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(54) Title: METHOD OF PRODUCING CYSTATIN C OR MODIFICATIONS HEREOF AND DNA-SEQUENCE FOR USE WHEN CARRYING OUT THE METHOD			
(57) Abstract Cystatin C or a modified cystatin C is produced biosynthetically by culturing microorganisms or cells into which cDNA extracted from human cells and with codons coding for cystatin C or a modified cystatin C is introduced.			

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Method of producing cystatin C or modifications hereof and DNA-sequence for use when carrying out the method.

5 The present invention relates to a method of producing cystatin C (previously called δ -trace or post- δ -globulin). The invention also relates to a DNA-sequence and a plasmid as well as a microorganism for use when carrying out the method.

10

Mature cystatin C is a known protein with 120 amino acids. The amino acid sequence is as follows:

15 Ser-Ser-Pro-Gly-Lys-Pro-Pro-Arg-Leu-Val-Gly-Gly-Pro-Met-Asp-Ala-Ser-Val-Glu-Glu-Glu-Gly-Val-Arg-Arg-Ala-Leu-Asp-Phe-Ala-Val-Gly-Glu-Tyr-Asn-Lys-Ala-Ser-Asn-Asp-Met-Tyr-Mis-Ser-Arg-Ala-Leu-Gln-Val-Val-Arg-Ala-Arg-Lys-Gln-Ile-Val-Ala-Gly-Val-Asn-Tyr-Phe-Leu-Asp-Val-Glu-Leu-Gly-Arg-Thr-Thr-Cys-Thr-Lys-Thr-Gln-Pro-Asn-Leu-Asp-Asn-Cys-Pro-20 Phe-His-Asp-Gln-Pro-Mis-Leu-Lys-Arg-Lys-Ala-Phe-Cys-Ser-Phe-Gln-Ile-Tyr-Ala-Val-Pro-Trp-Gln-Gly-Thr-Met-Thr-Leu-Ser-Lys-Ser-Thr-Cys-Gln-Asp-Ala

25 Native human cystatin C is a mixture of two types differing in that type I has the amino acid sequence Ser-Ser-OHPro-Gly...Ala, while type II, called 3-des-OH-cystatin C, has the following sequence: Ser-Ser-Pro-Gly...Ala (1). Thus, the two types only differ in the
30 third amino acid residue. As the relative ratio between the two types may vary somewhat, dependent on the origin of the preparations and the extraction method, the biological activity of the preparations may also vary somewhat.

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Cystatin C prepared according to the invention is of the type 3-des-OH-cystatin C and thus is considered to have

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constant activity.

As is known from the literature, cystatin C is an efficient inhibitor for cystein-proteinases, i.a. papain and cathepsin B.

Cystein-proteinases participate in the intracellular catabolism of proteins and peptides, in the proteolytical conversion of prohormones, in the extracellular degradation of collagen and in the penetration of normal tissue with malignant cells.

As cystatin C inhibits cystein-proteinases which accelerate cancer growth or metastasis-formation, it is a potential cancer-inhibiting agent.

Furthermore, cystatin C possesses antiviral and possibly antibacterial activity.

Cystein-proteinases, e.g. chymopapain, may be used for treatment of slipped disc. If these cystein-proteinases diffuse into the cerebrospinal fluid, cerebral hemorrhage will occur as a serious side-effect of the therapeutic treatment of slipped disc. As cystatin C inhibits such side-effect, it has therapeutic use in this connection.

Particularly important is the activity of cystatin C to counteract hereditary inclination to cerebral hemorrhage with amyloidosis. It is deposited as amyloid in the arterial walls. The metabolism of cystatin C in this disease which attacks young people is changed, and the sequence for deposited cystatin C deviates at one position from what originally is described regarding cystatin C isolated from people without the disease.

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In certain types of hereditary cerebral hemorrhage cystatin C is precipitated after splitting off the 10 amino-terminal amino acids in the form of an amyloid in the cerebral arteries which burst. In case of these types of hereditary cerebral hemorrhage a cystatin C molecule is present which at position 68 has a glutamine acid residue instead of a leucine residue.

In the decomposition of collagen in tissue participate i.a. cystein-proteinases, and cystatin C may therefore probably inhibit such decomposition of tissue.

The cystatin C amino acid sequence isolated from human urine has been characterized and appears to be somewhat homologous to c-Ha-ras oncogene products.

The invention is based on the use of a novel DNA-sequence containing codons which code for cystatin C or modifications thereof.

One embodiment of the invention relates to a DNA-copy or a cDNA-sequence formed on the basis of human cells or placenta. Thus, the invention also relates to such DNA-sequence, the structure of which is stated in claim 2.

Further, the invention relates to modified cystatin C, in which one or more amino acids at positions 5-17, 55-59 and/or 68 have been replaced by another amino acid. An example of such modified cystatin C is a derivative in which Leu at position 68 is changed to Gln.

The invention also relates to a method of producing biosynthetic cystatin C or a modified cystatin C. In such a method a host organism is used, such as bacterium, a yeast cell or a mammal cell line, in which a vector is included, such as a plasmid having the structure suited for expressing cystatin C or a modified

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cystatin C.

5 The DNA-sequence in question is inserted together with signal sequences, promoters etc in a plasmid incorporated in the microorganism which is subsequently cultured in a manner known per se.

10 In the method according to the invention a host organism containing a DNA-sequence coding for cystatin C is cultured in a substrate, the cystatin C formed subsequently being extracted from the culture medium. The preferred host organism is E. coli, but in principle any microorganism may be used, including yeast cells such as *Saccharomyces Cerevisiae*, or culturing can
15 proceed by means of mammalian cells.

The cystatin C formed is extracted from the culture medium in a manner known per se, e.g. by extraction by way of solvents and purification by chromatography.
20 Affinity chromatography with monoclonal or polyclonal anti-body substances has turned out particularly suited for isolating cystatin C in pure state.

