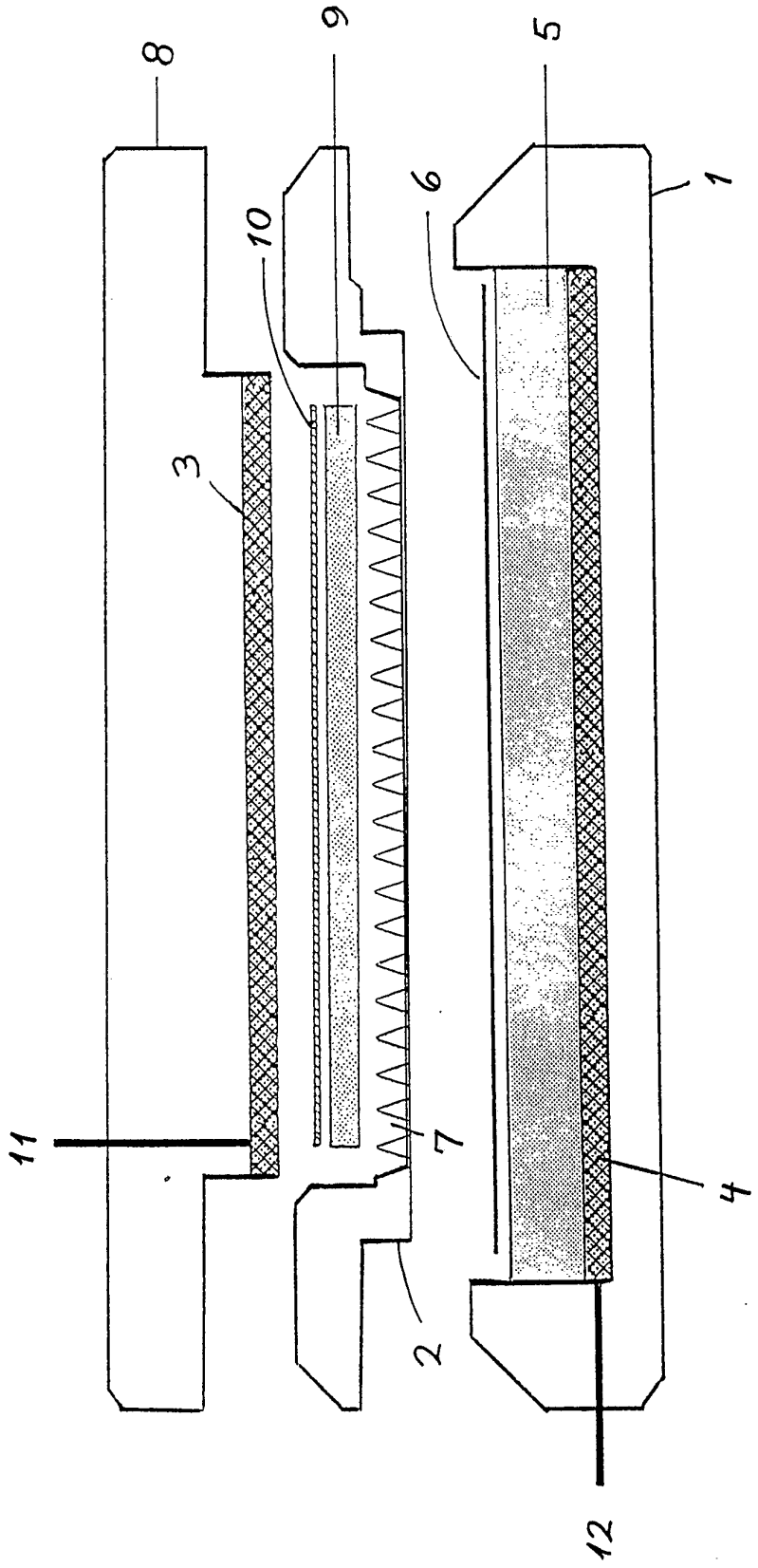


Fig. 1

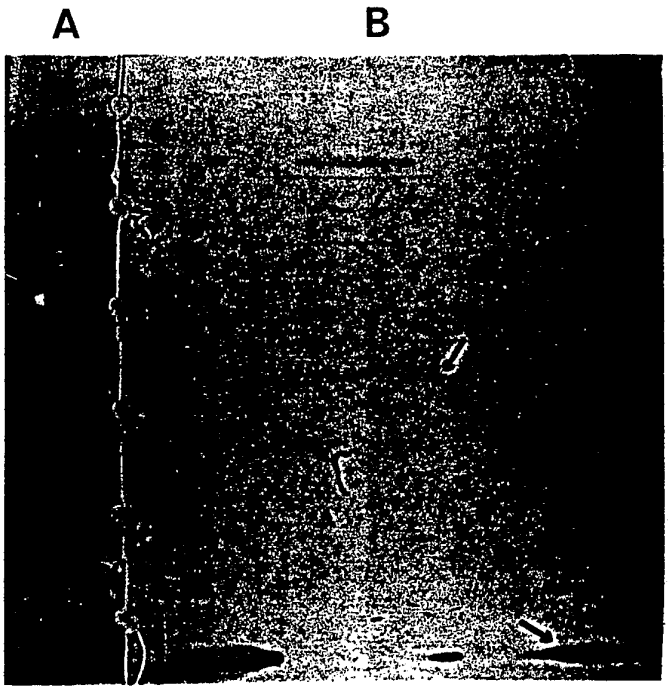


Fig. 2

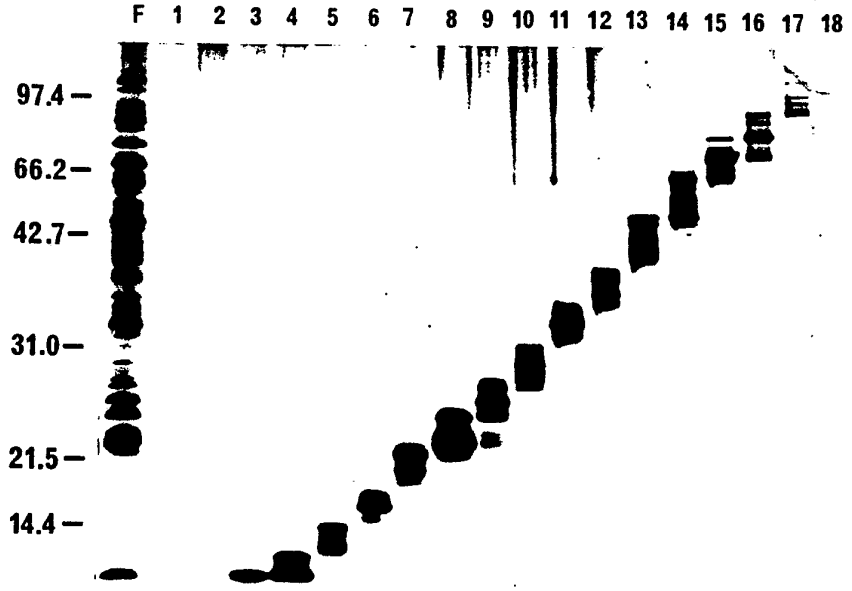


Fig. 3

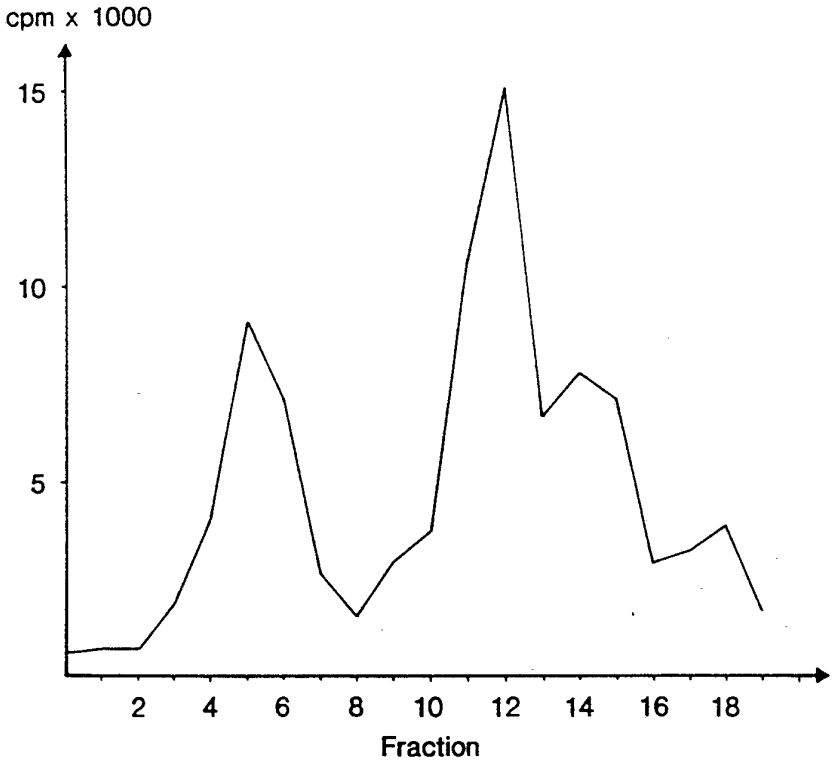


Fig. 4

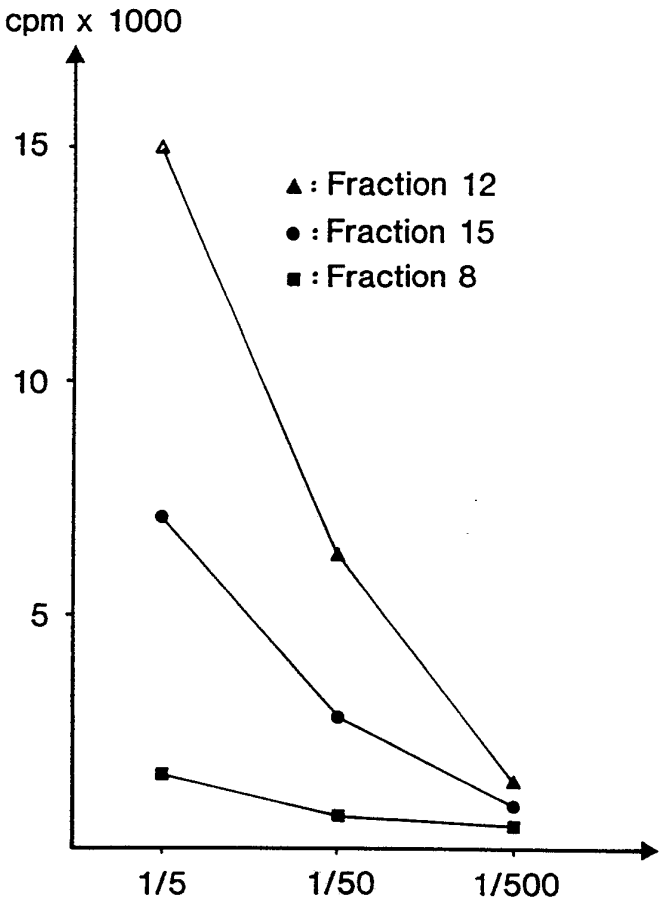

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●: Fraction 15
■: Fraction 8
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Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 92/00058

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

IPC5: B 01 D 57/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	B 01 D; G 01 N

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

SE,DK,FI,NO classes as above

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]
A	US, A, 4608147 (ANDREAS CLAD) 26 August 1986, see the whole document --	1-12
A	EP, A2, 0361062 (HOECHST AKTIENGESELLSCHAFT) 4 April 1990, see the whole document --	1-12
A	US, A, 4181594 (NABIL I. RIZK ET AL) 1 January 1980, see the whole document --	1-12

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21st September 1992	28 -09- 1992
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Inger Löfgren

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 92/00058

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 Swedish Patent Office EDP file on 28/08/92
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4608147	86-08-26	DE-A-C- 3337669	85-05-02
		DE-A- 3513897	86-10-30
		FR-A- 2553295	85-04-19
		GB-A-B- 2148326	85-05-30
		JP-A- 60098347	85-06-01
EP-A2- 0361062	90-04-04	DE-A- 3829111	90-03-01
		JP-A- 2107960	90-04-19
		US-A- 4948481	90-08-14
US-A- 4181594	80-01-01	NONE	