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(57) Abstract

The invention relates to a process for enriching the presence of *H.pylori* NAP in a mixture or proteins, comprising the step of salting–out other proteins. NAP has been found to remain soluble at ammonium sulphate concentrations of 80 % and above. The process preferably comprises the further step of metal–chelate chromatography. The combination of salting–out and metal–chelate chromatography results in very pure NAP. The NAP preferably has the same sequence as NAP as naturally occurring in *H.pylori* and is free from sequences typically associated with recombinant protein production. The processes and NAP of the invention can be used in diagnostic and therapeutic products and processes.

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ANTIGEN

This invention relates to the NAP protein of *Helicobacter pylori*.

BACKGROUND

Helicobacter pylori is a Gram-negative spiral bacterium which infects the human stomach. It is believed that over 50% of the world's population harbour the bacterium.

Clinical isolates of *H.pylori* can be characterised by the expression of a vacuolating cytotoxin (VacA), which induces vacuole formation in epithelial cells, and an immunodominant cytotoxin-associated antigen (CagA). Type I strains, which predominate in patients with ulcers or cancer, express both of these proteins, whereas type II strains express neither.

Various antigenic proteins have been described for *H.pylori* [*eg.* references 1 & 2], including its urease, VacA, flagella proteins, and adhesins. A protein known as NAP (neutrophil activating protein [3,4]), which is found in both type I and II strains, appears to be protective when tested in the *H.pylori* mouse model [5].

NAP is a homodecamer of 15kDa subunits [6], and it has been proposed that the multimeric complex has a ring-shaped structure which spontaneously forms hexagonal paracrystalline structures. The assembled protein appears to interact with glycosphingolipid receptors of human neutrophils [7].

Based on homology with bacterioferritins, it has been suggested that NAP may act as an iron buffer [3]. However, the presence of neither iron nor heme has been detected to date. The protein has also been reported to be a Na^+/H^+ antiporter [8].

As its name suggests, NAP promotes activation and adhesion of neutrophils to endothelial cells. Whilst it is has been suggested that this function is unlikely to be related to its intracellular function [3], the proadhesive activity can be neutralised by antiserum [6]. Since neutrophil activation and adhesion to endothelial cells constitute inflammation mechanisms, and since *H.pylori* is responsible for stomach inflammation, it seems likely that NAP represents the factor, or a factor, of *H.pylori* responsible for inflammation, probably at an early stage of gastric ulcer disease when an abundant accumulation of neutrophils in the superficial gastric mucosa is observed.

A protocol for the purification of the NAP decamer from *H.pylori* has been described [6], involving agarose chromatography, molecular sieving and ion-exchange chromatography. This

gave a yield of 72%. Recombinant NAP production in *E.coli* has also been reported [7]. The gene was cloned into plasmid pTrxFus to produce a thioredoxin fusion protein. Protein was then purified in the same way as the native protein. The N-terminal thioredoxin was reported not to affect the biological activity of NAP.

There remains, however, a need for pure NAP without the presence of cloning artefacts, fusion domains or the like. It is therefore an object of the invention to provide a process for the purification of native NAP. It is a further object that this process should be straightforward, easily scalable and economically feasible. It is a further object that the process should provide a highly pure protein.

We have now found that NAP has a surprisingly high aqueous solubility, remaining soluble even at 80% ammonium sulphate saturation.

DESCRIPTION OF THE INVENTION

According to the present invention, there is provided a process for enriching the presence of *H.pylori* NAP in a mixture of proteins, comprising the step of salting-out other proteins.

The salting-out step leaves the majority of NAP in soluble form. Although any suitable salt can be used, it is preferred to use ammonium sulphate. The final salt concentration is preferably 50% saturation or more (*eg.* 60%, 70%, 80% or more). The salting-out step preferably precipitates at least 50% of the proteins present in the mixture (*eg.* 60%, 70%, 80% or more).

Because of the surprisingly high solubility of NAP, this salting-out step alone removes the majority of proteins, considerably enriching the presence of NAP.

The resulting mixture of soluble proteins, enriched for NAP, may then be subjected to further enrichment. It is preferred to clarify the mixture first, however, in order to remove material precipitated in the salting-out step. This is typically achieved by filtration or, preferably, by centrifugation.

A suitable step for further enrichment utilises metal chelate chromatography [9]. Any suitable immobilised metal ion can be used (*eg.* zinc, cobalt, copper), but nickel is preferred.

In a preferred embodiment, therefore, the invention provides a process for the purification of *H.pylori* NAP from a protein mixture, comprising the steps of salting-out and metal chelate chromatography. This provides a simple two-step purification scheme for NAP.

By "purification" it is meant that NAP forms at least 75% (by weight) of the resulting mixture (*eg.* 80%, 85%, 90%, 95%, 97%, 99% or more).

The protein mixture may be any suitable source of NAP protein. Examples include *H.pylori* bacteria themselves, or other hosts which express the gene encoding *H.pylori* NAP (such as transformed bacteria). These are preferably lysed or disrupted prior to NAP enrichment/ purification in order to allow access to their cytoplasmic components (*eg.* sonication, French press, Hughes press, enzymatic lysis, grinding, freeze/thaw *etc*).

Preferred conditions and reagents for performing the processes of the invention are those set out in the examples below (*eg.* bacterial strains, vectors, restriction enzymes, culture media, temperatures, buffers, analytical methods *etc.*). For instance, in order to remove low molecular weight components, it is preferred to include at least one step of dialysis during the enrichment/purification process.