Isolation and analysis of cDNA clones have been effected
25 by screening recombinant phages. Thus λ gt11 cDNA library from human placenta and liver mRNA have been used. Screening was at a density of 50.000 plaques pr. 130 mm petri-dishes with affinity-purified anti-body substance by a method according to Young and Davis. Bound anti-
30 body substance was demonstrated by alkaline phosphatase-conjugated anti-substance (Promega Bioteknik).

λ -phages produced by the plate lysat method were isolated by centrifugation in a CsCl gradient. DNA was
35 extracted by standard methods. A mixed oligonucleotide probe being specific for cystatin C was hybridized to the product of phage DNA reacted with EcoRI in a

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Southern blot experiment. Hybridized cDNA insertion was ligated in EcoRI-linearized pUC18 plasmid vectors which were then used for transforming E. coli JMB83 cells. Plasmids were produced by alkaline lysis method. DNA-sequencing of insertions, the sub-clone in M13mp8, was carried out using a modified dideoxy-chain terminator technique.

In the following the invention is described by way of examples.

Example 1

cDNA library from human cells from liver, prostata and placenta were screened for cystatin C coding clones. About 1.2×10^6 recombinants of liver-cells and 3×10^5 recombinants from prostata cells were screened with anti-substances and a mixed oligonucleotide probe. The result of this screening was negative. On the other hand screening of 6×10^5 recombinants of placenta cells yielded nine clones which reacted with the anti-substance.

Three of these clones, designated C5, C6a and C12 respectively, were used in the following. Their inserts of 800, 800 and 700 base pairs, respectively, were specifically hybridized by Southern blot experiments to a mixed oligonucleotide, constructed from protein-sequence-data.

By sequencing inserts from clones C6a and C12, the sub-clone in EcoRI-cut M13mp8, it was disclosed that the inserts shared the same 3'-sequence, containing the polyadenyl-ring signal AATAAA.

The entire sequence of both strings of the clone C6a cDNA insert was determined by sequencing randomly

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overlapping fragments, the sub-clone in M13mp8. Each nucleotide was determined on an average 5.63 times. Data achieved by sequencing cDNA inserts of clones C6a and C12 from the ends revealed that the sequence of C12 inserts starts at position 91 of the C6a, see fig. 1.

The C6a insert contains 777 base pairs, including 77 base pairs of 5'-non-coding sequence and 262 base pairs of 3'-non-coding sequence. The polyadenyl signal AATAAA at position 756-761 is succeeded by 15 nucleotides. A possible translation initiation in agreement with the sequence discovered by Kozak is found 6 bp downstream of a stop codon. Initiation at this position and translation stop at the TAG-code at positions 516-518 will finally result in a 120 amino acid long cystatin C with a signal sequence of 26 amino acid residues. The protein in question will be identical with cystatin C, isolated from urine.

Fig. 1 below illustrates a sequencing strategy for human cystatin C cDNA-inserts. Inserts of the clones C12 and C6a were sequenced from their ends by the chain-terminator-method. A "shotgun" sequencing-analysis technique was used for sequencing both DNA-strings of C6a inserts. The horizontal arrows indicate the direction and extension of each sequencing analysis. The protein-coding area for inserted C6a is boxed and the hatched part indicates the area which codes for mature protein.

The DNA-structure coding for cystatin C comprises nucleotides derived from amino acid sequence for the clone C6a cDNA which codes for human precystatin C. The numbering of the nucleotide sequence starts at the first nucleotide and proceeds in the direction from 5' to 3'. The numbering of the amino acid starts with the first amino acid in the mature protein. The amino acid

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substituted in the cystatin C fragment deposited as amyloid in patients suffering from hereditary cerebral hemorrhage with amyloidosis is boxed. The Kozak-initiation and the polyadenyl-ring signal are underlined.

Example 2

Cytoplasmatic expression of methionin extended Cystatin C in E.coli:

The plasmid pUC18/C6a containing the cDNA sequence for Cystatin C was cut by the restriction enzymes NcoI/HindIII, whereafter the plasmid fragment was purified. A synthetic DNA linker containing the code sequence for the amino acid methionin and the approx. first 40 bases of mature Cystatin C were subsequently introduced between the NcoI and the HindIII sites (fig. 2). Instead of the codons occurring in the cDNA of cystatin C codons were used in the linker which result in high protein yield in E.coli. This plasmid was cut by the restriction enzymes ClaI/EcoRI, whereafter a fragment of about 600 bp containing the code sequence for methionin-extended Cystatin C was purified.

The plasmid pHD162 containing a synthetic promotor and Shine and Dalgarno sequence was cut by the restriction enzymes ClaI/EcoRI, whereafter the plasmid fragment was purified. This plasmid fragment was subsequently combined with the above 600 bp fragment to form the Cystatin C expression plasmid pHD262 (coding for methionin Cystatin C) (fig. 3). E.coli MC1061 containing the above expression plasmid was cultured as described below.

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Example 3Periplasmatic expression of Cystatin C in E.coli:

5 The plasmid pUC18/C6a mentioned in example 2 was cut by
the restriction enzymes NcoI/HindIII, whereafter the
plasmid fragment was purified. A synthetic DNA linker
containing the code sequence for Outer membrane protein
A (OmpA) from E.coli and the first approx. 40 bases of
10 mature Cystatin C were subsequently inserted between
the NcoI and HindIII sites. Optimal E.coli codons were
used. This plasmid was subsequently cut by the
restriction enzymes ClaI/EcoRI, whereafter a fragment of
about 700 bp containing the code sequence for the OmpA
15 signal peptide and Cystatin C were purified.

