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(54) Title: COMPOSITION CONTAINING COLLAGENASE AND CHYMOPAPAIN FOR ISOLATING HEPATOCYTES AND PANCREATIC ISLET CELLS (57) Abstract Proteolytic enzyme compositions and processes for isolating hepatocytes and pancreatic islet cells from tissue containing them are provided. The enzyme compositions include collagenase, which is essentially free of toxins and non-collagen specific components, and chymopapain, which is essentially free of toxins. The enzyme compositions preferably contain an aqueous mixture of collagenase having an activity of about 25 nkat/ml to about 120 nkat/ml, and chymopapain having an activity of about 0.15 nkat/ml to about 0.55 nkat/ml.		

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COMPOSITION CONTAINING COLLAGENASE AND CHYMOPAPAIN FOR
ISOLATING HEPATOCYTES AND PANCREATIC ISLET CELLS

BACKGROUND OF THE INVENTION

This is a continuation-in-part of copending application Serial No. 08/049,015, filed on April 16, 1993.

Field of the Invention

5 The present invention generally relates to proteolytic enzyme compositions and procedures for digesting connective tissue. More particularly, the present invention is directed to proteolytic enzyme compositions which can be used for reliably and reproducibly isolating hepatocytes
10 and pancreatic islet cells and to the processes used for such cell isolation.

Description of Relevant Art

Proteolytic enzymes have found wide utility in a variety of laboratory and clinical applications. Typically
15 these applications involve cell dissociation and related therapeutic procedures which are benefitted by the ability of proteolytic enzymes to hydrolytically break-up or loosen connective tissue networks. For example, bacterial collagenase derived from Clostridium histolyticum has been
20 used to disperse cells in laboratory tissue culture applications. Additionally, collagenase has demonstrated utility in cell isolation procedures such as those associated with isolating pancreatic islets and dispersing a variety of tumor cells. Other uses for collagenase involve its
25 topical use in clinical applications in which collagenase compositions are applied in the treatment of burns or ulcers and wound healing. Other uses include the treatment of Peyronie's disease and as an adjunct to cryoprostectomy for the removal of retained cryoslough, inter-
30 vertebral discolysis, and in ophthalmic surgery.

Like collagenase, chymopapain, the major proteolytic component of the crude latex of Carica papaya, has been utilized in the treatment of abnormal or herniated discs to selectively dissolve the nucleus pulposus of the disc.

5 Other uses associated with chymopapain include its utility in cryosurgical healing processes.

Combinations of proteolytic enzymes such as compositions of bacterial collagenase and hyaluronidase are reportedly particularly useful for digesting or dissolving

10 prostatic tissue in the treatment of benign prostatic hypertrophy. The combination of these two proteolytic enzymes apparently dissolves prostatic tissue in order to relieve the obstructive symptoms of prostatic hypertrophy.

Recently, bacterial collagenase derived from Clostri-

15 dium histolyticum has found utility in procedures involving the dissociation and isolation of microvessel cells embedded in fatty tissues. These procedures generally involve combining fatty tissues having embedded microvessels, such as liposuctioned fat, with collagenase under

20 conditions which cause the collagenase to disrupt and digest the connective tissue. By carefully separating the cells from the digested tissue, viable microvessel cells are recovered.

These viable and intact microvessel cells have found

25 particular utility as a coating on the interior of synthetic small diameter vascular grafts implanted in humans and animals to replace blood vessels. Similarly, microvessel cells are useful as deposits on the surface of biomedical implant devices in general where they provide an

30 improved biocompatibility to the implant. Apparently the microvessel cells contribute to the prevention of protein deposits and related cellular deposits on the implants which are known to occur when foreign materials are placed in contact with blood and tissue. In the case of vascular

35 grafts these deposits can quickly cause the vessel to occlude, resulting in the functional failure of the graft.

One problem associated with the use of commercial sources of crude collagenase to digest fatty tissues, as well as connective tissue in general, is that the degree to which tissue digests or hydrolyzes is unpredictable.

5 Moreover, cells which are isolated from tissue digestion procedures utilizing crude collagenase can be inferior in quality and have a low degree of viability and efficacy. Even when viable cells are successfully isolated, the yield and degree of viability is unpredictable.

10 The unpredictable nature of these procedures may be attributed to the lot variations inherent in commercial sources of crude collagenase. Another factor which may contribute to the lack of reproducibility in these procedures is the nature of the mixture of tissues being di-
15 gested. While connective tissues are formed largely of collagen, for which collagenase is specific in its hydrolytic activity, significant amounts of other proteins and glycoproteins are additionally found in connective tissue matrices. Thus, collagenase alone may not effectively
20 hydrolyze all of the tissue mixtures.

Further, collagenase derived from native bacteria differs widely in its collagen specific hydrolytic activity and the amount and character of impurities, including other proteases and toxins. The protease impurities in
25 crude collagenase contribute to the hydrolysis of minor proteins in connective tissue and actually aid in the digestive process. However, unfortunately protease impurities are active with proteins generally and can damage cells. Proteases can also react with collagenase, causing
30 the crude collagenase to be subject to catalytic degradation. The toxin impurities associated with crude collagenase can be a serious problem for procedures involving both in vivo and in vitro applications. Toxins can disrupt cell membranes, destroy cell viability and generally
35 lower cell yield. Additionally, impurities can contain variable amounts of bacterial DNA, which may cause cell

damage and possible immunological problems when isolated cells or tissue digestion procedures involve in vivo applications. Finally, the non-collagenase impurities found in crude collagenase may act as sensitizing antigens which
5 can cause anaphylactic shock if administered to patients.