According to the invention, there is also provided a process for enrichment or purification of NAP from a recombinant host, wherein said NAP has the same sequence as NAP as naturally occurring in *H.pylori*. The purified or enriched protein is free from amino acid sequences typically introduced during the process of recombination or recombinant expression (*eg.* poly-histidine tags, thioredoxin fusions, GST fusions, intein-terminal sequences *etc.*).

According to the invention, there is further provided a process for the preparation of a diagnostic agent or therapeutic agent (*eg.* an immunogenic composition or vaccine), comprising enrichment/purification of NAP as described above, followed by suitable formulation. For agents to be administered to animals, for instance, this might involve formulating the NAP into a physiologically acceptable buffer. For an immunogenic composition or vaccine, this might include the addition of an adjuvant, for instance. For a diagnostic reagent, this might involve the addition of a detectable label to NAP (*eg.* a radioactive or fluorescent label). These formulation steps are well within the capability of the skilled worker.

According to a further aspect of the invention, there is provided NAP obtainable according to any of the above processes.

There is also provided NAP having the amino acid sequence shown in Figure 3 as SEQ ID 2.

In addition, the invention provides fragments of NAP according to the invention, wherein said fragments retain one or more of the following functions: (a) the ability to activate neutrophils;

(b) the ability to bind a NAP-specific antibody (*eg.* the fragment retains one or more epitopes of full-length NAP).

Furthermore, the invention provides nucleic acid (*eg.* DNA or RNA) encoding said NAP or said fragments of NAP.

There is also provided a therapeutic or diagnostic agent comprising NAP (or fragments of NAP) according to the invention. This is preferably an immunogenic composition, such as a vaccine.

In preferred embodiments, the diagnostic or therapeutic agents of the invention comprise additional *H.pylori* proteins or antigens. For example, the compositions might comprise VacA (vacuolating cytotoxin) and/or CagA (cytotoxin-associated antigen) and/or urease proteins in addition to NAP.

Vaccines according to the invention may either be prophylactic (*ie.* to prevent infection) or therapeutic (*ie.* to treat disease after infection).

Such vaccines comprise antigen or antigens, usually in combination with "pharmaceutically acceptable carriers," which include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminium salts (alum), such as aluminium hydroxide, aluminium phosphate, aluminium sulphate, *etc*; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides or bacterial cell wall components), such as for example (a) MF59™ (WO 90/14837), containing 5% Squalene, 0.5% Tween™ 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE, although not required) formulated into submicron particles using a microfluidizer (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), containing 2% Squalene, 0.2% Tween 80, and one or more bacterial

cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Freund's complete and incomplete adjuvants (CFA & IFA); (5) cytokines, such as interleukins (*eg.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, *etc.*), interferons (*eg.* IFNγ), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; and (6) other substances that act as immunostimulating agents to enhance the efficacy of the composition. Alum and MF59™ are preferred.

As mentioned above, muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), *etc.*

The immunogenic compositions (*eg.* the antigen, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, *etc.* Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvanticity effect, as discussed above.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (*eg.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The immunogenic compositions are conventionally administered parenterally *eg.* by injection, either subcutaneously or intramuscularly. They may also be administered to mucosal surfaces

(*eg.* oral or intranasal), or in the form of pulmonary formulations, suppositories, or transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

NAP according to the invention can also be used in immunoassays to detect antibody levels (or, conversely, anti-NAP antibodies can be used to detect antigen levels). Immunoassays based on well defined, recombinant antigens can be developed to replace invasive diagnostics methods. Antibodies to NAP within biological samples, including for example, blood or serum samples, can be detected. Design of the immunoassays is subject to a great deal of variation, and a variety of these are known in the art. Protocols for the immunoassay may be based, for example, upon competition, or direct reaction, or sandwich type assays. Protocols may also, for example, use solid supports, or may be by immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the probe are also known; examples of which are assays which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

The invention also provides kits suitable for immunodiagnosis. These contain the appropriate labeled reagents and are constructed by packaging the appropriate materials, including the compositions of the invention, in suitable containers, along with the remaining reagents and materials (*eg.* suitable buffers, salt solutions, *etc.*) required for the conduct of the assay, as well as suitable set of assay instructions.

According to the invention, there is also provided a process for detecting antibodies against NAP in a biological sample, comprising the step of contacting NAP according to the invention with said biological sample.

According to the invention, there is further provided a method of immunising an animal, comprising the administration of NAP according to the invention. This NAP is preferably in the form of a vaccine composition.

As an alternative to protein-based vaccines, DNA vaccination may be employed [*eg.* Robinson & Torres (1997) *Seminars in Immunology* 9:271-283; Donnelly *et al.* (1997) *Annu Rev Immunol* 15:617-648]. Accordingly, rather than comprise NAP, the vaccines of the invention might comprise nucleic acid encoding NAP.

The invention further provides a protein comprising the amino acid sequence shown in Figure 3 as SEQ ID 2. This protein may be used in the same way as NAP according to the invention.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the complete sequence of the gene encoding NAP in strain CCUG, which was cloned into plasmid pSM214G to give pSM214-NAP. The Figure also shows the sequence in the plasmid vector flanking the 5' end of the gene (lower case) and the deduced amino acid sequence.

Figure 2 shows a comparison of the nucleotide sequence of the cloned NAP with that in references 6 and 8. Differences can be seen which lead to amino acid differences (**Figure 3**) at residues 8, 58 & 80 (in comparison with reference 6) and residues 8, 73, 97, 101 & 140 (in comparison with reference 8, deduced from the whole genome sequence).

