REGULATION 9

COMMONWEALTH OF AUSTRALIA
PATENTS ACT 1952-1973
APPLICATION FOR A PATENT

597568

LODGED AT SUB-OFFICE
10 DEC 1985
Sydney

We, LABORATORNI PRISTROJE, KONCERNOVY PODNIK

of Praha, CZECHOSLOVAKIA

hereby apply for a grant of a Patent for an invention entitled:

METHOD FOR PRODUCING HIGH-ACTIVE BIOLOGICALLY EFFICIENT COMPOUNDS IMMOBILIZED ON A CARRIER

which is described in the accompanying complete specification.
This Application is a Convention Application and is based on the Application(s) numbered : PV-9621-84

for a Patent or similar protection made in Czechoslovakia

on 11 December 1984

Our address for service is care of GRIFFITH HASSEL & FRAZER, Patent Attorneys of 71 York Street, Sydney 2000, in the State of New South Wales, Commonwealth of Australia.

Dated this 10th day of December 19 85

LABORATORNI PRISTROJE, KONCERNOVY PODNIK
By their Patent Attorney

GRIFFITH HASSEL & FRAZER

APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 16.3.90

To : THE COMMISSIONER OF PATENTS
COMMONWEALTH OF AUSTRALIA

DECLARATION FORM FOR COMPANY APPLICATIONS, AUSTRALIA

Form 7, 8.

Regulations 12 (1) and 12 (2)
COMMONWEALTH OF AUSTRALIA
PATENTS ACT 1952 - 69

## DECLARATION IN SUPPORT OF AN APPLICATION FOR A PATENT

In support of the Application made by LABORATORNI PRISTROJE, KONCERNOVY PODNIK (Name of Applicant Company)

of Praha, Czechoslovakia

for a patent for an invention entitled: METHOD FOR PRODUCING HIGH-ACTIVE BIOLOGICALLY EFFICIENT COMPOUNDS IMMOBILIZED ON A CARRIER

I, Ing. Zbysek Safarik, 13, Skolska, Praha 1, of the Applicant's (Full Name of Individual Declarant) Czechoslovakia address, do solemnly and sincerely declare as follows:

1. I am authorized by the applicant for the patent to make this Declaration on its behalf.

2. Peter HERMANN ; Jiri COUPEK ; Kornelia' SMALLA ; Ingo WILLHARDT and Jaroslava TURKOVA

(Full Name of Inventor)

of Mozartstrasse 21, Halle/S ; 1580 Kotorska, Praha 4, ; Ringfurtherweg 19, Magdeburg ; Block 762/03, Halle-Naustadt and 820 Julia Fucika, Ceský Brod, respectively

~~are~~ are the actual inventor(s) of the invention and the applicant is the assignee of the said inventor(s). for the said invention

3. The basic application(~~s~~) as defined by Section 141 of the Act, was/~~were~~ made in

Czechoslovakia (County) on the 11th

day of December 1984 by Laboratorni Pristroje, Koncernovy Podnik (Name of Applicant for Basic Application)

4. The basic application(~~s~~) referred to in paragraph 3 of this Declaration was/~~were~~ first application(s) made in a Convention country in respect of the invention, the subject of the application.

Declared at Praha, Czechoslovakia (City and Country) this 20th

day of November 1985.

Laboratorní PŘÍSTROJE koncernový podnik

162 03 Praha 6, Nárokrajní 335

(Signature)

To:
The Commissioner of Patents,
CANBERRA.

NOTE: Initial all Deletions and Alterations.
No-witnessing and no legalisation.
For a Non-Convention application delete paragraphs 3 and 4 and initial the deletion.
For Multiple Priorities incorporate details of all basic applications in paragraph 3.
For application for a Patent of Addition add "of Addition" after the word "patent" wherever the word occurs, and initial each such insertion.
For Joint Application by a Company and an Individual amend form so that the individual declares he is one of the applicants and is authorised by the other applicant to make this declaration upon the latter's behalf (Paragraph 1), and that the Company is an Assignee of a part interest in the invention from him (Paragraph 2.)

