(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 March 2003 (27.03.2003)

PCT

(10) International Publication Number
WO 03/024482 A1

(51) International Patent Classification[7]: A61K 39/395, A01K 67/00, G01N 33/00, A01K 67/027, A01N 43/04

(21) International Application Number: PCT/US02/29081

(22) International Filing Date:
13 September 2002 (13.09.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/318,867 14 September 2001 (14.09.2001) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 03/024482 A1

(54) Title: CRUSTACEANS AS PRODUCTION SYSTEMS FOR THERAPEUTIC PROTEINS

(57) Abstract: The present invention relates to the production of food products containing the biomass of a crustacean expressing a heterologous protein. The food product can be administered as a means of delivering therapeutic proteins through passive immunity.

# CRUSTACEANS AS PRODUCTION SYSTEMS FOR THERAPEUTIC PROTEINS

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

[001] This invention is directed to the production and use of proteins, nucleic acids, viruses and virus-like particles in crustaceans. Such products may be of important therapeutic potential in humans, as well as in animals produced via agricultural or aquacultural practices. These products can be delivered to the humans or animals directly by consumption of the crustacean itself, or following purification of the active material which was produced by the crustacean. The crustacean produces the material as a consequence of its infection by a virus containing a gene or genes for the production of the material in the crustacean. In this way the genetic material of the crustacean itself is not changed.

### 2. Description of Related Art

[002] Certain human therapeutic proteins are now being expressed directly in plants by direct modification of the plant's genome so that it produces a protein that a plant may never have produced before. Such plants are referred to as genetically modified organisms (GMO's). There have been concerns raised about the use of GMO's in conventional foods because of the potential for novel allergens being produced from the incorporated genes. Nevertheless, plants provide an excellent and inexpensive mode of producing certain human therapeutic proteins.

[003] Modem agricultural biotechnology has also established a mode of using plants as the production system for certain human therapeutic proteins without directly modifying the genome of the plant itself. In such cases the plant (*e.g.* Tobacco) is infected with a virus (*e.g.* tobacco mosaic virus; TMV) which canies the

gene for the human therapeutic protein. In such cases the expression of the plant is modified without directly changing the plant's DNA. The human therapeutic protein is then isolated, purified and used for human therapeutic purposes.

[004] Recombinant microbes including bacteria, yeast and fungi have been used to produce human therapeutic proteins. However, such recombinant microbes generally do not produce exact mimics of such proteins that are made in mammalian cells due to changes in post-translational modifications. Furthermore, such recombinant microbes have not been used for agricultural purposes incorporating ingestion of the whole organism. In both the plant and microbial cases, the recombinant organism has simply been used as a factory, and the therapeutic protein is then isolated and purified prior to use.

[005] Certain recombinant proteins have been produced in insect cells using an insect virus expression system (Baculovirus). Such proteins are also produced in intact insect larvae following infection with modified Baculoviruses. In both cases, the insect cells or larvae are used as factories to produce the protein of interest, and the recombinant protein is then purified for pharmaceutical purposes. Proteins produced by insect cells are generally closer mimics to those produced in mammalian cells due to a closer approximation of post-translational modifications and the process is generally much less costly than the production in mammalian cells. However, the proteins produced by insect cells or insect larvae still require many steps of purification before they can be used therapeutically.

[006] Certain crustaceans (*e.g.*, brine shrimp) are used as feeds for various aquaculture crops (*e.g.*, fish or shrimp) as they represent live feed for important first

larval stages. In many cases the Artemia is "loaded" with certain microalgae, which are carried into the larval animal through the consumption of the artemia. In this way certain beneficial nutrients can be provided from the algae to the larvae through the feed.

## SUMMARY OF THE INVENTION

[007] It is an object of the present invention to provide to a composition of matter which is an animal feed or feed component comprising a therapeutic protein or peptide and the use of this feed for the delivery of a therapeutic dose of a bioactive peptide or protein to an animal consuming such a feed.

[008] In one embodiment, this invention provides a feed comprising a crustacean (*e.g.* artemia) which has been infected with a recombinant virus (*e.g.*, Baculovirus) which expresses or carries the genes to express a protein of therapeutic value (*e.g.*, an antibody, or a protein that will convey an immunological response, or an antimicrobial capability). Such a feed could provide an oral vaccination for the consuming species.