The above fragment was then introduced in an expression
plasmid containing the λ PR promotor, an optimal Shine
and Dalgarno sequence, the polylinker region from the
20 plasmide pUC18 and the temperature sensitive CI
repressor gene from λ . The plasmid pHD313 (fig. 4)
hereby formed was then introduced in E.coli MC1061. The
expression of Cystatin C and purification of Cystatin C
were carried out as herein described.

25

Example 4Periplasmatic expression of cystatin C in E.coli:

30 The plasmid pUC18 was cut by ApaI/EcoRI, and a fragment
of approx. 680 base pairs was purified and ligated onto
a synthetic ApaI/ClaI linker containing the code
sequence for about the first 3 amino acids of pre-
cystatin C. The fragment was subsequently introduced
35 into the expression plasmid, mentioned in example 3,
containing the temperature inducing λ PR promotor.

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Example 5

5

Expression of periplasmatic modified Cystatin C in
E.coli:

10 A HindIII/EcoRI fragment of about 700 bp was isolated
from the expression plasmid pHD313 (example 3). The
fragment was introduced in M13 MP18 and exposed to in
vitro mutagenesis so that the code sequence for Cystatin
C was changed to contain one or more of the modifica-
tions appearing from table 1 below. After in vitro
15 mutagenesis a number of clones were cultured and
sequence determined. Clones with correct sequence were
subsequently cut by ClaI/EcoRI, whereafter a fragment of
about 700 bp was isolated and introduced into the
expression plasmid pHD313. The plasmides hereby formed
20 were cultured, and the product purified as described
above.

Table 1

25 CYSTATIN C MUTANTS WITH MODIFIED DNA-SEQUENCE

1. Leu⁶⁸→Gln
2. Del.Ser¹-Val¹⁰
3. 1+2
- 30 4. Gly¹¹→pos. charged, e.g. Arg
5. Gly¹¹→neg. charged, e.g. Glu
6. Gly¹¹→strongly hydrofob., e.g. Trp
7. 4.Gly¹¹→Ala¹¹, 5Gly¹¹→Ser¹¹
8. Substitution in single amino acids, e.g.
35 a.) Arg⁶→Glu
b.) Leu⁹→Arg,Glu
c.) Val¹⁰→Arg,Glu

- 10 -

- d.) Gly¹²→Arg,Glu
e.) Pro¹³→Arg,Glu,Trp
f.) Arg¹⁴→Arg,Glu
- 5 9. Substitution of the sub-regions, e.g. Arg⁸-Met¹⁴→
Gly-Gly-Gly-Gly-Gly-Gly
10. 10. Deletion Arg⁸-Met¹⁴
11. 11. Cys⁸³→Gly
12. 12. Cys⁹⁷→Gly
13. 13. Deletion Asn⁶¹→Ala¹²⁰
10 14. Deletion Asp⁸¹→Ala¹²⁰
15. 15. Deletion Ile¹⁰¹→Ala¹²⁰

Example 6

- 15 Production of Cystatin C on a technical scale by batch
fermentation.

E.coli MC1061 pHD 313-1-1, which expresses Cystatin C
with correct N-terminal, is cultured on LB petri dish
20 containing ampicilline. A single colony is transferred
to a 100 ml shaker flask containing LB medium and 50
mg/l ampicilline. This is incubated at 30°C, 200 rpm for
20 hours.

- 25 The fermentation is carried out in 10 l laboratory
fermentor under the following conditions: pH = 7.2,
aeration = 1 VVM, stirring = 1000 rpm and temperature =
30°C in the first phase. The medium used consists of
yeast extract, casamino acids and various salts.

30 The fermentor is inoculated with 100 ml culture from the
shake flask and the dosing of glucose starts when the
biomass contentration is OD_{525nm} = 5. The feed rate
must be 3.5 g glucose/l.h, and it is maintained through-
35 out the process. The formation of Cystatin C is induced
by raising the temperature in the fermentor to 40°C in
the growth phase (OD_{525nm} = 50) Cystatin C is formed

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quickly - 2 hours after the temperature was raised the fermentation is ended by lowering the temperature to 20°C and raising pH to 9.0. The entire fermentation process lasts about 12 hours.

5

By the process are achieved final concentrations of Cystatin C of approx. 1000 mg/l (Cystatin C constitutes approx. 10% of the total protein in the cell).

10 Extraction of Cystatin C

Extraction I of Cystatin C from E.coli

15 The cell suspension from the fermentation is centrifuged. The cells are resuspended in 20-25% w/w sucrose, 0.1 M EDTA, 0.2M Tris pH 9.0 and are agitated for 20 min. Cells are removed by centrifugation and the pellet is quickly resuspended in 0°C 10mM Tris pH 9 under vigorous agitation for 10 min.

20

The supernatant herefrom contains Cystatin C from the periplasmatic phase (70% of total extraction by mechanical homogenization).

25 **Extraction II of Cystatin C from E.coli**

Cell suspension from fermentation is adjusted to pH = 10.5 with 5M NaOH. After stirring for 20 min. the suspension is centrifuged. The supernatant herefrom
30 contains Cystatin C.

Extraction III of Cystatin C from E.coli

35 Cell suspension from fermentation is centrifuged and resuspended in 0.5M Tris pH = 10.5. After stirring for 20 min. the suspension is centrifuged. The supernatant herefrom contains Cystatin C.

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Example 7

5 The extract is dialyzed against 20 mM ethanolamine pH = 10.0 with a dialyze membrane of the type Spectropor® (mw cut 3500) and is then subjected to a Q-Sepharose column equilibrated in 20 mM ethanolamine, pH = 10.0. Fractions containing Cystatin C (3-des-OH cystatin C) are combined and concentrated on a filter of the type Diaflo®YM2.