Thus, in view of the varying and unpredictable nature of crude collagenase compositions which contain a host of proteolytically active and unreactive compounds as well as toxins, the use of crude collagenase compositions for
10 therapeutic digestion procedures and cell dissociation techniques can be unreliable. Alternatively, using purified collagenase having essentially only collagen specific hydrolytic reactive components in these tissue digestion procedures has not been successful. The failure of
15 purified collagenase in these procedures is apparently due to the tissue containing non-collagen proteins which are not digested by collagen specific collagenase. Crude collagenase will digest these tissues because it contains other proteolytic enzymes. However, it does so to a
20 varying and unpredictable degree.

It has been suggested that bacteria genetically engineered to produce limited forms of collagenase having known molecular weights and hydrolytic activity may be advantageous when utilized in tissue digestion procedures.
25 However, even when a wide range of isomeric forms of collagenase are utilized in tissue digestion procedures, the narrow specificity of collagenase in general precludes effectively hydrolyzing all of the tissue. This is because the wide spectrum of proteolytic activity and non-collagen
30 specificity supplied by crude collagenase derived from native bacteria is not available in these genetically engineered sources of collagenase. Even when toxins are removed from genetically engineered sources of collagenase, the resulting collagenase compositions do not provide
35 hydrolytic characteristics suitable for efficacious tissue digestion and/or cell dissociation procedures.

Accordingly, it is a principal object of the present invention to provide proteolytic enzyme compositions capable of dissociating tissue containing viable hepatocytes and pancreatic islet cells with predictable and reproducible yields and quality.

Another object of the present invention is to provide proteolytic enzyme compositions capable of digesting connective tissue in a reproducible and predictable manner.

It is another object of the present invention to provide tissue digestion procedures and associated therapeutic procedures which provide reproducible and predictable results.

It is a further object of the present invention to isolate viable and efficacious hepatocytes and pancreatic islet cells for various medical uses.

SUMMARY OF THE INVENTION

The present invention accomplishes the above objectives by providing proteolytic enzyme compositions capable of predictably and reproducibly digesting tissue containing hepatocytes and pancreatic islet cells. Further, the present invention provides processes for digesting tissue and dissociating cells from the connective tissue, providing efficaciously viable hepatocytes and pancreatic islet cells in high yield. The viable cells thereby provided have utility in a variety of in vivo and in vitro applications.

The present invention is based upon the discovery that, although neither collagenase nor chymopapain alone is effective to digest tissue, a mixture of collagenase and chymopapain can be used to safely, reproducibly and reliably digest connective tissue formed of a variety of proteins and glycoprotein extracellular matrix material. As a feature of the present invention, compositions of purified collagenase and chymopapain have been found to effectively digest connective tissue and reproducibly dissociate and isolate cells embedded in the tissue,

providing the isolated cells in high yield. Moreover, because the isolated cells have been processed with compositions of purified enzymes, the cell suspensions are essentially free of the harmful effects of toxins and unknown unreactive materials, making them highly viable and safe for in vivo use.

More particularly, the present invention provides novel enzyme compositions and associated methodologies useful for hydrolyzing connective tissue in biological systems. The enzyme compositions of the present invention principally comprise a combination of collagenase, in an amount sufficient to hydrolyze collagen in the biological system, and chymopapain in an amount sufficient to hydrolyze chymopapain active tissue in the biological system. Preferably, as a feature of the present invention the collagenase and chymopapain are purified and essentially free of toxic components, such as bacterial DNA and sensitizing antigens, and the collagenase is essentially free of non-collagen specific components.

It is also within the scope of the present invention to provide associated processes utilizing these enzyme compositions of collagenase and chymopapain. These processes effectively hydrolyze connective tissue systems and dissociate cells embedded in the tissue to produce highly efficacious and viable cells in high yield. An exemplary process of the present invention includes enzymatically digesting connective tissue by providing an enzyme composition of collagenase, essentially free of collagen inactive components and toxins and in an amount sufficient to hydrolyze the collagen present in the connective tissue, and chymopapain essentially free of toxins and in an amount sufficient to hydrolyze chymopapain active tissue in the connective tissue. Contacting the enzyme composition with the connective tissue produces a turbid-appearing system indicating substantial tissue hydrolysis.

More specifically, a preferred process of the present invention utilizes the above steps to hydrolyze connective

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tissue in order to dissociate and isolate hepatocytes and pancreatic islet cells embedded in the tissue. Advantageously, hepatocytes and pancreatic islet cells which form part of the mixture are dissociated from the connective
5 tissue and isolated in higher yield and have improved viability when compared with cells isolated from tissue hydrolyzed according to prior art procedures which utilize crude collagenase. The higher yield of such cells provided by the processes of the present invention is characterized by the increased number of viable healthy cells.
10

The increased yield as well as increased viability and integrity of cells isolated according to the processes of the present invention are readily demonstrated by laboratory testing techniques. More particularly, cell-counting
15 techniques provide cell size information and information relating to the distribution of cell sizes in a given batch of isolated cells. Similarly, cell function and efficacy are demonstrated by their biological function, such as production of insulin by pancreatic islet cells in response to glucose concentration change in culture media.
20 This response is characterized by the ratio of insulin production in the presence of glucose to the base-line value.

The greater viability and number of useful cells isolated according to the teachings of the present invention
25 are particularly important for applications which involve various medical procedures such as transplanting hepatocytes or pancreatic islet cells into individuals suffering from liver or pancreatic disease.