[009] In another embodiment of this invention, the artemia are used as a low-cost, large-scale production system for a therapeutic protein of interest. Such a protein may be consumable by human or other animals as part of the whole artemia biomass or, alternatively, the protein can be extracted and purified to be provided as a conventional pharmaceutical product either orally, by injection, or by transdermal or nasal delivery.

[010] The marine environment is filled with bacteria and viruses that can attack fish and/or shellfish. Infection by such bacteria or viruses can devastate intensive marine-based farms very quickly. The same is true for terrestrial

environments where viral or bacterial infections can also dramatically limit farm productivity. One of the major disease control problems in shrimp aquaculture today is infection by certain viruses (*e.g.*, White Spot, Taura, etc.). Neither antibiotic, nor probbitic strategies will work in this situation, and shrimp cannot be vaccinated by methods analogous to those used for fish. Shrimp, like all crustaceans have only a rudimentary immune system so they are particularly susceptible to devastation by viral attacks.

[011] This invention provides a solution to this problem by providing a nutritional control method using the target animal's feed as the vector to deliver anti-viral antibodies or fragments thereof, directly to the shrimp. These "Designer feeds" would deliver a therapeutic dose of antibody directly to the gut of the shrimp. This approach is known as "passive immunity" because the antibody remains outside the host organism and simply prevents viral infestation through the gut wall. The invention envisions the use of transgenic multicellular organisms (plants, animals, insects, etc) to deliver the antibody to the gut of the target animal through the consumption of the transgenic multicellular organism. Alternatively, the feed source itself may be infected with a host-specific virus that is engineered to produce the antibody, or fragment thereof, of interest in a multicellular organism that can be fed to the target animal. Alternatively, the feed material may deliver a portion of the virus (*e.g.* a coat protein) or fragment thereof in order to actively immunize the shrimp, other shellfish or finfish or terrestrial animal that consumes the feed.

## BRIEF DESCRIPTION OF THE DRAWINGS

[012] Figure 1 represents a Pacific white shrimp (*Penaeus vannamei*) that was transfected orally with an engineered Baculovirus (AcNPV-eGFP) to express GFP as a fusion protein. The recombinant GFP-tagged Baculovirus observed at 72 hours post-infection was located specifically within the hepatopancreas area in the shrimp's cephalothorax.

## DETAILED DESCRIPTION OF THE EMBODIMENTS

[013] This invention provides advantages in production and delivery of antimicrobial compounds, antibodies or therapeutic proteins by producing and packaging them in a crustacean source compared to a microbial source. Sterile fermentation is important for Drug-GMP compliance whereas the less-pure sources will be perfectly fine for food or feed use. Crustaceans are common elements in the food chain for either aquatic species or terrestrial species (including Aquaculture or Agriculture). One of the major problems with bacterial production of human proteins is that the microbially produced recombinant proteins are ineffective because of incorrect post-translation modifications. Eukaryotic processing (*e.g.*, by crustacean species) provides post-translational modifications that may be more "native" compared to recombinant products from bacteria.

[014] Antibodies or antibody fragments to desired targets, such as White Spot virus or Taura virus, may be prepared by routine immunization and selection of monoclonal antibody-producing hybridomas, or by screening viral or bacterial expression libraries of immunoglobulin genes and gene fragments. See "Current Protocols in Immunology," Collagen, et al., eds, Wiley-Interscience, 1991, and periodic supplements. Nucleic acid sequences encoding the binding sites of the

selected antibodies can be cloned using standard methods (see "Current Protocols in Molecular Biology," Ausubel, et al., eds, Wiley-Interscience, 1987, and periodic supplements), and antibodies may be expressed from recombinant plants, animals or insects or cloned into viruses that infect the desired feed materials.

[015] There are a number of well known bactericidal and bacteriostatic peptides which will inhibit microbial growth and include, but are not limited to cecropins, peneadins, bactenecins, callinectins, myticins, tachyplesins, clavanins, misgurins, pleurocidins, parasins, histones, acidic proteins, and lysozymes. According to this invention, these peptides may be made in a crustacean host using recombinant methods well known to those in the art, and optionally provided as a feed component to convey resistance or tolerance to infestation. Crustaceans are the foodstuffs for many aquaculture species, and this invention contemplates recombinant production of therapeutic proteins in the natural or farm diet of juvenile fish (*e.g.*, half-grown catfish) as well as fish larva. Thus, within the contemplation of this invention are host organisms that make up part of the food chain for the feeding of larvae, juveniles and adults in aquaculture, as well as the same life sequence in the terrestrial animal feeds (*e.g.* pigs, chickens, and cows).