GRIFFITH, HASSEL & FRAZER,
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SYDNEY 2001. AUSTRALIA.

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METHOD FOR PRODUCING HIGH-ACTIVE BIOLOGICALLY EFFICIENT COMPOUNDS IMMOBILIZED ON A CARRIER

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(57) Claim

1. Method for producing highly active immobilized biologically efficient compounds selected from the group comprising enzymes, enzyme inhibitors and immunoactive proteins, useful for chemical transformation of compounds by enzymatic catalysis and for preparative and analytical separation techniques, by means of a hydrolytically stable covalent linking of said biologically efficient compounds to macroporous polymeric carriers selected from the group comprising copolymers of hydroxyalkyl methacrylates ($C_2$ to $C_6$ - alkyl) with alkylene dimethacrylate ($C_2$ to $C_4$ -alkylene), copolymers of styrene with divinylbenzene or alkylene dimethacrylate ($C_2$ to $C_4$ - alkylene), copolymers of glycidyl methacrylate with alkylene dimethacrylate ($C_2$ to $C_4$ - alkylene), porous glass, and silica gel, comprising linking said biologically efficient compounds to an activated macroporous polymeric carrier in the presence of solutions of inorganic salts added in a concentration from 0,5 to 3 mol/l, the given concentration of inorganic salt present in the bonding reaction causing a

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two-step reaction, whereby a hydrophobic adsorption of the biologically efficient compound on carrier occurs in the first step and then, in the second step, the biologically efficient compounds react with the carrier giving rise to a covalent linkage.

# COMPLETE SPECIFICATION

(ORIGINAL)

**FOR OFFICE USE**

Short Title:

Int. Cl:

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Complete Specification—Lodged:
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Related Art:

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

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TO BE COMPLETED BY APPLICANT

Name of Applicant: LABORATORNI PRISTROJE, KONCERNOVY PODNIK

Address of Applicant: Praha, CZECHOSLOVAKIA

Actual Inventor: Peter HERMANN ; Jiri COUPEK ; Kornelia SMALLA ; Ingo WILLHARDT and Jaroslava TURKOVA

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Complete Specification for the invention entitled: METHOD FOR PRODUCING HIGH-ACTIVE BIOLOGICALLY EFFICIENT COMPOUNDS IMMOBILIZED ON A CARRIE

The following statement is a full description of this invention, including the best method of performing it known to me:—*

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* Note: The description is to be typed in double spacing, pica type face, in an area not exceeding 250 mm in depth and 160 mm in width, on tough white paper of good quality and it is to be inserted inside this form.

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The invention pertains to a universal method for producing high-active biologically efficient compounds by immobilization with a covalent linkage on macroporous polymeric carriers in the presence of higher concentrations of inorganic salts.

Research and application of immobilized biologically efficient compounds represents nowadays an extensive field comprising both development and application projects of considerable practical importance.

The preparation and application of insoluble enzymes by bonding a soluble enzyme to a carrier enables to use the catalyst system repeatedly in a batch or through-flow arrangement of reaction in which the insoluble enzyme has a role of the specific heterogeneous catalyst. Such arrangement of the catalytic enzymatic reaction facilitates the subsequent separation of the enzyme from substrate and reaction products and lowers the cost and enables to automate the process in the multiple or continuous performance.

The rate of enzymatically catalyzed reaction depends, in the interaction of a substrate with the covalently immobilized enzyme, also on the character of linkage enzyme-carrier, on physical and chemical properties of the carrier (the size and distribution of pores, hydrophilicity of surface, etc.). Hydrodynamic parameters of the system, the flow rate of substrate, temperature, and others also significantly influence the final yield of reaction. The detailed knowledge of the factors affecting enzymatically catalyzed reactions utilizing the immobilized enzymes was obtained also in investigation the processes in preparative scale (penicilinacylase) (I. CHIBATA, Immobilized Enzymes, J. Wiley and Sons, New York 1970).