30 Further objects, features, and advantages of the present invention will become apparent to those skilled in the art from a consideration of the following detailed description, taken in conjunction with the associated drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the particle size distribution of rat hepatocyte cells isolated using crude collagenase in accordance with the prior art;

5 Fig. 2 illustrates the particle size distribution of rat hepatocyte cells isolated using purified collagenase-chymopapain enzyme compositions in accordance with the present invention;

10 Fig. 3 illustrates the static incubation glucose response by porcine pancreatic islet cells isolated using crude collagenase according to the prior art; and

Fig. 4 illustrates the static incubation glucose response by porcine pancreatic islet cells isolated using purified collagenase-chymopapain enzyme compositions
15 according to the present invention.

DESCRIPTION OF EXEMPLARY EMBODIMENTS

The present invention provides proteolytic enzyme compositions and processes capable of predictably and reproducibly digesting physiological connective tissue in
20 a variety of therapeutic and laboratory applications. These applications range from in vivo therapeutic treatment procedures to techniques which involve dissociating and isolating cells embedded in connective tissue for subsequent laboratory or clinical applications.

25 The compositions and processes of the present invention are suitable for reproducibly hydrolyzing or digesting a wide variety of collagens, non-collagenous connective tissue proteins, glycoproteins, and extracellular matrix materials. Those skilled in the art
30 will appreciate that the ability to hydrolyze a wide range of proteins and protein mixtures makes the teachings of the present invention widely applicable in a number of in vivo as well as in vitro tissue digestion procedures.

The compositions and processes of the present invention
35 find particular application in cell dissociation procedures including laboratory cell culture methods and re-

lated cell isolation techniques. As a feature of the present invention, cells can be effectively and reproducibly isolated from a host of different proteinaceous connective tissues and harvested in higher yield with improved preservation of the cell membranes. Moreover, these cells have better viability and are free of toxins and contaminants, when compared with cells isolated using prior art processes. For this reason, the compositions and processes of the present invention are particularly suitable for isolating hepatocytes and pancreatic islet cells embedded in connective tissues for subsequent utility in various clinical procedures such as transplanting hepatocytes or pancreatic islet cells into individuals suffering from liver, pancreatic, or other types of diseases.

More particularly, the enzyme compositions of the present invention include purified collagenase in an amount sufficient to hydrolyze collagen present in the system, and chymopapain in an amount sufficient to hydrolyze chymopapain active tissue in the system. The collagenase and the chymopapain are purified and essentially free of toxic components such as bacterial components and sensitizing antigens. Additionally, the collagenase is free of collagen inactive components.

Preferred exemplary embodiments of the present invention are solutions of collagenase and chymopapain in a physiologically compatible liquid. Suitable physiologically compatible liquids include phosphate buffered saline solutions and similar buffered electrolyte solutions having osmolalities which are compatible with physiological tissue. A particularly suitable commercially available electrolyte solution is Plasmalyte® electrolyte solution available from Baxter-Hyland, having a buffered pH of 7.4 and an osmolarity of 294 mOsmol/L obtained with controlled concentrations of sodium, potassium, magnesium, chloride, acetate, and gluconate ions. As illustrated below, addi-

tives such as human serum albumin and serum are preferred in many applications.

Those skilled in the art will appreciate that the concentration or amount of each enzyme present in the solutions will vary with the amount and the type of tissue to be hydrolyzed. The well-known principles of enzyme activity are applicable and basic experimentation involving techniques designed to optimize enzyme concentration and total activity provide necessary information to assure the effective hydrolysis of the amount and type of selected connective tissue. For applications directed toward digesting connective tissue and isolating hepatocytes and pancreatic islet cells embedded in the tissue, exemplary compositions of the present invention include a solution of from about 25 nkat/ml to about 120 nkat/ml purified collagenase and from about 0.15 nkat/ml to about 0.55 nkat/ml purified chymopapain in a suitable pH buffered physiologically compatible liquid. The nkat/ml unit is defined as nanomoles of substrate hydrolyzed per second by 1 ml of enzyme solution under the assay condition used. For purposes of the present invention, enzyme activity assays were conducted at 37°C. In a preferred embodiment of the present invention, the enzyme composition is a solution of 30 nkat/ml purified collagenase and 0.3 nkat/ml chymopapain in a solution of about 0.4 wt% human serum albumin in Plasmalyte® electrolyte solution for the isolation of hepatocytes and a solution of from about 25 nkat/ml to about 120 nkat/ml of purified collagenase and about 0.15 nkat/ml to about 0.55 nkat/ml chymopapain for pancreatic islet cells.

Most commercial collagenase is derived from the bacterium Clostridium histolyticum and in its crude form differs from batch to batch in hydrolytic activity and purity. Uncontrolled amounts of impurities found in crude collagenase may include contaminating bacterial components, pigment, pyrogens, proteases, and peptidases, including clostripain, trypsin, and caseinase. On the

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other hand purified collagenase, suitable for use in the compositions of the present invention, is substantially free of pigment, bacterial components, and nonspecific enzyme activity. Crude collagenase is readily available from a number of commercial sources including Sigma Chemical Company of St. Louis, Missouri, and Boehringer Mannheim Biochemicals of Indianapolis, Indiana. Commercial sources of crude forms of collagenase are typically packaged in a lyophilized powder form which helps maintain its stability. Alternative sources of collagenase which are substantially free of toxins and collagen inactive compounds include collagenase purified according to the process described in Bond et al. "Purification and Separation of Individual Collagenases of Clostridium histolyticum Using Red Dye Ligand Chromatography", Biochemistry Vol. 23, No. 13, 3077-3091, 1984. By removing non-specific enzyme activities and minimizing or eliminating sensitizing antigens in order to minimize immunogenicity, purified collagenase suitable for use in accordance with the present invention exhibits a batch-to-batch uniformity in specificity for collagen as well as toxin-free characteristics.