[016] Edible materials can be any materials that are ingested. One embodiment of this invention would be where crustaceans are genetically modified to produce the exogenous peptide and/or antibody or antibody fragments directly, and modified crustaceans are ingested.

[017] Post-harvest processing of some sort may be required to prepare the material for use. This invention contemplates normal (known) processes for

converting the crustacean material into feeds. Such normal process include homogenization followed by extrusion into pellets of various sizes depending on the application (*e.g.*, larval, juvenile or adult). Other modes of preparation would include spray drying, fluid bed drying, or even providing the material as a liquid suspension. Alternatively, crustaceans which express therapeutic proteins may be grown and harvested, followed by isolation of a fraction containing the therapeutic protein from the crustacean. Optionally, additional processing steps may be applied to further purify the therapeutic protein as is known in the art.

**Examples**

[018] The invention as contemplated herein, is described in the following examples, but its utility is not limited to the examples provided.

[019] **Example 1. Production of viral antigen in artemia.** Infectious pancreatic necrosis virus (IPNV) is an example of a virus that can cause a high mortality in juvenile trout and salmon. In these animals, it is best to vaccinate as early as possible. Thus, it may be beneficial to deliver an oral vaccine at an early larval stage. The IPNV genome contains two segments; the larger segment contains the genes for the VP2, VP3 and VP4 viral coat proteins. A full length cDNA clone containing one or more of the viral coat protein genes, and a transfection marker (*e.g.*, Green Fluorescent Protein - GFP), is prepared using conventional molecular biology techniques. This fragment is then ligated into a Baculovirus transformation vector, such as pAcUW21, at the cloning site behind the P10 promotor region. The recombinant Baculovirus, containing both the viral coat protein gene and the GFP gene, is then used to transfect artemia. The infection and expression of the product of interest (*e.g.*, VP2) is monitored by the appearance of green fluorescence in the

artemia. Once expression is satisfactory, the artemia can be harvested and fed to the fish larvae. Ingestion of the VP2-expressing artemia by fish larvae will provide a mode of oral vaccination that is both inexpensive and effective.

[020] **Example 2. Production of antibodies to White Spot Virus (WSV) in artemia.** WSV contains three main viral coat proteins, and all three proteins can be used to prepare antibody fragments (Fabs), and the genes coding for those Fabs, using recombinant phage and expression in *E. coli*. The genes for these Fabs can be prepared and isolated by technology known to those of skill in the art. The gene will then be spliced into a Baculovirus vector and used to infect the artemia.

[021] Starting with 1-5 mg of antigen (each of the three viral proteins), two balb/c mice are immunized and the spleens are removed and splenocytes isolated. Messenger RNA is isolated from the splenocytes and purified on an oligo dT column. A cDNA library is prepared and amplified by PCR before gel analysis. Bands are isolated from the gels, and Vh and V, chains are precipitated with ethanol. Following assembly and fill-in reactions, a second amplification may be required prior to purification and isolation of the purified ScFv. The DNA is then digested with Sfi and Not1 and ligated into a plasmid such as pCANTAB5E. *E. coli* is then transformed and the recombinant phage library is rescued and triple panned to select the antigen-positive recombinant phage antibodies. The ScFv gene, coding for a Fab of highest specificity, is then isolated from the plasmid and ligated into a Baculovirus expression system. Such a virus may also contain an expression marker for transfection such as green fluorescent protein (GFP). Artemia can then be infected with the Baculovirus, and the extent of infectivity can be easily monitored by the intensity of the green

color. In such a case, the infected shrimp will also produce an antibody against WSV. Using biomass as a feed additive will introduce the antibody or antibody fragment directly into the animal, thus providing passive immunity.