Figure 4 shows SDS-PAGE (A) and Western blot (B) of total cell proteins from *E.coli*. Lane 1: total extract from transformed cells; lane 2: negative control; lane 3: low MW markers.

Figure 5 shows Poinceau staining (A) and Western blot (B) of transformed *E.coli*. Lane 1: soluble extract; lane 2: insoluble extract; lane 3: low MW markers.

Figure 6 shows Poinceau staining (A) and Western blot (B) of transformed *B.subtilis*. Lanes 1 & 2: strain SMS118, soluble & insoluble extracts, respectively; Lanes 3 & 4: strain SMS300, soluble & insoluble extracts, respectively; lane 5: negative control (*B.subtilis* transformed with pSM214 without the NAP insert).

Figure 7 illustrates the effect of salting-out by ammonium sulphate. Lanes 1 & 2: 60% saturation, pellet & supernatant (from *E.coli*), respectively; lanes 3 & 4: increased saturation from 60% to 80% (pellet & supernatant, respectively); lane 5: purified NAP; lane 6: markers.

Figure 8 shows the purity of the final NAP product. Lanes 1-3 contain material purified from *E.coli*; lanes 507 contain material purified from *B.subtilis* (SMS118). From left to right, these lanes contain 1μg, 2μg & 3μg protein, respectively. Lanes 4 & 8 are markers.

EXAMPLES

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature *eg.* *Molecular Cloning; A Laboratory Manual, Second Edition* (Sambrook, 1989); *DNA Cloning,*

Volumes I and ii (ed. Glover 1985); *Oligonucleotide Synthesis* (ed. Gait 1984); *Nucleic Acid Hybridization* (ed. Hames & Higgins 1984); *Transcription and Translation* (ed. Hames & Higgins 1984); *Animal Cell Culture* (ed. Freshney 1986); *Immobilized Cells and Enzymes* (IRL Press, 1986); *A Practical Guide to Molecular Cloning* (Perbal, 1984); the *Methods in Enzymology* series (Academic Press, Inc.), especially volumes 154 & 155; *Gene Transfer Vectors for Mammalian Cells* (ed. Miller & Calos 1987, Cold Spring Harbor Laboratory); *Immunochemical Methods in Cell and Molecular Biology (ed.* Mayer & Walker, 1987); *Protein Purification: Principles and Practice* (Scopes, 1987); *Handbook of Experimental Immunology, Volumes I-IV* (ed. Weir & Blackwell 1986).

Cloning of the gene encoding NAP

The gene shown in Figure 1 was amplified from the CCUG chromosome using the following PCR primers, which also introduced *Sac*I and *Hind*III restriction sites:

5′-ctcgagctctagagggtattaataatgaaaacattgaat-3′

5′-cccttaagcttttaagccaaatgagcttc-3′.

The amplification product was digested with *Sac*I and *Hind*III and ligated into plasmid pSM214G [10] which had been digested with the same two enzymes. This plasmid is a shuttle expression vector between *E.coli* and *B.subtilis*. As can be seen from Figure 1, the recombinant gene is expressed under the control of a constitutive promoter and a ribosome binding site, which function in both *E.coli* and *B.subtilis*.

The ligated plasmid was used to transform *E.coli* and positive clones were selected on chloramphenicol plates. A plasmid from one positive clone ("pSM214-NAP") was isolated and characterised. Glycerol batches of this clone were stored at –80°C.

In addition, the plasmid was used to transform *B.subtilis*, which was also stored as glycerol batches at -80°C.

Preliminary expression analysis

Single colonies of transformed *E.coli* or *B.subtilis* strains were inoculated into 4ml LB-CAP medium (*ie.* LB medium +20μg/ml chloramphenicol) and cultured to 14 hours at 37°C. Control strains were grown containing the transformation vector without the NAP insert.

E.coli cultures were harvested and resuspended in SDS-PAGE loading sample buffer. *B.subtilis* cultures were harvested, treated with 0.3mg/ml lysozyme (30 minutes, 37°C), and then 3xSDS-PAGE loading sample buffer was added. The samples were incubated for 5

minutes at 95°C and separated by SDS-PAGE, after which the proteins were analysed by Coomassie blue staining and Western blot. The blot was visualised with an antiserum obtained by immunising a rabbit with a NAP-thioredoxin fusion (Figure 4).

The transformed bacteria clearly express a 15kDa protein not present in the non-transformed strains, as shown by rabbit antiserum.

Cell culture and lysis

Single colonies of transformed *E.coli* or *B.subtilis* were inoculated in 5ml LB-CAP and incubated for 37°C for 10 hours. The 5ml cultures were then used to inoculate 2 litre flasks containing 500-ml LB-CAP. After 14 hours incubation at 37°C on a rotary shaker (250 cycles/min), the cells were harvested by centrifugation at 6000*g* for 20 minutes at 4°C. Cell pellets were disrupted using either sonication or a French press.

For sonication, the pellets were resuspended in 8ml buffer A (20mM Tris-HCl, pH 7.8) supplemented with 0.3mg/ml lysozyme. After incubation on ice (10 minutes) and then at 37°C (7 minutes), 35µl of a 2mg/ml DNaseI solution (Sigma D-4263) was added. The samples were put on ice and sonicated extensively until disappearance of viscosity (Branson sonifier 450, medium tip, duty cycle 50, output control 5, approx. 25x2 minute cycles of 1 minute sonication/1 minute on ice). The lysate was brought to 14ml with buffer A and centrifuged at 20000*g* for 20 minutes at 4°C. Supernatant (soluble total extract) and pellets (insoluble total extract) were separated and either used immediately or stored at –20°C.