10 Then the material is subjected to a column of the type Bio-Gel®P60, equilibrated in 0.05 M ammonium hydrogen carbonate. Fractions containing Cystatin C are collected. By this isolation procedure the yield is typically 50-60%

15

Construction of a synthetic gene coding for Cystatin C by using optimal E.coli codons:

20 It is known from the literature that highly expressed E.coli proteins make the use of certain condons for the individual amino acids. Simultaneously with the production of cDNA for Cystatin C a Cystatin C gene was synthesized on the basis of the published amino acid sequence, the gene contained the codons preferred in

25 highly expressed E.coli proteins. The gene was also modified in such a way as to allow a fusion with the signal sequence from either outer membrane protein A from E.coli or the signal sequence from the fibria-protein K88/99 from E.coli. After synthesis the above

30 gene was step-cloned in the plasmid pUC18. The gene was hereafter isolated and introduced in the expression plasmid mentioned in example 3 in combination with one of the above signal sequences. The plasmid was introduced into E.coli MC1061 and cultured as described

35 above.

OmpA-cystatin C gene with optimal E.coli codons.

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CG-ATG-AAA-AAA-ACT-GCT-ATC-GCT-ATC-GCT-GTT-GCT-CTG-GCT-
 GGT-TTC-GCT-ACT-GTT-GCT-CAG-GCT-TCT-TCT-CCT-GGT-AAA-CCG-
 CCT-CGT-CTG-GTT-GGT-GGT-CCG-ATG-GAC-GCT-TCT-GTT-GAA-GAA-
 GAA-GGT-GTT-CGT-CGT-GCT-CTG-GAC-TTC-GCT-GTT-GGT-GAA-TAC-
 5 AAC-AAA-GCT-GTT-AAC-GAC-ATG-TAC-CAC-TCT-CGT-GCT-CTG-CAG-
 GTT-GTT-CGT-GCT-CGT-AAA-CAG-ATC-GTT-GCT-GGT-GTT-AAC-TAC-
 TTC-CTG-GAC-GTT-GAA-CTG-GGT-CGT-ACC-ACC-TGC-ACC-AAA-ACC-
 CAG-CCG-AAC-CTG-GAC-AAC-TGC-CCG-TTC-CAC-GAC-CAG-CCG-CAC-
 CTG-AAA-CGT-AAA-GCT-TTC-TGC-TCT-TTC-CAG-ATC-TAC-GCT-GTT-
 10 CCG-TGG-CAG-GGT-ACC-ATG-ACC-CTG-TCT-AAA-TCT-ACC-TGC-CAG-
 GAC-GCT-TAA-TAG

Example 8

15 Production and isolation of 3-des-OH-cystatin C

Bacteria media containing 3-des-OH-cystatin C were
 centrifuged and bacteria-free media concentrated on a
 DiafloTM YM 2 filter. The concentrated material was
 20 dialyzed against 20 mM ethanol amine, pH 10.0 in
 SpectraporTM dialysis membrane (mw cutoff 3.500) and
 subsequently applied to a Q-SepharoseTM column
 equilibrated in 20 mM ethanol amine, pH 10.0. Fractions
 containing 3-des-OH-cystatin C were pooled and
 25 concentrated on a DiafloTM YM 2 filter. The material was
 then applied to a Bio-GelTM column equilibrated in 0.05
 M ammonium bicarbonate. Fractions containing 3-des-OH-
 cystatin C were combined. By this isolation procedure
 the 3-des-OH-cystatin C yield is typically 50-60%.

30

Characterization of 3-des-OH-cystatin C:

3-des-OH-cystatin C isolated as described above from
 culture of pHD 313-transformed E.coli, was physically-
 35 chemically characterized with a view to biological
 activity. The results from the characterization were
 compared with result from corresponding analysis of

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human cystatin C.

Analysis with Ouchterlony's double immune diffusion technique showed that all cystatin C-epitopes recognized by a polyclonal anti-serum raised against human cystatin C are also present in 3-des-OH-cystatin C. Agarose gel electrofores at pH 8.6 and SDS-PAGE under reducing or non-reducing conditions showed that 3-des-OH-cystatin C and human cystatin C are identical as regards charge and molecular weight. Amino acid analysis showed that the amino acid composition in 3-des-OH-cystatin C is almost identical with that of human cystatin C. Automated sequence determination showed that 3-des-OH-cystatin C has the N-terminal sequence.

SSPGKPPRLVGGPMDASVEEEGV--ALDFAVGEYNKASNDMY at each position identical with that found in human cystatin C (cf. ref. 1) and in addition 100% of the prolin residue at position 3 in 3-des-OH-cystatin C lacked a hydroxyl group. Amino acid analysis of disulfide bound peptides showed that 3-des-OH-cystatin C has disulfide bridges between Cys⁷³-Cys⁸³ and Cys⁷³-Cys⁸³, i.e. the same as is the case in human cystatin C (cf. ref. 15).

Biological activity in 3-des-OH-cystatin C is determined through titration against concentration determined papain. 3-des-OH-cystatin C was 83% active, which is higher than corresponding figures for human cystatin C (55%). The strength of binding 3-des-OH-cystatin C on the cystein proteinases papain (EC 3.4.22.2), cathepsin B (EC 3.4.22.1) and dipeptidyl peptidase I (EC 3.4.14.1) was determined. The equilibrium constant for dissociation of 3-des-OH-cystatin C enzyme complex (< 0.005 nM, 0.50 nM and 3.5 nM) was within the experimental error limit identical with the equilibrium constant for human cystatin C enzyme complex (< 0.005 nM, 0.27 nM and 3.5 nM).

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Thus physical-chemical characterization and analysis performed of the interaction of 3-des-OH-cystatin with cystein proteinases shows that 3-des-OH-cystatin C has full biological activity and is identical with human cystatin C with the exception that it completely lacks hydroxyl group on residue 3.