[022] **Example 3. Expression of a bactericidal or bacteriostatic protein in artemia.** A bactericidal or bacteriostatic protein is chosen for the particular application. Suitable examples include proteins of the penaeidin class for pathogenic control in shrimp. Penaeldins are members of a family of antimicrobial peptides isolated from crustaceans (*e.g.*, Penaeus shrimp). Antimicrobial peptides may also come from insects and chelicerates and may include but are not limited to cecropins, peneadins, bactenecins, callinectins, myticins, tachyplesins, clavanins, misgurins, pleurocidins, parasins, histones, acidic proteins, and lysozymes. The gene for the chosen protein or peptide is either isolated from the original source, or an amplification source, or it can be made synthetically. The gene is spliced into a bacculorvirus vector which may also contain the gene for Green Fluorescent Protein (GFP). The virus is then used to tranfect artemia and the production of the bacteriostatic or bacteriocidal protein is monitored by the intensity of expression of GFP. Upon isolation of artemia producing the material at the appropriate level, the artemia are added to the diet of the fish or shrimp. In this way the bateriostatic or bacteriocidal proteins are delivered directly to the gut of the animal in the form of the artemia itself. The entire artemia can be used as a feed or feed component. Alternatively, the crustacean may be homogenized and extruded into pellets suitable for feed applications. The Baculovirus vectors can be inactivated by high temperature or other procedures familiar to those experts in the field prior to use as feeds.

[023] **Example 4. Expression of Human Insulin in Artemia.** Human insulin is a human therapeutic protein that may be produced in crustaceans. The gene for human insulin (or any other human therapeutic protein) is obtained, or synthetically produced, and ligated into a Baculovirus expression vector. The Baculovirus expression vector may also contain a transfection marker such as Green (or Red) Fluorescent Protein (GFP or R-FP). The Baculovirus is then used to infect a culture of a crustacean such as artemia. The level of expression of the therapeutic protein will be determined by the intensity of the fluorescence caused by the GFP or RFP. At the point of maximal production of the therapeutic protein, the crustaceans are harvested. Brine shrimp may be used directly as a wet paste, or dried by some appropriate process (*e.g.* spray drying, extrusion, etc.) which retains the integrity of the product. Alternatively, the brine shrimp may be broken and the protein of interest isolated and purified by conventional biochemical methodologies. The purified protein may be used in an oral, nasal, dermal or injectable delivery form.

[024] **Example 5. Production from Other Crustaceans and Rotifers.** Using the engineered Baculoviruses described in any of examples 1-4, other crustaceans (*e.g.*, crab, crawfish, lobster, etc) can also be infected. Such an infection might occur at a much later stage in the life cycle, such that the fully mature crustacean may carry the pharmaceutical product in a much more food acceptable form. Alternatively, certain other crustaceans (*e.g.*, crab, crawfish) may be used as a vector for delivery of the therapeutic proteins following the procedures in examples 1-4. Alternatively, rotifers may be used as a vector for delivery of the therapeutic proteins following the procedures in examples 1-4.

[025] **Example 6. Incorporation of a Gene for a Therapeutic Protein into Baculovirus and the Use of the Infected Material as Feed.** Pacific white shrimp (*Penaeus vannamei*) were transfected orally with an engineered baculovirus (AcNPV-eGFP) to express GFP as a fusion protein. The Bacmid Bac-to-Bac® Baculovirus Expression system (Invitrogen) was utilized for cloning and transfection. A 720 kb fragment containing GFP was fused to the polyhedron (pPolh) promoter and flanked by Xho I sites 3' to pPolh. Using methods described in the Invitrogen product literature, Sf9 insect cells were transfected with the recombinant baculovirus. After 72 hours, plaque formation was visually confirmed, and 70 ml culture fluid medium was pelleted at 100 g for 5 minutes at 4°C. The resulting cell pellet was maintained at 4°C until it was subsequently used for oral infection. The corresponding resulting supernatant fluid was centrifuged for 2 hours at 80,000 g at 4°C on a 27% sucrose gradient to yield purified virus. This sucrose-purified virus pellet was maintained at 4°C until it was subsequently used for oral infection.

[026] Shrimp isolation chambers consisting of 3-qt containers filled with 30 ppt salinity dechlorinated water were provided with air stones for oxygenation. Three one-gram shrimp were placed in each container and allowed to acclimatize overnight.