For French press disruption, cells were resuspended in 15ml buffer A and lysed by three passages in the press. The soluble proteins were collected by centrifugation at 12000*g* for 30 minutes at 4°C and the supernatant was brought to 28ml with buffer A.

SDS-PAGE analysis of the soluble and insoluble total extracts (Figures 5 & 6) show that the serum raised against NAP reacts only with the soluble fraction, indicating that NAP is fully soluble in buffer A.

Protein purification

B.subtilis total soluble proteins were diluted with buffer A to give a total protein concentration of 8mg/ml (Bradford). Ammonium sulphate was added to a final concentration of 60% saturation and the salting-out process was left overnight at 4°C with gentle stirring. Precipitated proteins were removed by centrifugation at 12000*g* for 30 minutes at 4°C and the supernatant was dialysed overnight against buffer A.

The dialysed solution was loaded onto a nickel-activated chelating sepharose FF column (1x8 cm) equilibrated with buffer A. The column was washed with buffer A+200mM NaCl. Protein elution was carried out with a 46ml linear gradient of 0-40mM imidazole, followed by a second 10ml gradient of 40-100mM imidazole (flow rate 0.5ml/min). Elution was then continued with 25ml 100mM imidazole.

Fractions were analysed by SDS-PAGE, and those containing NAP were pooled and dialysed against PBS buffer (pH 7-7.5).

NAP was purified from *E.coli* in the same way, except that the salting-out procedure used 80% saturation.

Purity

From 1 litre of culture, the results of the purifications were:

Bacterium	NAP (mg)	Purity (SDS-PAGE)
B.subtilis	10	90%
E.coli	30	95%

An indication of purity, by Coomassie Blue staining, is given in Figure 8. The material from *E.coli* appears to slightly purer. A yield of 80% is estimated, by densitometric analysis.

Salting-out

NAP appears to be soluble even at very high concentrations of ammonium sulphate. As shown in Figure 7, NAP remains soluble even at 80% saturation. Lane 4 of the gel shows that salting-out alone gives a high degree of purification.

Multimeric assembly

The ability of purified NAP to assemble into a multimeric form was investigated using size exclusion chromatography (non-reducing, non-denaturing). NAP was loaded on a Sepharose 12 HR 10/30 column equilibrated in buffer (25mM Tris-HCl, 150mM NaCl, pH 7.8) and eluted at 0.5ml/min. Regardless of the source of the NAP, the protein eluted in a single peak having the same retention time as yeast alcohol dehydrogenase (MW 150kDa), indicating a decameric structure [6].

N-terminal sequencing

N-terminal sequencing of purified NAP was carried out using a Beckmann LF 3000 protein sequencer equipped with on-line RP-HPLC analysis of PTH amino acids. The 10 amino acids sequenced were identical to those deduced from the gene sequence shown in Figure 1.

Comparison with native protein

H.pylori CCUG cells were collected from the surface of blood agar plates, washed in ice-cold PBS, and resuspended in lysis buffer (20mM Tris-HCl, 2.5mM EDTA, 0.3mg/ml lysozyme, pH 7.8). The cell suspension was incubated at 37°C for 20 minutes, sonicated, and centrifuged at 20000*g* for 40 minutes. The supernatant (soluble proteins) was stored at –20°C until use.

The retention time in gel filtration chromatography for NAP purified from *E.coli* or from *B.subtilis* was identical to that in the *H.pylori* soluble protein extract.

It will be understood that the invention is described above by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

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CLAIMS

1. A process for enriching the presence of *H.pylori* NAP in a mixture of proteins, comprising the step of salting-out other proteins.

2. A process according to claim 1, wherein the final salt concentration is at least 50% saturation.

3. A process according to claim 1 or claim 2, further comprising a step of metal chelate chromatography.

4. A process according to claim 3, wherein said metal is nickel.

5. A process for the purification of *H.pylori* NAP from a protein mixture, comprising the steps of salting-out and metal chelate chromatography.

6. A process according to any preceding claim, wherein said NAP is produced by a recombinant host and wherein said NAP has the same sequence as NAP as naturally occurring in *H.pylori*.

7. A process for the preparation of a diagnostic agent or therapeutic agent, comprising the steps of a process according to any preceding claim, followed by suitable formulation.