Similarly, chymopapain, a proteolytic enzyme extracted from papaya latex, is commercially available in a dry lyophilized state from a number of sources including Sigma Chemical of St. Louis, Missouri. Chymopapain is available in crude, partially purified, and more highly purified forms which differ in the amount of papain, lysozyme peptidase A and sensitizing antigens found in the preparation. Chymopapain suitable for use in the present invention is characterized as having essentially no immunogenicity and essentially no toxicity as a result of purification processes. Chymopapain from most commercial sources, which has been purified using known chromatographic purification processes, provides chymopapain suitable in the practice of the present invention. Alternatively, puri-

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fied chymopapain can be prepared using, for example, the process described in U.S. Patent No. 4,719,108.

An exemplary tissue system demonstrating the features of the present invention is connective tissue. Generally, connective tissue, which holds cells together, is a complex mixture of collagen, other extracellular proteins, glycoproteins, and mucopolysaccharides. Purified collagenase alone will not effectively hydrolyze all of this extracellular matrix material. However, it has been discovered by the present inventors that by combining purified collagenase with toxin-free chymopapain a wide range of connective tissue systems and biologically derived raw materials can be predictably digested. Moreover, the hydrolyzed tissues and cells isolated during these hydrolysis processes are free of antigenic components which can cause anaphylactic shock if present in cells or tissues implanted or digested in vivo. Accordingly, cells isolated in accordance with the present invention for subsequent implantation do not present toxic health hazards to their recipients. Similarly, the compositions of the present invention can be utilized for in vivo procedures with little risk of anaphylactic shock.

The enzyme compositions of the present invention can be prepared according to processes known in the art. Typically, these processes involve mixing the two enzymes in a selected physiologically compatible liquid such as phosphate buffered normal saline solution or Plasmalyte® electrolyte solution containing human serum albumin (HSA) and CaCl_2 . Then lyophilizing the resulting aqueous solution provides a stable dry enzyme preparation which can be reconstituted with deionized water. Preferably, the compositions are reconstituted just prior to their use in order to minimize any degradation that may occur once the enzymes are placed in solution. Similarly, it is preferred that the prepared enzyme compositions are maintained at reduced temperatures in the range of about $4^\circ\text{C} \pm 2^\circ\text{C}$ until their use. Alternatively, separate stock

solutions of each enzyme, which typically include concentrated forms of the enzyme in a buffered saline solution, can be prepared in advance and stored frozen at about -80°C. Just prior to use, the solutions are thawed, 5 diluted with a suitable physiologically compatible diluent to a desired enzyme activity or concentration, and then combined to form the enzyme composition. Suitable diluents include aqueous based solutions buffered to a pH of about 7.4 and having a physiologically compatible 10 osmolarity.

It is additionally within the scope of the present invention to utilize these compositions in processes for digesting connective tissue. Thus, the processes of the present invention broadly include providing a composition 15 of the present invention and causing the composition to contact selected tissue for a length of time and at a temperature sufficient to substantially hydrolyze the tissue and permit isolation of hepatocytes and pancreatic islet cells.

20 Preferred exemplary processes in accordance with the teachings of the present invention include digesting connective tissue for the purpose of dissociating and isolating cells embedded in the connective tissue. When used in accordance with processes known in the art for 25 dissociating and isolating cells, the compositions of the present invention provide highly viable cells which are particularly useful for gene therapy and transplanting into humans or animals for therapeutic purposes. For example, pancreatic cells can be isolated from donor 30 pancreases and transplanted into humans or animals for purposes of treating pancreatic related diseases. Additionally, hepatocytes can be isolated from liver in accordance with known procedures utilizing compositions of the present invention.

35 A most preferred process of the present invention includes providing an appropriate enzyme composition of the present invention and contacting the enzyme composition

with connective tissue for a length of time and at a temperature sufficient to substantially hydrolyze the connective tissue and to isolate hepatocytes and pancreatic islet cells embedded in the tissue. For purposes of dissociating and isolating cells, an exemplary preferred process for digesting tissue includes the steps of providing an enzyme composition of a Plasmalyte® electrolyte solution of about 25 nkat/ml to about 120 nkat/ml purified collagenase, about 0.15 nkat/ml to about 0.55 nkat/ml chymopapain and about 0.4 wt% human serum albumin. Then, the enzyme composition at about 37°C is perfused into the organ containing the tissue to be digested, for example, a liver or pancreas, by means of cannulation in an amount of about 1-10 ml of enzyme composition per gram of organ weight until the organ is sufficiently expanded to permit dissection, incubation of the combination of cells and partially hydrolyzed tissue, and isolation of the desired viable hepatocytes or islet cells.

Further separation of viable cells from the incubated combination can be accomplished by shaking the incubated combination until hydrolyzed tissue separates from a supernatant containing cells. The cells are then separated from the supernatants by centrifugation and pipeting off the supernatants. Preferred exemplary processes further include rinsing the cells with a physiologically compatible liquid prior to their evaluation and use.

Tissues which are subject to digestion in accordance with the present invention should have good contact with the enzyme solution. Accordingly, large pieces of tissue should be minced with a pair of scissors prior to further treatment. Additionally, the tissue is preferably rinsed with a physiologically compatible rinsing solution in order to remove visible blood contaminants including clotted blood. Suitable rinsing solutions include those having pH ranges and osmolarity ranges which are compatible with cellular material, such as phosphate buffered saline and Plasmalyte® electrolyte solution. A preferred

method for rinsing the mixture involves transferring the tissue into a sieve-tissue grinder cup and adding phosphate buffered saline solution to the mixture while stirring. Excess liquids and blood contaminants are removed
5 by the rinsing and sieving process. The homogenized and rinsed mixture is then prepared for the above-described digestion and cell isolation procedures.