Example 9

10

Therapeutic use of 3-des-OH-cystatin C as a CNS-protective agent at chymopapain-treatment of sciatica

Just before chymopapain treatment of patients with sciatica a molar amount of 3-des-OH-cystatin C three times higher than the molar amount of chymopapain to be used is injected into the cerebrospinal fluid. For example, if 8 mg of chymopapain (the active component of the currently available drugs: Discase®; Travenol Laboratories Ltd., Thetford, UK and Chymodiactin®; Smith Laboratories, Inc., Rosemont, IL, U.S.A.) is to be used for intradiscal injection, 12 mg of 3-des-OH-cystatin C is injected into the cerebrospinal fluid. The 3-des-OH-cystatin C might be composed of 5 mg 3-des-OH-cystatin C per ml of sterile physiological saline. This will protect against any side effects due to proteolytic breakdown of nervous tissue caused by improper injection of chymopapain into the cerebrospinal fluid.

Alternatively, if clinical or radiographic signs of improper chymopapain injection into the cerebrospinal fluid are observed at, or after, execution of the intended intradiscal chymopapain injection, a molar amount of 3-des-OH-cystatin C three times higher than the molar amount of chymopapain used, is immediately injected into the cerebrospinal fluid in order to inhibit the tissue-damaging effect of chymopapain.

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Directly after the injection of 3-des-OH-cystatin C the patient is gently moved in a way which brings about a rapid distribution of the injected solution of 3-des-OH-cystatin C with the cerebrospinal fluid of the patient.

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

1. DNA-sequence characterized in that it
5 comprises codons which code for cystatin C or modifica-
tions hereof.

2. DNA-sequence according to claim 1, characterized
10 in that it is a DNA-copy or cDNA having
the structure

		22
	GGGCGCAGCGGGTCCTCTCTAT	
15		95
	CTAGCTCCAGCCTCTCGCCTGCGCCCCAGTCCCGCGTCCCGCTCCTAGCGACC	
	Met Ala Gly Pro Leu Arg	
	-26	-21
20		155
	GCC CCG CTG CTC CTG CTG GCC ATC CTG GCC GTG GCC CTG GCC GTG AGC CCC GCG GCC GGC	
	Ala Pro Leu Leu Leu Leu Ala Ile Leu Ala Val Ala Leu Ala Val Ser Pro Ala Ala Gly	
		-1
25		215
	TCC AGT CCC GGC AAG CCG CCG CGC CTG GTG GGA GGC CCC ATG GAC GCC AGC GTG GAG GAG	
	Ser Ser Pro Gly Lys Pro Pro Arg Leu Val Gly Gly Pro Met Asp Ala Ser Val GLU Glu	
	1	20
30		275
	GAG GGT GTG CCG CGT GCA CTG GAC TTT GCC GTC GGC GAG TAC AAC AAA GCC AGC AAC GAC	
	Glu Gly Val Arg Arg Ala Leu Asp Phe Ala Val Gly Glu Tyr Asn Lys Ala Ser Asn Asp	
		40
35		335
	ATG TAC CAC AGC CGC GCG CTG CAG GTG GTG CGC GCC CGC AAG CAG ATC GTA GCT GGG GTG	
	Met Tyr His Ser Arg Ala Leu Gln Val Val Arg Ala Arg Lys Gln Ile Val Ala Gly Val	
		60
40		395
	AAC TAC TTC TTG GAC GTG GAG CTG GGC CGA ACC ACG TGT ACC AAG ACC CAG CCC AAC TTG	
	Asn Tyr Phe Leu Asp Val Glu Leu Gly Arg Thr Thr Cys Thr Lys Thr Gln Pro Asn Leu	
		80
45		455
	GAC AAC TGC CCC TTC CAT GAC CAG CCA CAT CTG AAA AGG AAA GCA TTC TGC TCT TTC CAG	
	Asp Asn Cys Pro Phe His Asp Gln Pro His Leu Lys Arg Lys Ala Phe Cys Ser Phe Gln	
		100

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515
 ATC TAC GCT GTG CCT TGG CAG GGC ACA ATG ACC TTG TCG AAA TCC ACC TGT CAG GAC GCC
 Ile Tyr Ala Val Pro Trp Gln Gly Thr Met Thr Leu Ser Lys Ser Thr Cys Gln Asp Ala
 120
 5 593
 TAG GGGTCTGTACOGGGCTGGCCTGTGCCTATCACCTCTTATGCACACCTCCCACCCCTCTATTCCCACCCCTGGAC
 672
 10 TGGTGGCCCCCTGCCTTGGGGAAGGTCTCCCATGTGCCTGCACCAGGAGACAGACAGAGAAGGCAGCAGGCGGCCCTTTG
 751
 TTGCTCAGCAAGGGGCTCTGCCCTCCCTCCTTCCTTCTTGCTTCTCATAGCCCCGGTGTGCGGTGCATACCCCCACC
 15 777
 TCCTGCAATAAAATAGTAGCATCCCC

20

or a fraction of this structure.

25 3. DNA-sequence, characterized in that it consists of a synthetically produced DNA-sequence coding for cystatin C or modifications thereof.

30 4. DNA-sequence, characterized in that it comprises codons which are optimal at expression in microorganisms.

5. Recombinant cystatin C, characterized in that it consists of 3-des-hydroxy-cystatin C.

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6. Modified cystatin C, characterized in that it has the structure for cystatine C in which one or more amino acids at positions 5-17, 55-59 and/or 68 have been replaced by another amino acid.

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7. Modified cystatin C according to claim 6, characterized in that Leu at position 68 has been changed to Gln.