Immobilized biologically active comounds have found a very broad application in the affinity chromatography. This method employs the capacity of numerous biologically efficient compounds to form sorption complexes with other

compounds, while the character of interaction is very specific and reversible. If one of the components of a sorption complex is bonded to a solid carrier, only such compounds are selectively adsorbed from solution under suitable conditions which are noted for the specific affinity to the bonded compound. The sorption complex may be easily separated from other components in solution by means of the carrier. Dissociation of the sorption complex and separation of the soluble component from the component which remains chemically bonded to the carrier occur by changing the conditions (pH, ionic strength, temperature, addition of competitive sorbates, and the like). This procedure finds its use in the isolation and purification of enzymes, enzyme inhibitors, antidotes, antigens, soluble proteins, etc. Immobilization of bioactive compounds is advantageously used in biochemical analysis in the application of radioactive-labelled compounds or in optical labelling.

Numerous materials are used as carriers of biologically efficient compounds, for example, porous glass, silica gel, activated carbon, cellulose and its derivatives, starch, agarose, crosslinked polydextrans, synthetic polymers and copolymers as polyacrylamide, polystyrene, polyamides, poly(maleic anhydride), polyacrylates, polymethacrylates, etc. Some types are unsuitable because they carry ionogenic functional groups, other are noted for their strong nonspecific sorption properties for proteins. Other types have insufficient mechanical, hydrolytical, microbial or thermal stability or unsuitable distribution of pore size. These features considerably narrow their region of application. Homogeneous hydrophilic carriers of polysaccharide type mostly must not dry out, which fact makes difficult their storage and transportation.

Most of the above mentioned shortcomings are overcome in carriers prepared by copolymerization of 2-hydroxyalkyl methacrylates with alkylene dimethacrylates, which are

activated for the purpose of immobilization reaction by substitution on the hydroxyl group (J. Coupek, J. Turkova, O. Hubalkova, M. Krivakova; Czechoslovak Patent No. 167,530).

Further detailed studies concerning the mechanisms of immobilization of biologically efficient compounds revealed that the covalent linkage has to be as stable as possible towards hydrolysis, which cannot be satisfied, for example, by activation with cyanogen bromide or by amide, sulfide, ester or other bonding groups. Immobilization utilizing reactive epoxide groups according to the following scheme proved most suitable:

$$-CH_2-\underset{\diagdown O \diagup}{C}-CH_2 \;+\; H_2N\text{-Prot} \longrightarrow -CH_2-\underset{\displaystyle OH}{\overset{\displaystyle |}{C}}-CH_2\text{-NH-Prot},$$

where Prot means the residue of protein.

Experiments with immobilization on the copolymer of glycidyl methacrylate with ethylene dimethacrylate (J. Turkova, K. Blaha, M. Malanikova, D. Vajenerova, F. Svec and J. Kalal; Biochem. Biophys. Acta 5424 (1978) 162) revealed that the reactive glycidyl methacrylate groups enclosed in a strongly crosslinked matrix of copolymer cannot be wholly employed for immobilization and that their subsequent deactivation is difficult. Therefore, a macroporous copolymer of 2-hydroxyethyl methacrylate with ethylene dimethacrylate was advantageously used as a carrier which was activated with epichlorohydrine and contains epoxypropyl functional groups only on the inner surface of porous (J. Turkova, K. Blaha, J. Horacek, J. Vajcner, A. Frydrychova and J. Coupek; J. Chromatogr. 215 (1981) 165-179) and on the surface of particles.

However, the yield of immobilized activity was rather low in some cases of direct bonding of protein on the epoxypropyl derivatives of the hydroxyethyl methacrylate copolymer. This shortcoming, which occurs also in several other known activations of synthetic polymeric carriers, is removed by the subject of this invention.