[027] The following procedures were performed within 30 minutes prior to feeding the shrimp. A pellet matrix was prepared by first adding 100 mg of alginic acid (Sigma) to 10 ml of distilled deionized water ($ddH_20$) in a beaker and heating to 40 40°C while stirring. After the gel began to form, 150 mg of starch (Sigma) was added. The solution was allowed to mix for a minute before addition of 500 mg of krill meal. While continuing to stir the solution, the heat source was removed.

[028] An aliquot of pellet matrix (500 µl) was combined with either 5 µl of the infected cell pellet or 5 µl of sucrose-purified virus, and gently mixed with vortex a mixer. The infected pellet matrix was aspirated into a tuberculin syringe to which a 21-gauge needle was subsequently attached. A formation solution was formed by dissolving 5 grams of calcium chloride (J.T. Baker) and 1 gram of sodium chloride (Research Organics) into 100 ml of $ddH_2O$. While the formation solution was stirring slowly, the matrix was squeezed through the needle into the solution to form tubular pellets. Pellets formed in solution immediately upon impact and a spatula was used to clean the needle between pellets. Pellets appeared to be 25-30 µl in volume. The pellets were washed in 10% NaCl and added to the shrimp isolation containers. The shrimp immediately consumed the pellets, and were fed to satiation. Each shrimp consumed approximately one pellet.

[029] Seventy two hours after consuming the virally infected matrix, the shrimp were placed in a petri dish and observed on a Dark Reader® transilluminator (Claire Chemical Research). Shrimp expressing GFP exhibited a greenish glow (Figure 1). Uninfected shrimp demonstrated no fluorescence. The recombinant GFP-tagged Baculovirus observed at 72 h was located specifically within the hepatopancreas area in the shrimp's cephalothorax (Figure 1).

## REFERENCES

[030] The specification is most thoroughly understood in light of the following references, all of which are hereby incorporated in their entireties.

[031] Ausubel, *et al.*, eds., (1987 and periodic supplements) *Current Protocols in Immunology*, Wiley-Interscience.

[032] Bac-to-Bac Baculovirus expression systems manual. Invitrogen Life Technologies. Cat. No. 10359-016

[033] Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994) Green Fluorescent Protein as a Marker for Gene Expression. *Science.* 263: 802-805.

[034] Collagen, *et al.*, eds. (1991 and periodic supplements) *Current Protocols in Immunology*, Wiley-Interscience.

[035] Inoue, S., and Tsuji, F.I. (1994) *Aequorea* green-fluorescent protein: Expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Letters.* 341: 277-280.

[036] Lewis, David L., De Camillis, Mark A., Brunetti, Craig R., Halder, G., Kassner, Victoria A., Selegue, J.E., Higgs, S., and Carroll, S.B. (1999) Ectopic gene expression and homeotic transformations in arthropods using recombinant Sindbis viruses. *Current Biology* 9:1279-1287.

[037] Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J. (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene.* 111: 229-233.

CLAIMS

What is claimed is:

1. An aquaculture, animal or human feed containing a crustacean or parts thereof comprising one or more proteins, including antibodies, antibody fragments, virus-like particles or a combination thereof, wherein said proteins are non-native to the crustacean, and preferably wherein said proteins are non-native to the virus.

2. The aquaculture, animal or human feed containing a crustacean or parts thereof which have been infected with viruses containing genes which code for the expression of proteins, including antibodies, antibody fragments, virus-like particles or a combination thereof, and preferably wherein said proteins are non-native to the virus.

3. A feed containing a therapeutic protein wherein the therapeutic protein is produced by a crustacean.

4. A human therapeutic protein coded for by recombinant viral DNA and produced in a crustacean.

5. A method of delivering therapeutic proteins to a human or nonhuman animal comprising administering a food or feed comprising a crustacean expressing a non-native therapeutic protein.

6. The method of delivering therapeutic proteins wherein the non-human animal is subjected to intensive agricultural practices.

7. The method of delivering therapeutic proteins wherein the non-human animal is fish or shellfish in aquaculture.

8. The method of delivering therapeutic proteins to a human or nonhuman animal comprising administering a feed comprising a crustacean wherein the crustacean is infected by a recombinant virus such that the therapeutic protein is expressed recombinantly.

9. The method of delivering a therapeutic protein wherein the therapeutic protein is a protein which inhibits growth or replication of *Vibrio* species *in vitro*.

10. The method of delivering a therapeutic protein wherein the therapeutic protein is a protein which inhibits Taura or White spot virus infection in shrimp.