As generally mentioned above, hepatocytes and pancreatic islet cells isolated from hydrolyzed tissues in
10 accordance with the teachings of the present invention are isolated in higher yields and have greater viability than cells isolated by prior art processes. Moreover, since the enzyme compositions used in the processes of the present invention are free of toxins, in the event that iso-
15 lated cells are implanted for therapeutic purposes or are subjected to other in vivo uses, any residual cotransplanted enzyme composition will not pose the threat of an anaphylactic or other adverse response.

The superior physical and functional characteristics
20 of the cells isolated according to the process of the present invention are sometimes demonstrated by the higher yield of cells having expected characteristics as determined by known cell-counting methods. Other indicators of the superior results obtained by the present invention in-
25 clude the higher insulin production response to glucose concentration in culture media by pancreatic islet cells isolated in accordance with this invention. Furthermore, since intact and viable hepatocytes or pancreatic islet cells can be isolated by the process of the present inven-
30 tion, a higher yield of these cells, as produced by the present invention, is an indicator of a highly safe and efficacious process.

As described in more detail in the examples which follow, digesting porcine pancreatic tissue utilizing
35 purified collagenase without chymopapain being present resulted in no porcine islet cells being isolated. In contrast, a comparable procedure utilizing an enzyme

The invention will be better understood by reference to the following nonlimiting examples which illustrate the use of exemplary enzyme compositions of the present invention in processes for isolating hepatocytes and pancreatic islet cells from tissue containing them. In these examples the activity of collagenase is expressed as nkat/ml of enzyme composition as determined by assay using FALGPA, 15
furylacryloyl Leu-Gly-Pro-Ala synthetic substrate. The activity of chymopapain was determined by assay using BAPNA, 20
Benzoyl-L-arginine-p-nitroanilide synthetic substrate.

25 Example 1

Sprague Dawley of Fisher 344 male rats, 8-16 weeks
35 old, 125-350 G, were brought to an animal facility one

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week before being used as liver donors and were maintained on a 12-hour dark/light cycle. For liver perfusion, each rat was anesthetized following a standard animal operating procedure. Heparin, 500 u, was administered by intraperitoneal injection. The abdominal area was cleaned with betadine and opened through a midline incision. A 16-gauge catheter was inserted into the inferior vena cava and secured with a silk tie. While getting the animal ready, calcium-free HEPES-saline buffer and enzyme solution were warmed in a 37°C water bath. A tubing linked to a pump for perfusion was inserted into buffer or enzyme solution. The other end of the perfusate tubing was connected to a cannula. The flow of calcium-free HEPES-saline buffer, pH 7.4 was initiated at 25 ml/minute for 5-6 minutes. An incision was made on the diaphragm and the inferior vena cava was clamped in the thorax. The portal vein was cut to allow efflux of the buffer. The perfusion was switched, via stopcock, to the collagenase-chymopapain solution and continued for 5-10 minutes. At this time the liver, the average weight of which was about 20 g, was significantly distended and the internal structure began to loosen up and showed signs of collapsing. The liver was carefully dissected free and placed on ice in a sterile petri dish containing William's E. media. The liver capsule was peeled back and the hepatocytes were dispersed by gentle shaking. The liver cell suspension was filtered through a 300 μ m mesh filter into a 50 ml sterile centrifuge tube and allowed to settle by gravity for 15 minutes, followed by brief centrifugation. The cells were washed three times with sterile HEPES-saline, and the volume was reduced by 50% after each wash. A small aliquot of cell suspension was mixed into isotonic solution. The cell concentration and size distribution were analyzed with a Coulter Counter fitted with a multisizer unit.

A total of 8×10^7 hepatocytes of 90% viability were isolated using crude collagenase compared to a total of

12x10⁷ hepatocytes at 95% viability using the purified collagenase and chymopapain mixture.

Figs. 1 and 2 illustrate the particle size distribution of hepatocytes isolated using the enzyme composition of the present invention (Fig. 2) compared to using crude collagenase (Fig. 1).

The viable cells recovered using the collagenase-chymopapain mixture were less damaged and were isolated in a higher yield than those using crude collagenase, as can be seen by comparing the 20-30 μ range of the curve shown in Fig. 1, which represents the particle size distribution of hepatocytes isolated using crude collagenase with that of the curve shown in Fig. 2, which represents the particle size distribution of hepatocytes isolated using the purified collagenase-chymopapain enzyme composition of this invention.

The following example illustrates the isolation of porcine pancreatic islet cells.

Example 2

Enzyme solutions were prepared according to the procedure of Example 1 except that Hanks balanced salt solution (HBSS) containing CaCl₂ was used instead of HEPES-saline buffer solution.

Female farm pigs, six months of age, were brought to an animal facility one week before being used as pancreas donors. The pigs were fasted overnight prior to pancreatectomy. The tail and body of the pancreas will be removed surgically with no warm-ischemia time. After removal, the pancreas will be placed in cold University of Wisconsin (UW) solution on ice and immediately transported to the laboratory.

After removing the surrounding tissue, the pancreas was weighed and the duct cannulated. Warm enzyme solution in a volume of 4 ml/g of tissue was used to expand the organ by injecting through the duct using a 30 ml syringe.