It consists in a method for producing highly ~~high~~-active

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immobilized biologically efficient compounds useful for chemical transformation of compounds by the enzymatic catalysis and for preparative and analytical separation techniques, by means of a hydrolytically stable covalent linking of biologically efficient compounds to macroporous polymeric carriers, comprising linking said biologically efficient compounds to an activated microporous polymeric carrier in the presence of inorganic salts added in the concentration 0.5 to 3.0 mole/liter. The said concentration of inorganic salts in the bonding reaction causes reduction or elimination of a solvation envelope of protein molecules. In the first step, a hydrophobic adsorption of biologically efficient compounds on the carrier comes about, while, in the second step, the biologically efficient compounds react with functional groups of the carrier giving rise to the covalent linkage.

The macroporous polymeric carriers are selected from the groups comprising synthetic copolymers of hydroxyalkyl methacrylates ($C_2$ to $C_6$ alkyl) with alkylene dimethacrylate ($C_2$ to $C_4$ alkylene), copolymers of styrene with divinylbenzene or alkylene dimethacrylate ($C_2$ to $C_4$ alkylene), copolymers of glycidyl methacrylate with ethylene dimethacrylate, porous glass, and silica gel.

The polymeric carrier has to be activated prior to the immobilization reaction. Reactive functional groups are selected from the group comprising epoxides, aldehydes, primary or secondary amines, carboxyls and thiols. The macroporous polymeric carrier has advantageously a rigid structure and spherical shape. To confirm the effect of the invention, they were chosen the compounds comprised in the group of enzymes, enzyme inhibitors, and also immunoactive proteins.

Salts, the presence of which in bonding of bioactive compounds substantially increases the yield of bonded protein and the yield of activity, are selected from the group comprising ammonium and sodium sulfates and phosphates. Reaction temperature is given by the reactivity of functional groups of the carrier and the



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bioactive compound determined for immobilization and is in the interval -5°C to 30°C. The reaction proceeds at pH 3-10 for 0.5 to 40 hours.

The method for immobilization of biologically efficient compounds in the presence of higher concentrations of inorganic salts according to the invention is highly universal and its mechanism was studied in details and confirmed with numerous biologically efficient compounds of various nature. The subject of invention is further elucidated and substantiated in several examples, which, however, do not limit its scope by any means.

Example 1

Enzyme aminoacylase (3.5.1.14.)(10 mg) was dissolved in 5 ml of 0.2 M solution of phosphate buffer (pH 7). Ammonium sulfate was added, after the dissolution of protein had been completed, in such a way, that the final concentration of salt amounted to 1.75 mol/l of solution. Then, 100 mg of copolymer of 2-hydroxyethyl methacrylate with ethylene dimethacrylate (SEPARON 1000; exclusion limit $E_{max}$ = 2x10$^6$ Dalton, particle size 40-80 µm, capacity of surface-bonded epoxide groups 1.77 mmol/g) was added. To speed up penetration of the solution into the macroporous structure of carrier, the suspension was kept under vacuum of a water-jet pump (15 mm Hg column) for 5 minutes. Immobilization of acylase was carried out for 30 hours at 4°C under moderate shaking of the suspension. After completed immobilization, the carrier was washed with a buffer solution (phosphate, pH 7) and with 1 M NaCl solution. The content of bonded protein and its enzymatic activity were then determined. The yield of bonded activity was 10% with respect to the protein used and 20% with respect to the bonded protein.

Example 2

The reaction was carried out in the similar way as in example 1, with the distinction that it proceeded in the presence of an optimum amount of ammonium

monohydrogensulfate (1.5 mol/l). The yields of activity were the same as in Example 1.

Example 3

Enzyme thermitase (3.4.21.14.) (1 mg) was dissolved in 5 ml of 0.2 M phosphate buffer with pH 8.0. A solution of ammonium sulfate was added in such a way that the total concentration of salt amounted to 2.0 mol/l. Similarly as in example 1, 100 mg of a carrier was added, which contained epoxide groups (1.77 mmol/g) on the copolymer of 2-hydroxyethyl methacrylate with ethylene dimethacrylate, and the suspension was subjected to vacuum of a water-jet pump for 5 minutes. The reaction took 40 hours at 20°C under moderate shaking in this case. The yield of activity was 10%.