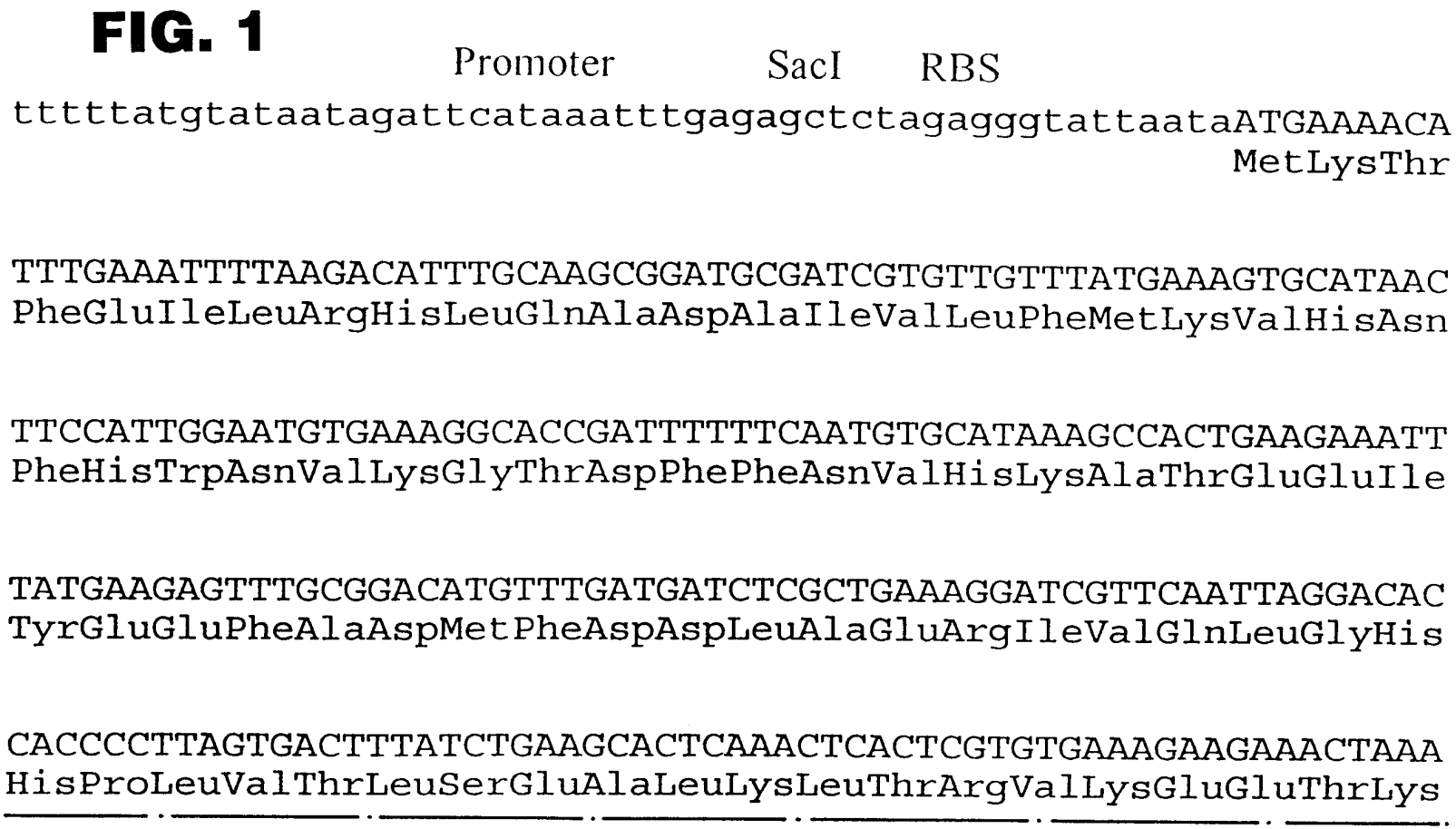
8. A process according to the claim 7, wherein said therapeutic agent is a vaccine, and wherein said formulation involves mixing said NAP with a physiologically acceptable buffer and/or an adjuvant.

9. NAP obtainable according to the process of any one of the preceding claims.

10. NAP according to claim 9, obtainable according to the process of claim 6.

11. Pure NAP.

FIG. 1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GCTtaaaagctt
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   HindIII
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FIG. 1 (CONTD.)

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GENOMA        1  ATGAAAACATTTGAAATTCTAAAACATTTGCAAGCGGATGCGATCGTGTTATTTATGAAA

SEQ ID 1     61  GTGCATAACTTCCATTGGAATGTGAAAGGCACCGATTTTTTCAATGTGCATAAAGCCACT
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SEQ ID 1    121  GAAGAAATTTATGAAGAGTTTGCGGACATGTTTGATGATCTCGCTGAAAGGATCGTTCAA
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SEQ ID 1    241  GAAACTAAAACGAGCTTCCACTCTAAAGACATCTTTAAAGAAATTCTAGGCGATTACAAA
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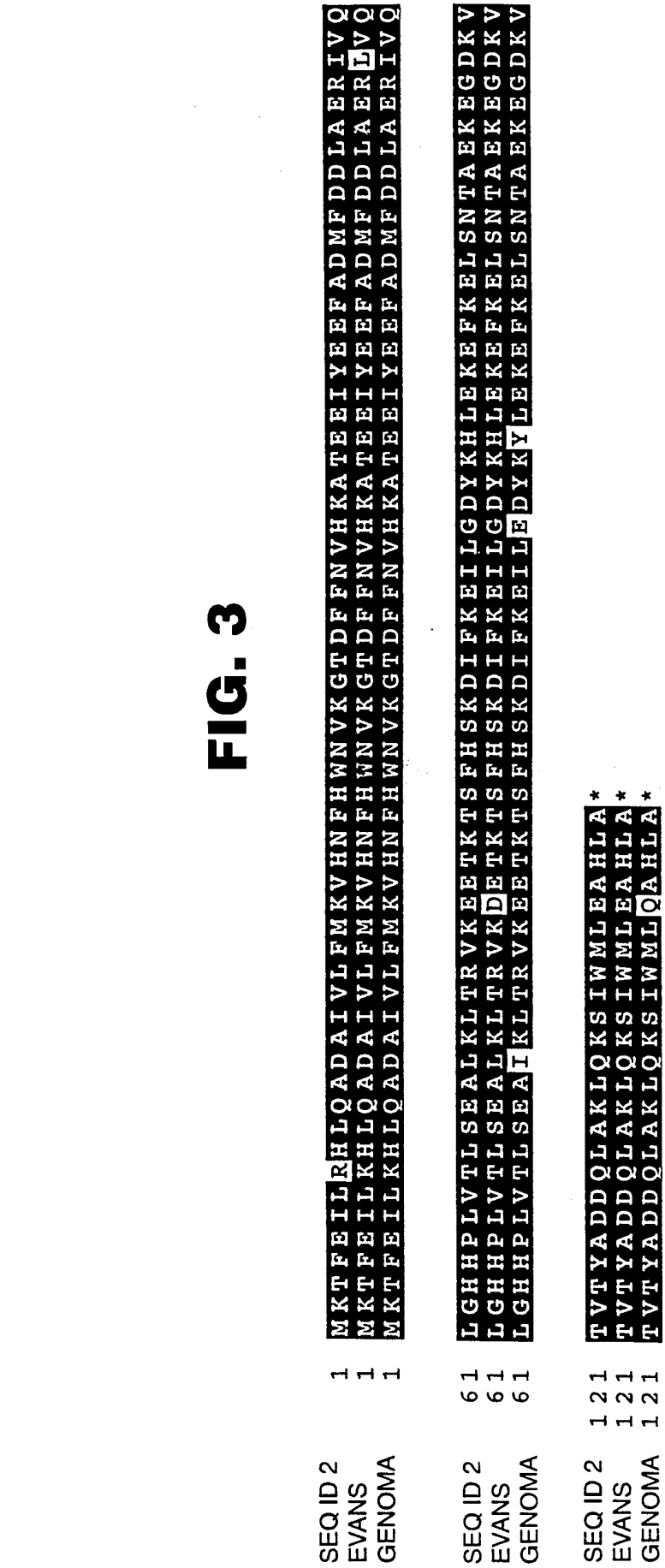
FIG. 2