Run-off solution was collected into a tray. The pancreas was then cut into large pieces approximately one inch in diameter and loaded into a container together with all the enzyme solution used for organ expansion. The container with tissue was carefully sealed and agitated by gentle mechanical shaking in a 37°C water bath for 12 minutes or until the majority of the tissue was digested into small fragments. The digested tissue was filtered through a 3 mm screen into another container, placed on ice, and digested further by gentle manual stirring. During cold digestion, small cell clumps, including free islets, were separated every three minutes by filtering the top half of the digest through an 800 μ m screen and stored in cold preservation solution until all tissues were digested. To the remaining digest, an equal amount of cold preservation solution was added and digestion continued until the majority of the islets were free. The islet suspensions obtained were pooled. The appearance of digested tissue and the presence of free islets were monitored microscopically, using dithizone (DTH) staining to identify β cells. The yield was estimated by the number of stained islets in suspension of known volume. The remaining undigested tissue was weighed to determine the weight of digested tissue. The results obtained are shown in Table I.

TABLE I.

Experiment	Collagenase Concentration (nkat/ml)	Chymopapain Concentration (nkat/ml)	Tissue Digested (g)	Total Islets
A	50	None	None	None
B	50	0.40	44	276,000
C	50	0.30	58	302,034
D	50	0.25	19	61,500
E	60	0.35	17	101,600
F	60	0.55	17	45,800
G	100	0.30	34	228,800
H	100	0.25	29	97,200
I	100	0.20	38	200,000
J	120	0.22	36	197,667
K	120	0.20	50	108,267

The variation in tissue digested and total islets released was due largely to the inherited differences among individual animals. In contrast to the data shown in Table I, when crude collagenase was used, it either did
5 not show any effectiveness, or needed to be used at widely varying concentrations for islet isolation. No reproducible yields were obtained using crude collagenase.

The following example illustrates the effect upon cell function of isolating porcine pancreatic islet cells using
10 the enzyme composition of the present invention compared to using crude collagenase.

Example 3

Using the porcine pancreatic cells isolated according to the procedure of Example 2, static incubation glucose
15 responses were plotted over time for cells isolated using crude collagenase, shown in Fig. 3, and for cells isolated using the purified collagenase-chymopapain enzyme composition of the present invention, shown in Fig. 4. The index for insulin production:

$$\frac{\text{Insulin (stimulated)}}{\text{Insulin (unstimulated)}}$$

20

which is a measure of cell function can be seen to have a higher value in the case of the curve plotted in Fig. 4 than in Fig. 3. This is an indication that the islet
25 cells isolated using the enzyme composition of the present invention function more normally than islet cells isolated using the crude collagenase of the prior art.

The following example illustrates the isolation of rat islet cells.

30

Example 4

The procedure of Example 2 was followed except that, due to the small quantities of purified enzyme used, the pancreas was expanded with HBSS solution, then cut into pieces approximately 0.5 cm in diameter. About 2.5 g of

tissue was suspended in 10 ml enzyme solution in a vial with screw cap. The vial was carefully sealed and agitated in a 37°C water bath until most of the tissue pieces appeared to be disintegrated. Digestion was also monitored by sampling of tissue suspension periodically and staining the islets with dithizone solution. Incubation and shaking were stopped when the majority of the islets was released but before damage to the islets was detected. The results are shown in Table II.

10

TABLE II.

Experiment	Collagenase Concentration (nkat/ml)	Chymopapain Concentration (nkat/ml)	No. of Islets	Viability
A	50	0.25	1089	94%
B	50	0.15	325	99%
C	50	0.40	933	91%

15 Although there has been hereinabove described a composition containing collagenase and chymopapain for hydrolyzing connective tissue to isolate cells in accordance with the present invention, for the purpose of illustrating the manner in which the invention may be used to advantage, it should be appreciated that the invention is not limited thereto. Accordingly, any and all modifications, variations, or equivalent arrangements which may occur to those skilled in the art, should be considered to be within the scope of the present invention as defined in
20
25 the appended claims.

WE CLAIM:

1. An enzyme composition useful for hydrolyzing tissue containing hepatocytes or pancreatic islet cells, said composition comprising:

5 an aqueous mixture of collagenase having an activity of about 25 nkat/ml to about 120 nkat/ml, and chymopapain having an activity of about 0.15 nkat/ml to about 0.55 nkat/ml, in a physiologically compatible electrolyte solution buffered to a pH of about 7.0 to 7.4, wherein the activity
10 of collagenase in nkat/ml is defined as the number of nanomoles of furylacryloyl Leu-Gly-Pro-Ala synthetic substrate (FALGPA) hydrolyzed per second by 1 ml of collagenase solution at 37°C and the activity of chymopapain in nkat/ml is defined as the
15 number of nanomoles of Benzoyl-L-arginine-p-nitroanilide synthetic substrate (BAPNA) hydrolyzed per second by 1 ml of chymopapain solution at 37°C, said collagenase being essentially free of toxins and non-collagen active components and said
20 chymopapain being essentially free of toxins.

2. The enzyme composition of claim 1 wherein said electrolyte solution is a phosphate buffered saline solution.

3. The enzyme composition of claim 1 wherein said electrolyte solution is a HEPES-saline buffer, pH 7.4 containing CaCl_2 .

4. The enzyme composition of claim 1 wherein said electrolyte solution is a Hanks balanced salt solution containing CaCl_2 .

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5. The enzyme composition of claim 1 wherein said collagenase has an activity of about 30 nkat/ml and said chymopapain has an activity of about 0.3 nkat/ml.