- 20 -

8. DNA-sequence, characterized in that it comprises codons coding for a modified cystatin C as stated in claim 6.

5 9. DNA-sequence, characterized in that it comprises codones coding for cystatin C with one or more of the following modifications:

1. Leu⁶⁸→Gln
- 10 2. Del.Ser¹-Val¹⁰
3. 1+2
4. Gly¹¹→pos. charged, e.g. Arg
5. Gly¹¹→neg. charged, e.g. Glu
6. Gly¹¹→strongly hydrofob., e.g. Trp
- 15 7. 4.Gly¹¹→Ala¹¹, 5Gly¹¹→Ser¹¹
8. Substitution in single amino acids, e.g.
 - a.) Arg⁶→Glu
 - b.) Leu⁹→Arg,Glu
 - c.) Val¹⁰→Arg,Glu
 - 20 d.) Gly¹²→Arg,Glu
 - e.) Pro¹³→Arg,Glu,Trp
 - f.) Arg¹⁴→Arg,Glu
9. Substitution of the entire region, e.g. Arg⁸-Met¹⁴→Gly-Gly-Gly-Gly-Gly-Gly-Gly
- 25 10. Deletion Arg⁸-Met¹⁴
11. Cys⁸³→Gly
12. Cys⁹⁷→Gly
13. Deletion Asn⁶¹→Ala¹²⁰
14. Deletion Asp⁸¹→Ala¹²⁰
- 30 15. Deletion Ile¹⁰¹→Ala¹²⁰

10. A method of producing biosynthetic cystatin C or a modified cystatin C, characterized in that a host organism containing a DNA-sequence coding for cystatin C or the modified cystatin C is cultured in a substrate, the cystatin C or modified cystatin C formed subsequently being extracted from the culture.

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11. A method according to claim 10, c h a r a c t e r -
i z e d in that the host organism used is a bacterium,
a yeast cell or a mammal cell line.
- 5 12. A method according to claim 11, c h a r a c t e r -
i z e d in that the microorganism used is E.coli or S.
cerevisiae.
- 10 13. A method according to claim 11 or 12,
c h a r a c t e r i z e d in that the DNA-sequence used
has the structure stated in claims 2-4 or 8-9.
- 15 14. Plasmid for introduction into a host organisme
which is used for biosynthetic fermentation of cystatin
C, c h a r a c t e r i z e d in that it contains the
DNA-structure stated in claims 2-4 or 8-9 as well as
promotor and signal sequences etc.
- 20 15. Microorganism, c h a r a c t e r i z e d in that
it contains a plasmid as stated in claim 14.
- 25 16. Therapeutic preparation containing the cystatin C
or modified cystatin C produced by the method according
to claims 10-13.
17. Therapeutic treatment of brain haemorrhage with
cystatin C.
- 30 18. Therapeutic treatment or prevention of tissue
damaging effect in relation to sciatica.

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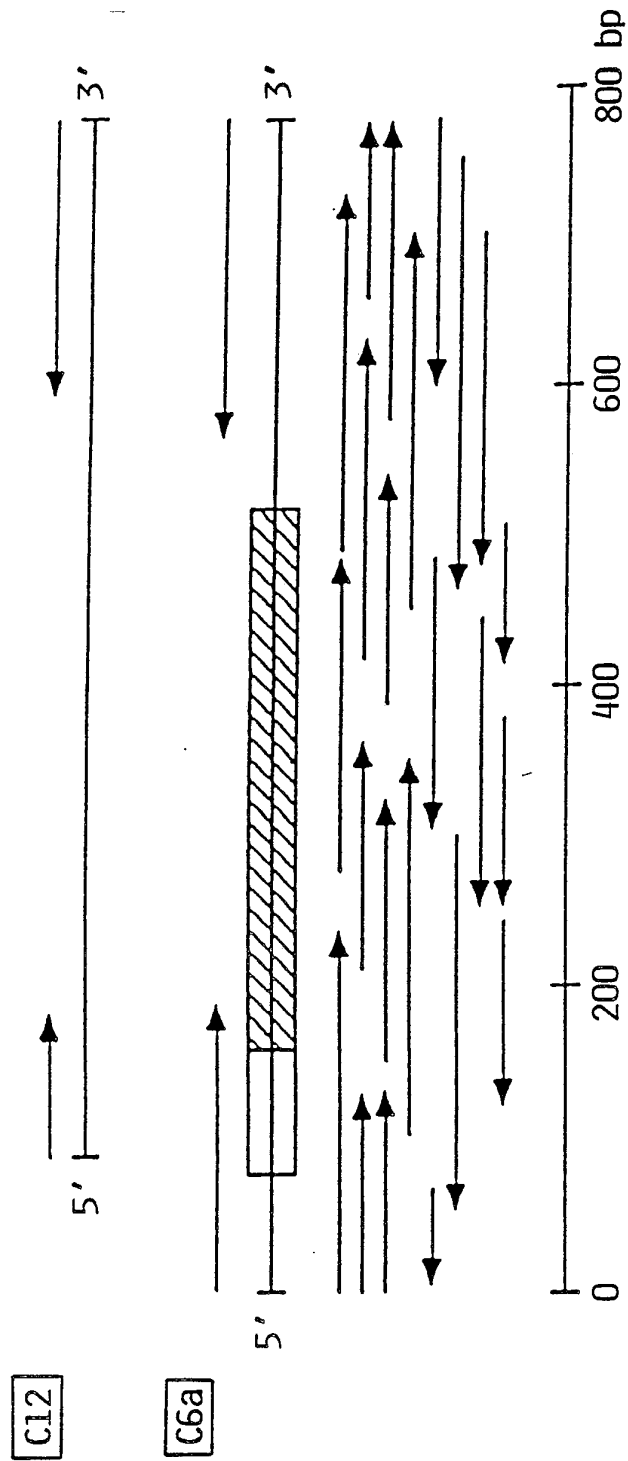