Example 4

The reaction was carried out analogously to example 3, with the distinction that an optimum concentration of added salt (sodium sulfate) was 1.25 mol/l. The reaction conditions and the yield of activity of the immobilized enzyme were the same as in example 3.

Example 5

To 1 mg of enzyme penicilinacylase (3.5.1.11.) in 5 ml of phosphate buffer (0.2 M, pH 8), it was added 100 mg of an activated carrier with particle size 100-200 µm and then stepwise a solution of ammonium sulfate in such a way, that the final concentration of salt amounted to 2.5 mol/l. The reaction was carried out at 40°C for 40 hours under moderate shaking. The reaction mixture was worked out in the same way as in example 1. The yield of activity was 45%.

Example 6

To 5 mg of enzyme elastase (3.4.21.36.) in 5 ml of 0.2 M phosphate buffer of pH 8.0, it was added 100 mg of a copolymer of 2-hydroxypropyl methacrylate crosslinked with butylene dimethacrylate (exclusion limit $8 \times 10^5$ Dalton; particle size 80-100 µm) activated with epoxy groups (capacity 1.25 mmol/g). A solution of sodium

hydrogensulfate was added up to the final concentration of 1.5 mol/l. The reaction took 20 hours at 4°C under moderate shaking. The yield of activity was 17%.

Example 7

Cystathion- -synthetase (4.2.1.22.) (10 mg) was dissolved in 5 ml of 0.2 M TRIS-HCl buffer solution at pH 8.7, 100 mg of a carrier, having the same composition as in example 1, was added followed by the solution of ammonium sulfate up to its optimum concentration of 2.5 mol/l. The reaction took 40 hours at -4°C. The reaction mixture was worked out according to example 1. The yield of activity was 35%.

Example 8

To 1 mg of carboxypeptidase A (3.4.17.1.) dissolved in 5 ml of 0.2 M phosphate buffer, it was added 100 mg of a carrier prepared by copolymerization of glycidyl methacrylate with ethylene dimethacrylate 1:2 (exclusion limit $1x10^6$ Dalton; particle size 100-200 µm). The bonding reaction was carried out in the presence of 2.0 mol/l of ammonium sulfate at 4°C for 20 hours. The product was worked out according to example 1 and the yield of activity related to the protein added was determined as 11.5%.

Example 9

Anti-cathepsin D-IgG (3 mg) was dissolved in 5 ml of 0.2 M phosphate buffer of pH 8.0 and bonded to 100 mg of carrier, which was the same as in example 1, in the presence of 1.5 mol/l ammonium sulfate for 24 hours at 25°C under moderate shaking. After the reaction had been completed, the carrier with the bonded protein was isolated by filtration, washed with the above mentioned buffer solution, packed into a column and tested for application in the affinity chromatography of Cathepsin D-IgG.

Example 10

The carrier according to example 1 (250 mg) was allowed to react at 50°C with 1 g of

hexamethylenediamine in 5 ml of water under moderate stirring for 3 hours. The reaction mixture was washed with water. The wet sorbent after washing was dispersed in 2 ml of 5% aqueous solution of glutaraldehyde, shaken for 20 hours and washed with water until the reaction with diphenylhydrazine was negative. The wet activated carrier was mixed with a solution of trypsin-inhibitor isolated from potatoes (1 mg/ml), 2 ml of 1.25 $Na_2SO_4$ was added, the reaction mixture was moderately shaken for 5 hours at 25°C and the carrier was then filtered and washed with water. The immobilized inhibitor was used for the affinity chromatography of trypsin.