6. A process for isolating viable hepatocytes or pancreatic islet cells from tissue containing viable hepatocytes or pancreatic islet cells, said process comprising the steps of:

- 5 providing an enzyme composition comprising an aqueous mixture of collagenase having an activity of about 25 nkat/ml to about 120 nkat/ml, and chymopapain having an activity of about 0.15 nkat/ml to about 0.55 nkat/ml, in a physiologically compatible electro-
10 lyte solution buffered to a pH of about 7.0 to 7.4, said collagenase being essentially free of toxins and non-collagen active components and said chymopapain being essentially free of toxins;
- 15 contacting tissue containing viable hepatocytes or pancreatic islet cells with said enzyme composition for a length of time and at a temperature sufficient to hydrolyze said tissue without adversely affecting the viability of said hepatocytes or pancreatic islet cells; and
- 20 isolating viable hepatocytes or pancreatic islet cells from the hydrolyzed tissue.

7. The process of claim 6 wherein said electrolyte solution is a phosphate buffered saline solution.

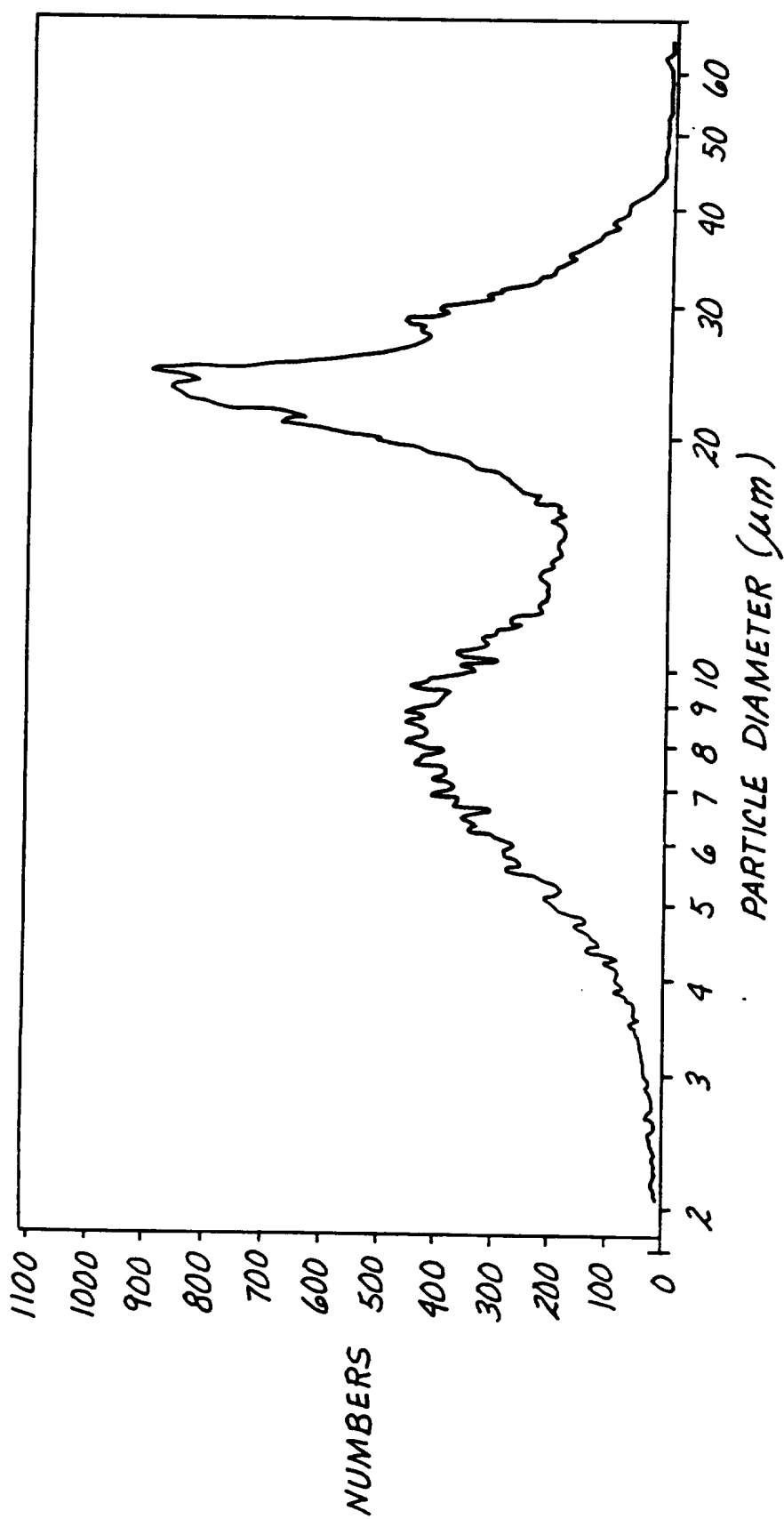
8. The process of claim 6 wherein said electrolyte solution is a HEPES-saline buffer, pH 7.4 containing CaCl_2 .