FIG. 3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EVANS 61 LGHHPLVTLSEALKLTRVKDETKTSFHSKDIFKEILGDYKHLEKEFKELSNTAEKEGDKV
GENOMA 61 LGHHPLVTLSEAIKLTRVKEETKTSFHSKDIFKEILEDYKYLEKEFKELSNTAEKEGDKV
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FIG. 4(A)

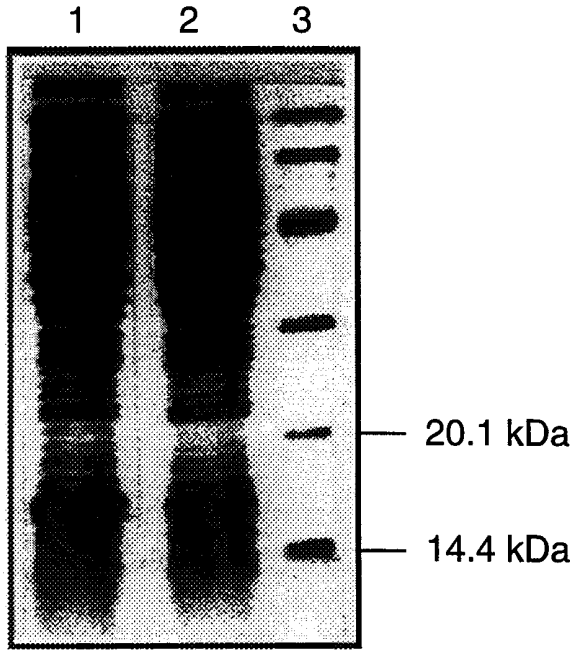


FIG. 4(B)

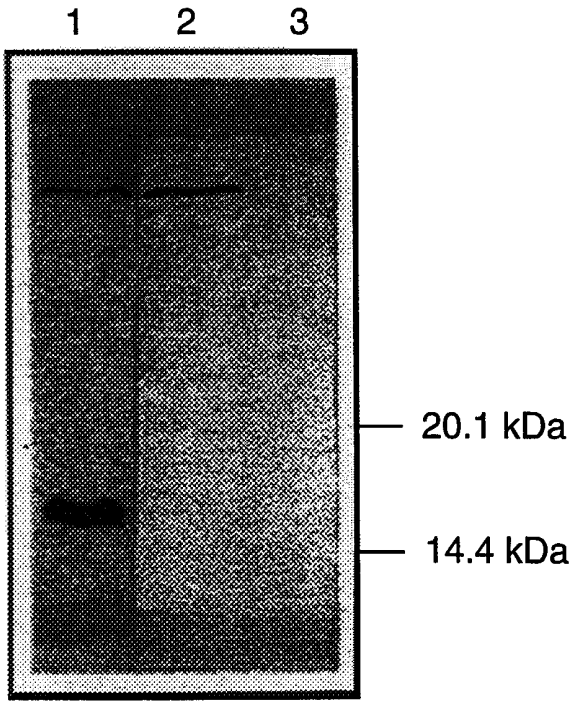


FIG. 5(A)

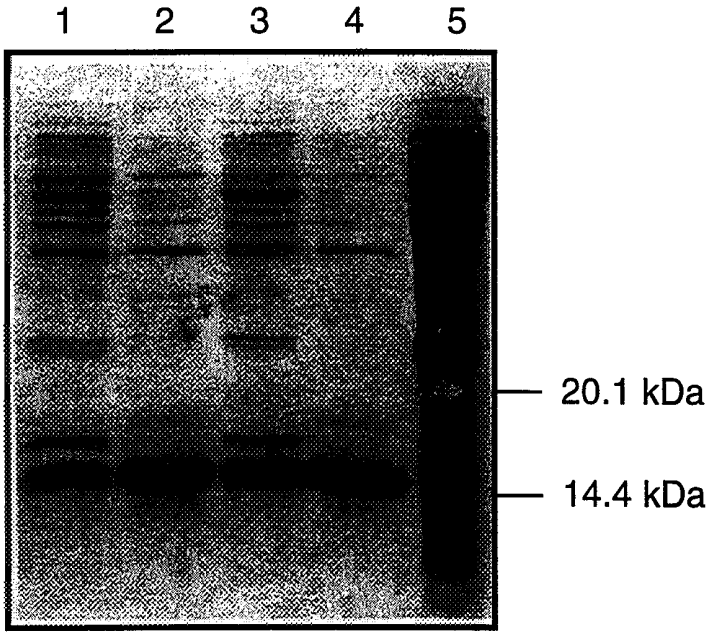


FIG. 5(B)