Fig.1

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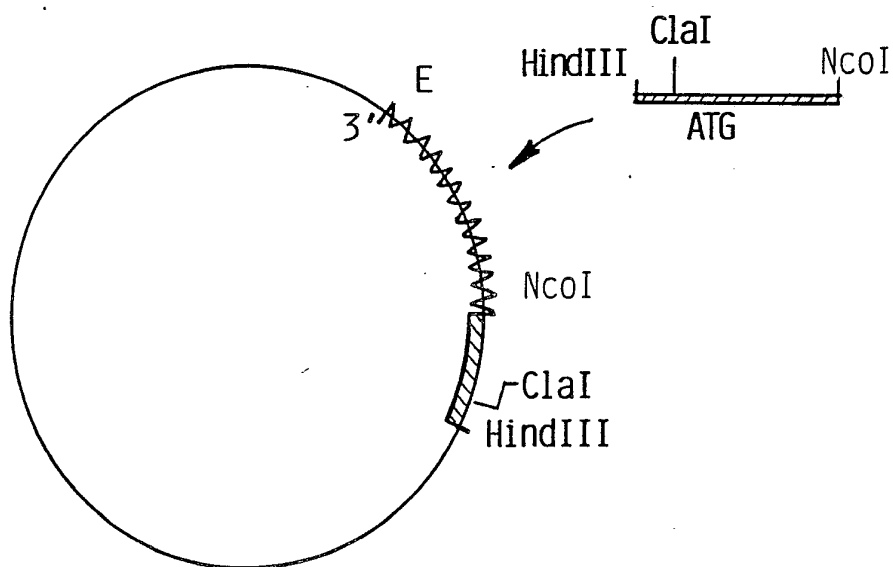
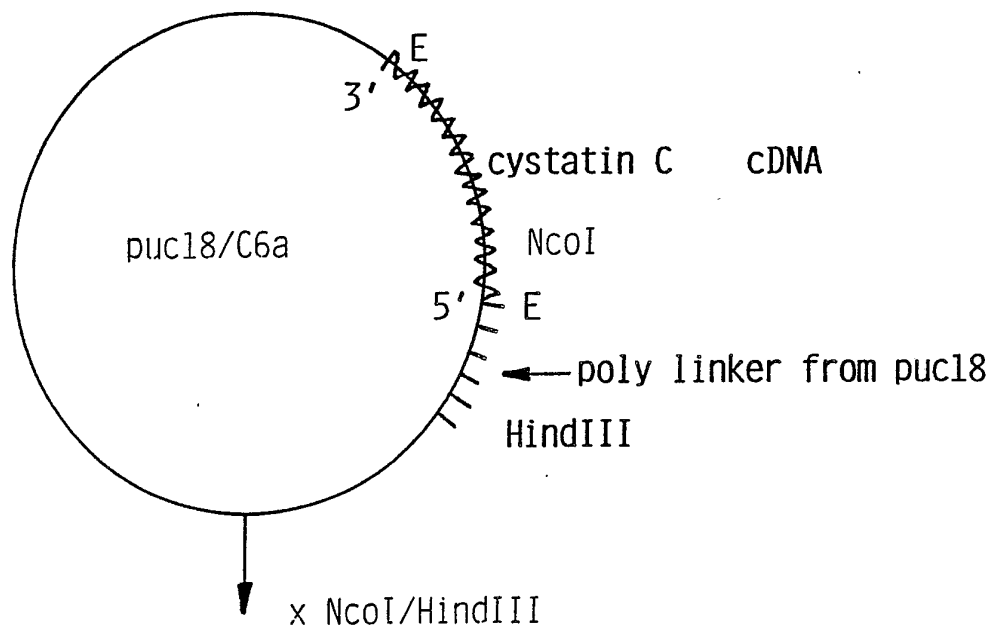


Fig.2

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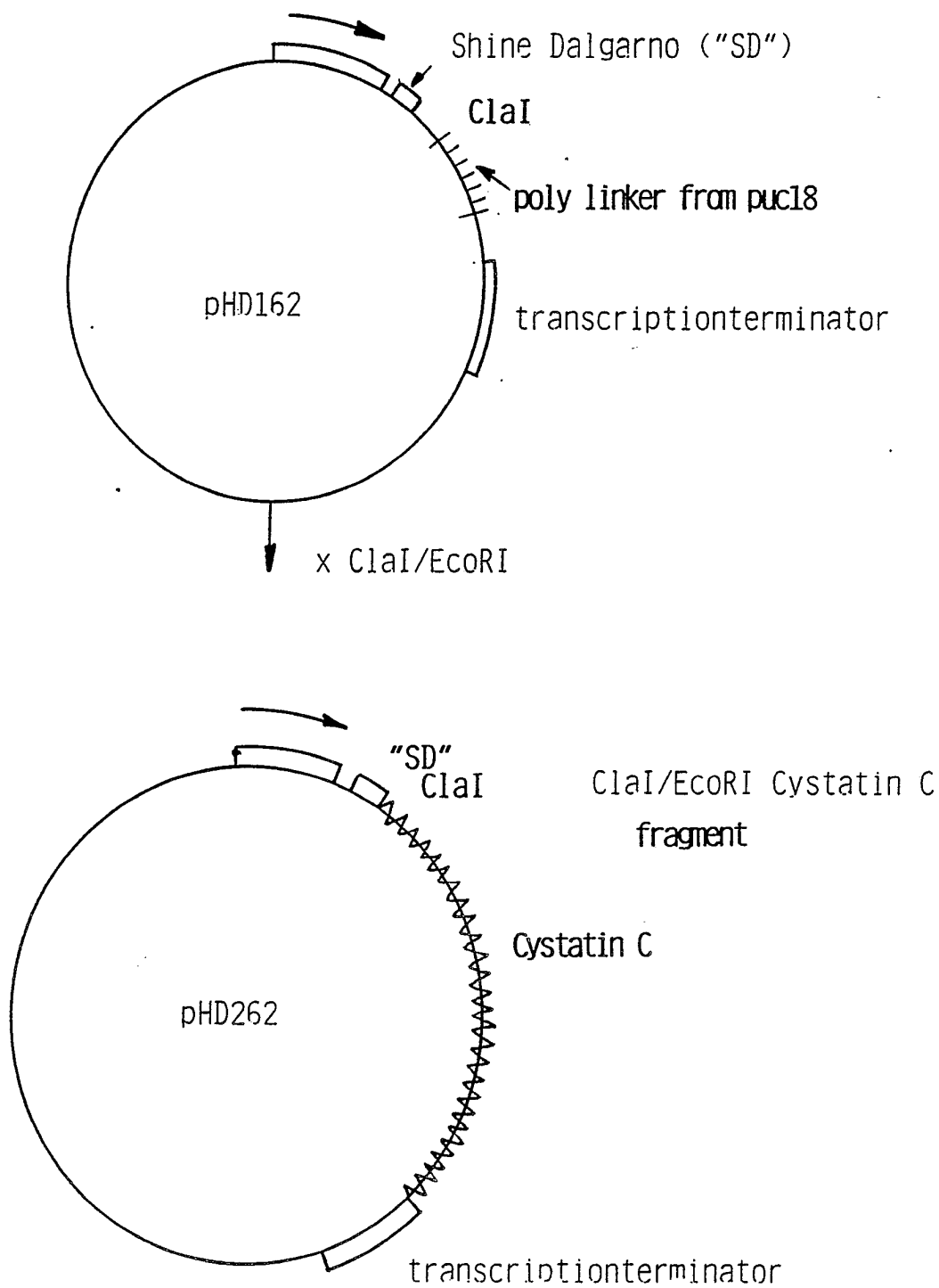