11. The method of delivering a therapeutic protein wherein the therapeutic protein is a recombinantly expressed antibody or fragment thereof.

12. A method of delivering a therapeutic protein to a human, wherein the therapeutic protein is produced by a crustacean.

13. The method according to any of the preceding claims wherein the therapeutic protein is a protein naturally expressed in humans.

14. The method according to any of the preceding claims wherein the therapeutic protein acts to supply a deficiency in a patient to whom the protein is administered.

15. The method of claim 12, wherein the organism is infected by a recombinant virus which encodes the therapeutic protein and the therapeutic protein is expressed recombinantly.

16. The method of claim 12, wherein the therapeutic protein is a protein which inhibits growth or replication of *Vibrio* species *in vitro*.

17. The method of claim 12, wherein the therapeutic protein is a protein which inhibits Taura or White spot virus infection in shrimp.

18. The method of claim 12, wherein the therapeutic protein is a recombinantly expressed antibody or fragment thereof.

19. The method of claim 12, wherein the recombinantly expressed antibody or fragment thereof specifically binds to an infectious agent of disease in the non-human animal.

20. A method of delivering therapeutic proteins to crustaceans comprising infecting said crustaceans with a recombinant *Autographa californica* nuclear polyhedrosis virus and feeding said crustaceans edible material containing said virus.

21. The method of claim 20, wherein the source of said virus is the supernatant of cultured cells infected with said virus.