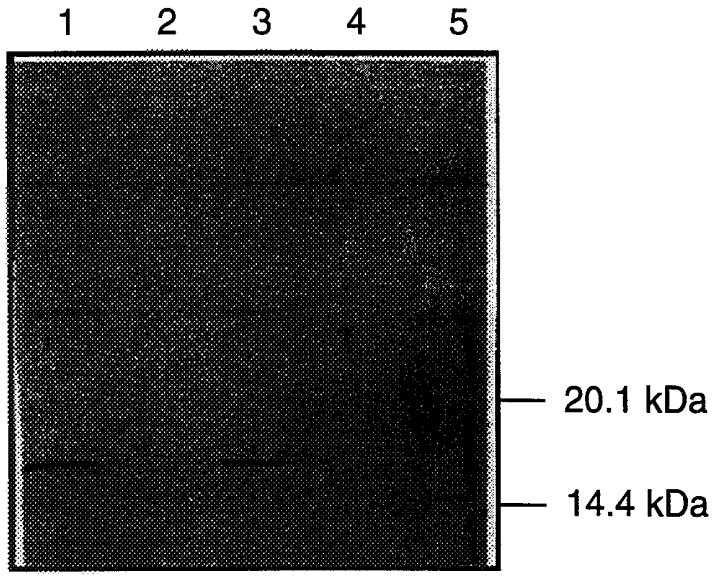


FIG. 6(A)

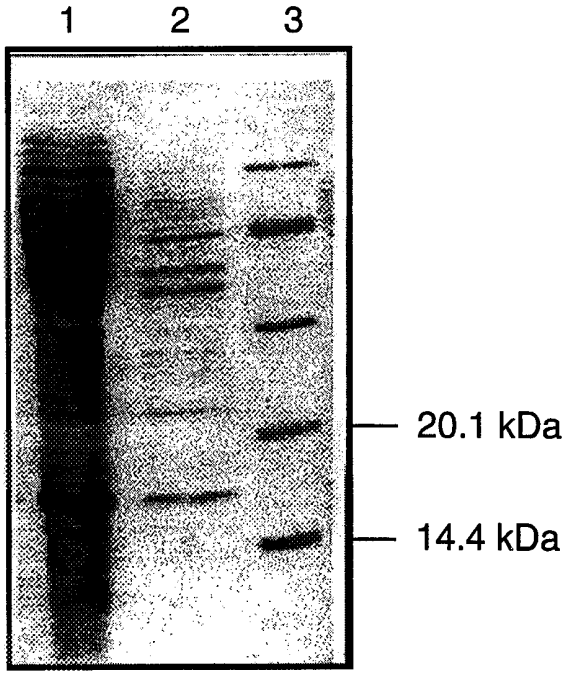


FIG. 6(B)