Fig.3

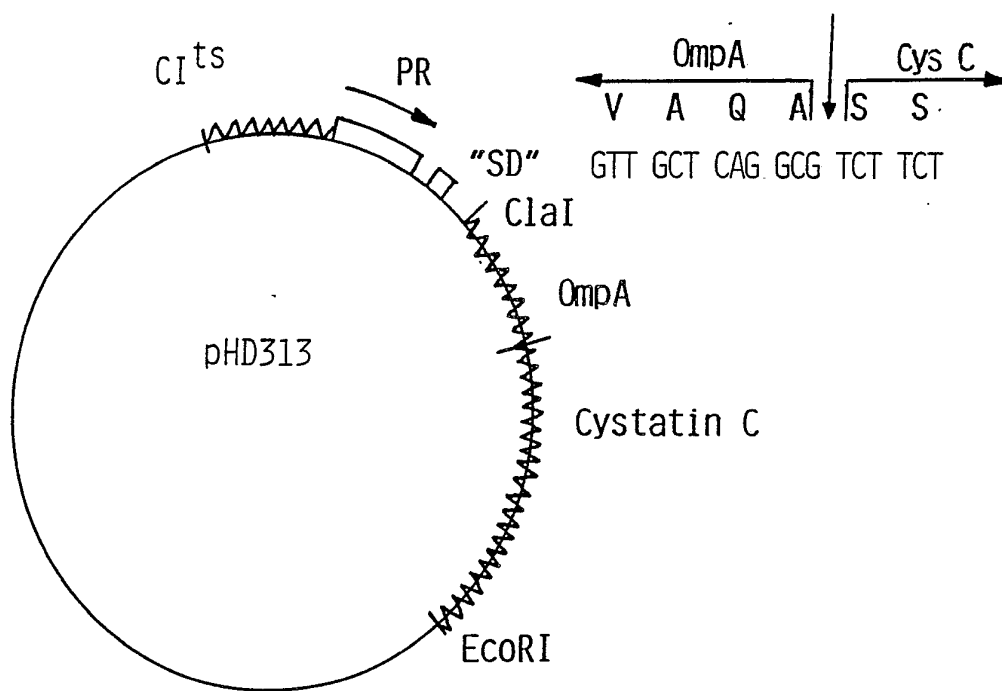


Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK88/00082

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 ⁴		
C 12 P 21/02, C 12 N 15/00, C 07 K 13/00, A 61 K 37/04		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC 4	C 12 N 15/00; C 12 P 21/02; A 61 K 37/04; C 07 K 13/00	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
SE, NO, DK, FI classes as above. Data base search: WPI, CA, Genbank, EMBL.		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P, X	FEBS letters, Volume 216, number 2, 229-233, June 1987, (Abrahamson et al) "Molecular cloning and sequence analysis of cDNA coding for the precursor of the human cysteine proteinase inhibitor cystatin"	1-16
X, Y	Proc.Natl.Acad.Sci. USA, Vol 83 pp 2974-2978, May 1986 (Ghiso et al), "Amyloid fibrils in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is a variant of y-trace basic protein (cystatin C)" see page 2978	1-16
X Y	Proc.Natl.Acad.Sci.USA, Vol 79. pp 3024-3027, May 1982, (Grubb et al) "Human y-trace, a basic microprotein: Amino acid sequence and presence in the adenohypophysis"	1-5 6-16
X Y	Biochemical and biophysical research communications, Vol 86, No 3, 1979 (Tonnellet et al) "Partial amino acid sequence of two forms of human post - Y - globulin" see summary page 1, pp 613-619	1-5 9-16
¹⁰ Special categories of cited documents: "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
1988-08-16		1988 -08- 30
International Searching Authority Swedish Patent Office		Signature of Authorized Officer Yvonne Siösteen

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

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

1. ☒ Claim numbers 17-18 because they relate to subject matter not required to be searched by this Authority, namely:

Methods for treatment of the human or animal body
by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers 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:

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

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

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 of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ 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 claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.