22. The method of claim 20, wherein the source of said virus is cultured cells infected with said virus.

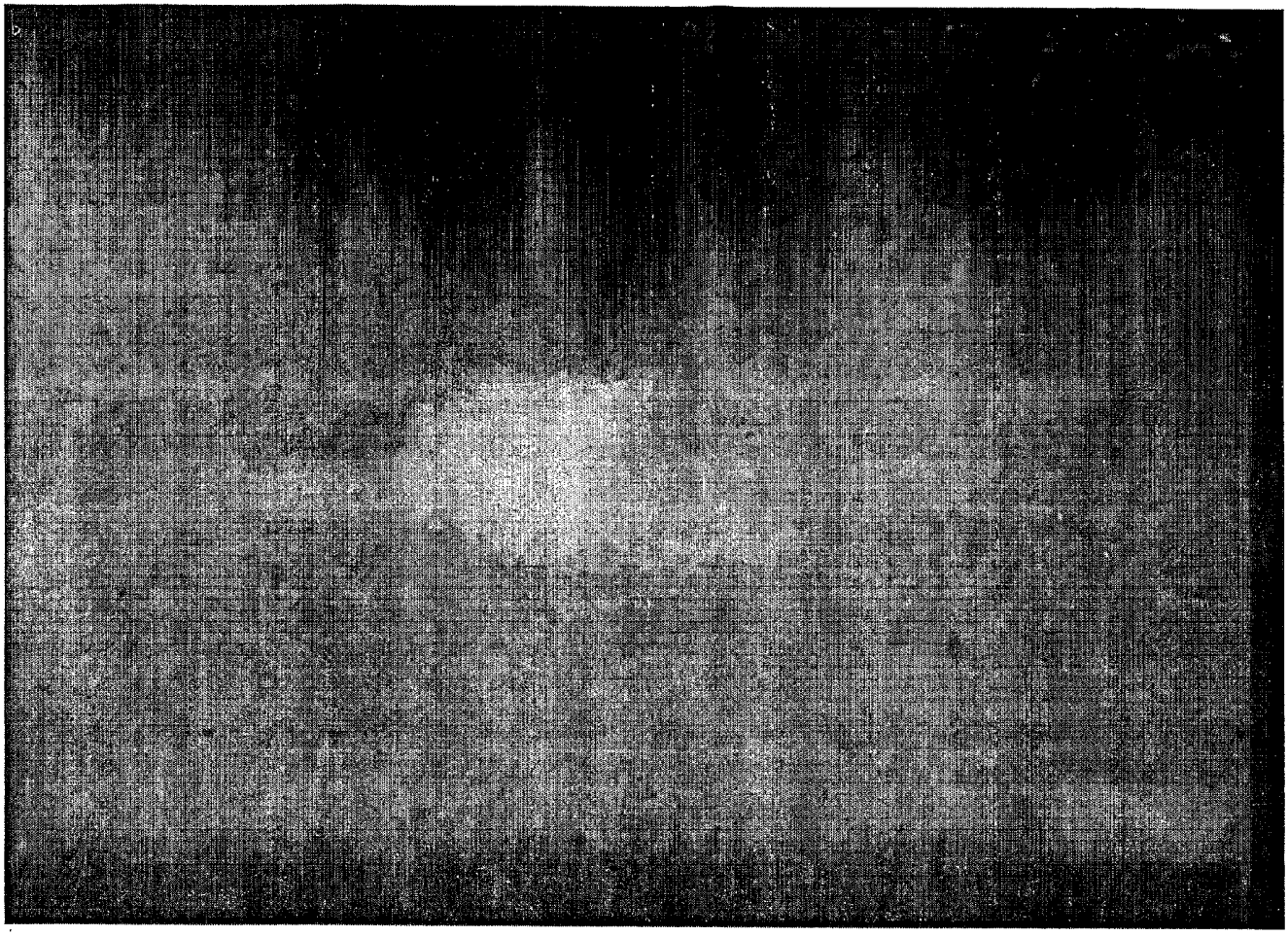

**Figure 1**

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/29081

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : A61K 39/395; A01K 67/00; G01N 33/00; A01K 67/027; A01N 43/04
US CL : 424/807; 800/8; 800/3; 800/20;514/44

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)
U.S. : 424/807; 800/8; 800/3; 800/20;514/44

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 practicable, search terms used)
EAST, STN-MEDLINE, CAPLUS, LIFESCI, EMBASE, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 4,141,986 (CASSIDY et al) 27 February 1979 (27.02.1979, entire document. | 9 |
| Y | WO 00/75288 A1 (REGENTS OF THE UNIVERSITY OF CALIFORNIA) 14 December 2000 (14.12.2000), page 4, lines 25-30, see entire document. | 1-8,12,15 |
| --- | | --- |
| A | | 20-22 |
| Y | MASON, H.S. et al. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. PNAS, May 1996, Vol. 93, pp. 5335- | 1,3-7,12 |
| Y | MODELSKA, A. et al. Immunization against rabies with plant derived antigen. PNAS, March 1998, Vol. 95, pp. 2481-2485. | 1-8,12,15 |
| Y | YU, J. A plant based multicomponent vaccine protects mice from enteric diseases. Nature Biotech. June 2001, Vol. 19, pages 548-552. | 1,3-7,12 |
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| Y | US 5,202,422 A (HIATT et al.) 13 April 1993 (13.04.1993), col. 3, lines 37-43; col. 19, lines 42-45. | 1,3-7,11,12,18,19 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* 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 application or patent 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 when the document is taken alone

"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

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 20 December 2002 (20.12.2002) | 27 JAN 2003 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703)305-3230 | Authorized officer<br>Valarie Bertoglio<br>Telephone No. (703) 308-1234 |

Form PCT/ISA/210 (second sheet) (July 1998)

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/29081

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 5,681,746 A (BODNER et al) 28 October 1997 (28.10.1997), column 3 lines 40-45. | 13-14 |
| A | US 2001/0006953 A1 (POET et al) 05 July 2001 (05.07.2001), entire document. | 9,10 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/29081

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

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

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

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

4. ☐ 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 claims Nos.:

**Remark on Protest**

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

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/29081

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-19, drawn to a feed containing biomass of a non-recombinant crustacean that expresses a therapeutic protein and a method of delivering said therapeutic protein to an organism by administering the feed.

Group II, claim(s) 20-22, drawn to a method of delivering therapeutic proteins to a crustacean comprising administering a virus via food containing said virus.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Unity of invention between different categories of inventions will only be found to exist if the specific combinations of inventions are present. Those combinations include:

1) A product and a special process of manufacture of said product.
2) A product and a process of use of said product.
3) A product, a special process of manufacture of said product, and a process of use of said product.
4) A process and an apparatus specially designed to carry out said process.
5) A product, a special process of manufacture of said product, and an apparatus specially designed to carry out said process.

The allowed combinations do not include multiple products, multiple methods of using said products, and methods of making multiple products as claimed in the instant application, see MPEP § 1850. Groups I and represent different methods with distinct method steps and and technical considerations.