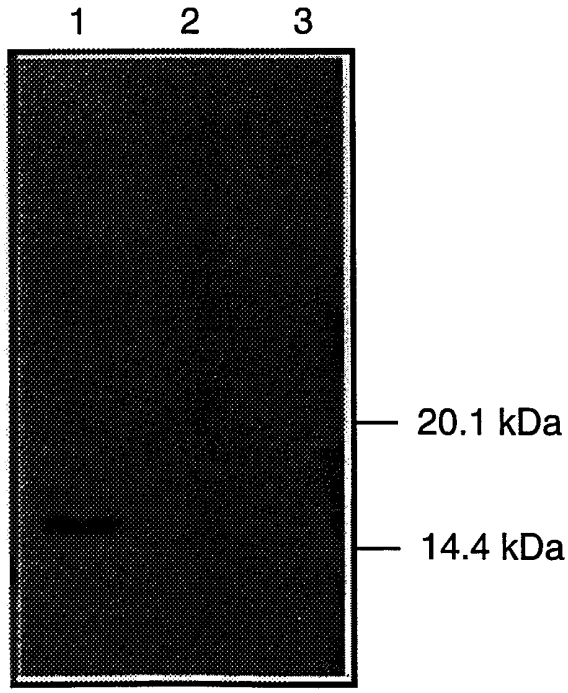


FIG. 7

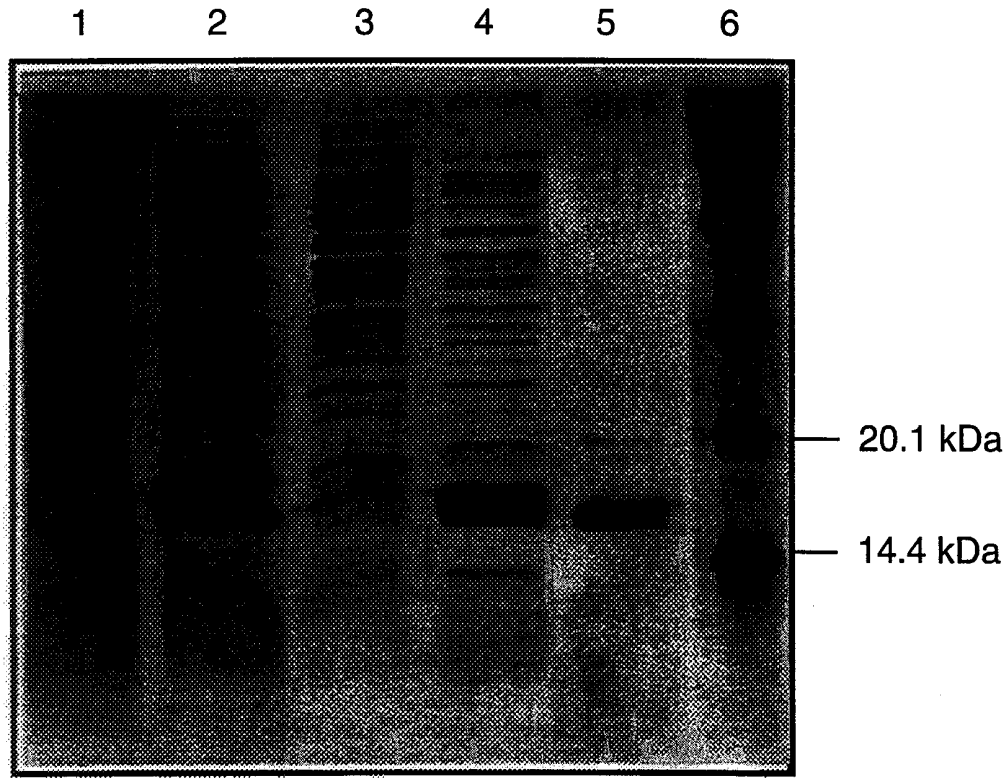
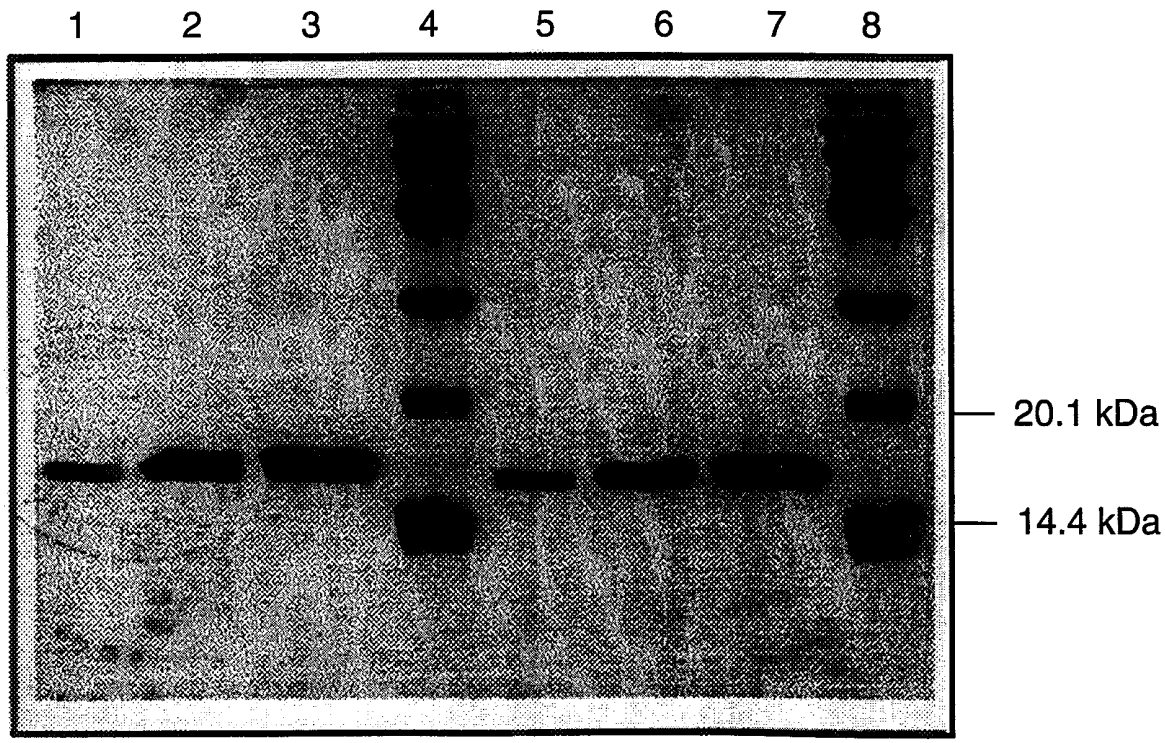


FIG. 8



INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/IB 99/00695

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N30/02 C07K14/205 C12N15/00 C07K16/12 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 06321 A (KOCHER THEODOR INST ;SANDOZ AG (CH)) 14 June 1990 (1990-06-14)	9-11
Y	the whole document	4-6

X	PATENT ABSTRACTS OF JAPAN vol. 006, no. 231 (C-135), 17 November 1982 (1982-11-17) & JP 57 132880 A (BANYU SEIYAKU KK), 17 August 1982 (1982-08-17)	1-3,7,8
Y	abstract	4-6

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 August 1999	02/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/IB 99/00695

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EVANS D J ET AL: "CHARACTERIZATION OF A HELICOBACTER PYLORI NEUTROPHIL-ACTIVATING PROTEIN" INFECTION AND IMMUNITY, vol. 63, no. 6, 1 June 1995 (1995-06-01), pages 2213-2220, XP002029039 ISSN: 0019-9567 cited in the application the whole document -----	9-11

1

INTERNATIONAL SEARCH REPORT

.nformation on patent family members

Inte onal Application No
PCT/IB 99/00695

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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