Example 11

Chymotrypsin (3.4.21.1.) (10 mg) was dissolved in 5 ml of 0.2 M phosphate buffer solution of pH 8 and 100 mg of carrier based on the epoxypropyl derivative of silica gel (exclusion limit $5x10^5$ Dalton, capacity 0.5 mmol/g) was added to the mixture. Ammonium sulfate (2.5 mol/l) was added and the bonding reaction was carried out for 20 hours at 30°C. After washing according to example 1, the yield of activity of the bonded chymotrypsin was determined as 8.5%.

Example 12

The experiment was carried out analogously as in example 11 with the distinction that porous glass with the mean pore diameter 500 Å was used as the carrier. The carrier was activated by reaction with triethoxyepoxypropylsilane. The yield of activity of the bonded chymotrypsim was 9.7%.

Example 13

Trypsin (3.4.21.4.) (10 mg) was dissolved in 5 ml of 0.2 M phosphate buffer with pH 8 and 100 mg of carrier according to example 1 was added. The reaction took 20 hours at the concentration of ammonium sulfate 2.5 mol/l and ambient temperature.

Example 14

Pepsin (3.4.23.1.) (10 mg) was mixed in 5 ml of 0.1 M

acetate buffer of pH 4.0 with 100 mg of carrier similarly as in example 1. The reaction proceeded for 24 hours at the optimum concentration of ammonium sulfate 1.25 mol/l and 25°C. After washing according to example 1, the yield of immobilized activity was found 15%.

Example 15

To 10 mg of insulin in 5 ml of 0.05 M phosphate buffer with pH 8, it was added 100 g of epoxy-activated carrier according to example 1. The bonding raction was carried out in the presence of 1.75 mol/l of sodium monohydrogenphosphate at 25°C for 50 hours. The bonded insulin was used for the affinity chromatography of antidotes against the insulin from an antiinsulin serum in 0.1 M sodium barbiturate with pH 8.8, which contained 3% of albumin. The antidotes were desorbed with 3 M aqueous HCl.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Method for producing highly active immobilized biologically efficient compounds selected from the group comprising enzymes, enzyme inhibitors and immunoactive proteins, useful for chemical transformation of compounds by enzymatic catalysis and for preparative and analytical separation techniques, by means of a hydrolytically stable covalent linking of said biologically efficient compounds to macroporous polymeric carriers selected from the group comprising copolymers of hydroxyalkyl methacrylates ($C_2$ to $C_6$ - alkyl) with alkylene dimethacrylate ($C_2$ to $C_4$ -alkylene), copolymers of styrene with divinylbenzene or alkylene dimethacrylate ($C_2$ to $C_4$ - alkylene), copolymers of glycidyl methacrylate with alkylene dimethacrylate ($C_2$ to $C_4$ - alkylene), porous glass, and silica gel, comprising linking said biologically efficient compounds to an activated macroporous polymeric carrier in the presence of solutions of inorganic salts added in a concentration from 0,5 to 3 mol/l, the given concentration of inorganic salt present in the bonding reaction causing a two-step reaction, whereby a hydrophobic adsorption of the biologically efficient compound on carrier occurs in the first step and then, in the second step, the biologically efficient compounds react with the carrier giving rise to a covalent linkage.

2. The method according to Claim 1 wherein the macroporous polymeric carriers contains functional groups selected from the group comprising epoxides, aldehydes, primary and secondary amines, carboxyls and thiols.

3. The method according to any one of Claims 1 or 2, wherein the macroporous polymeric carriers have a rigid structure and spherical form.

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4. The method according to Claim 1, wherein the salts are selected from the group comprising sodium phosphates, ammonium phosphates, sodium sulfates and ammonium sulfates.

5. The method according to Claim 1, wherein the bonding reaction is carried out in the temperature region from -5°C to 30°C, in the region of pH 3 to 10, and for 0.5 to 50 hours.

6. Method for producing high-activity immobilized biologically efficient compounds substantially as herein described with reference to any one of the examples.

DATED this 1st day of March, 1990

LABORATORNI PRISTROJE, KONCERNOVY PODNIK
By their Patent Attorneys
GRIFFITH HACK & CO.

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