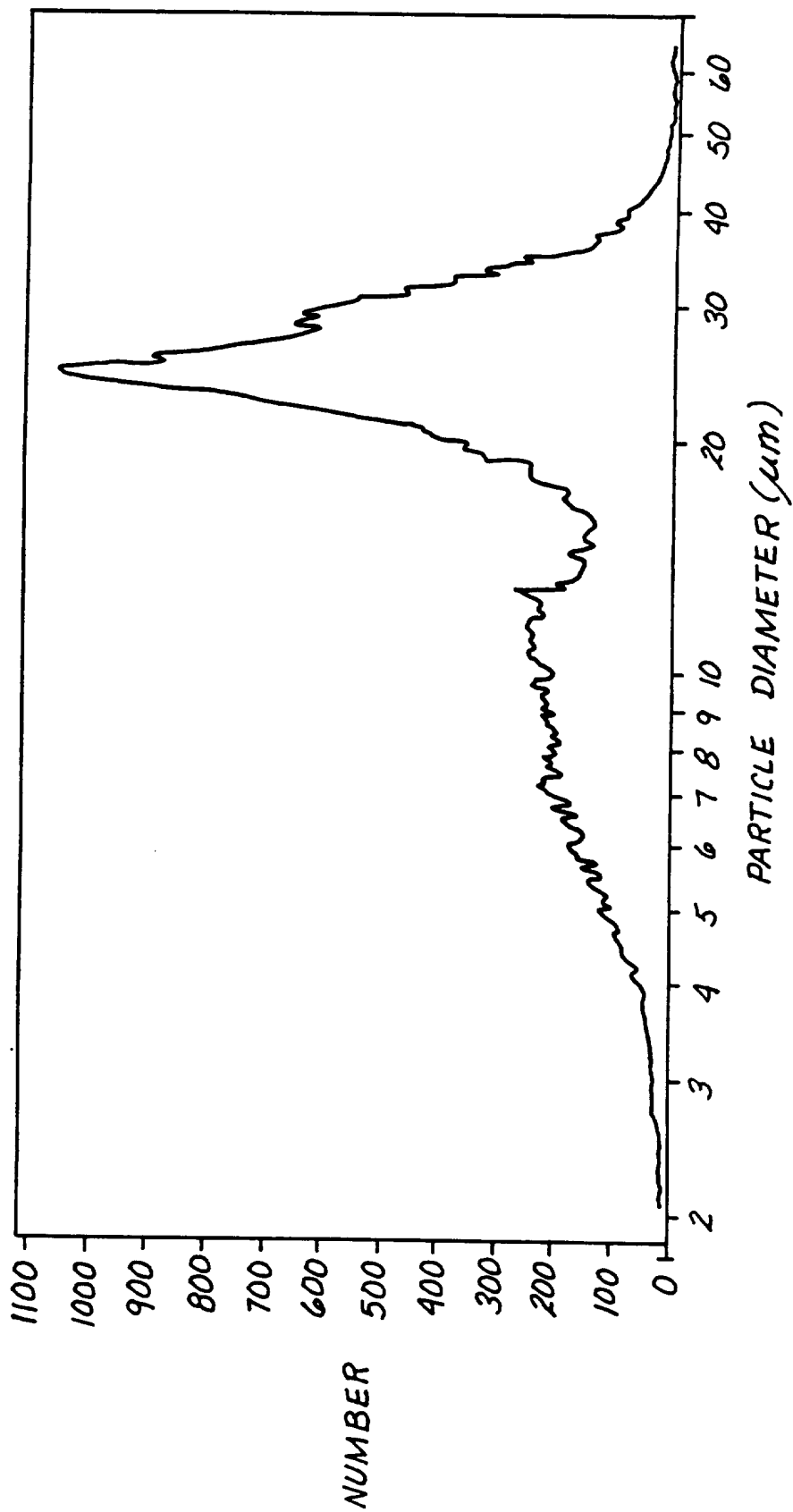
9. The process of claim 6 wherein said electrolyte solution is a Hanks balanced salt solution containing CaCl_2 .

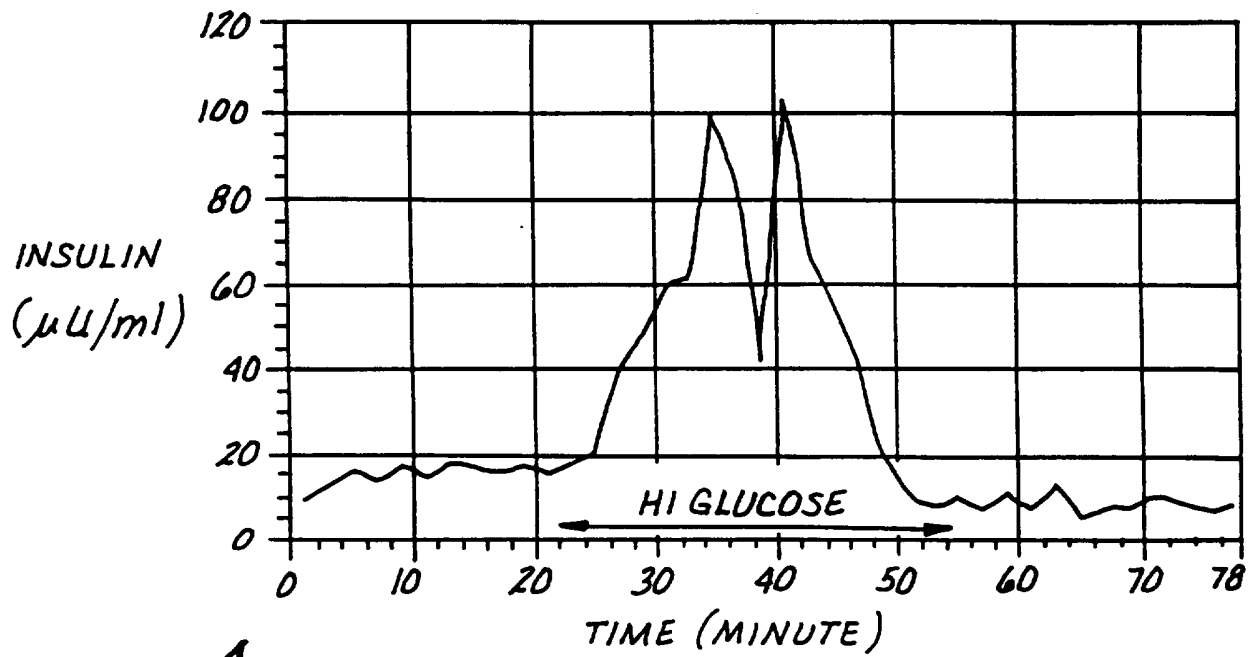
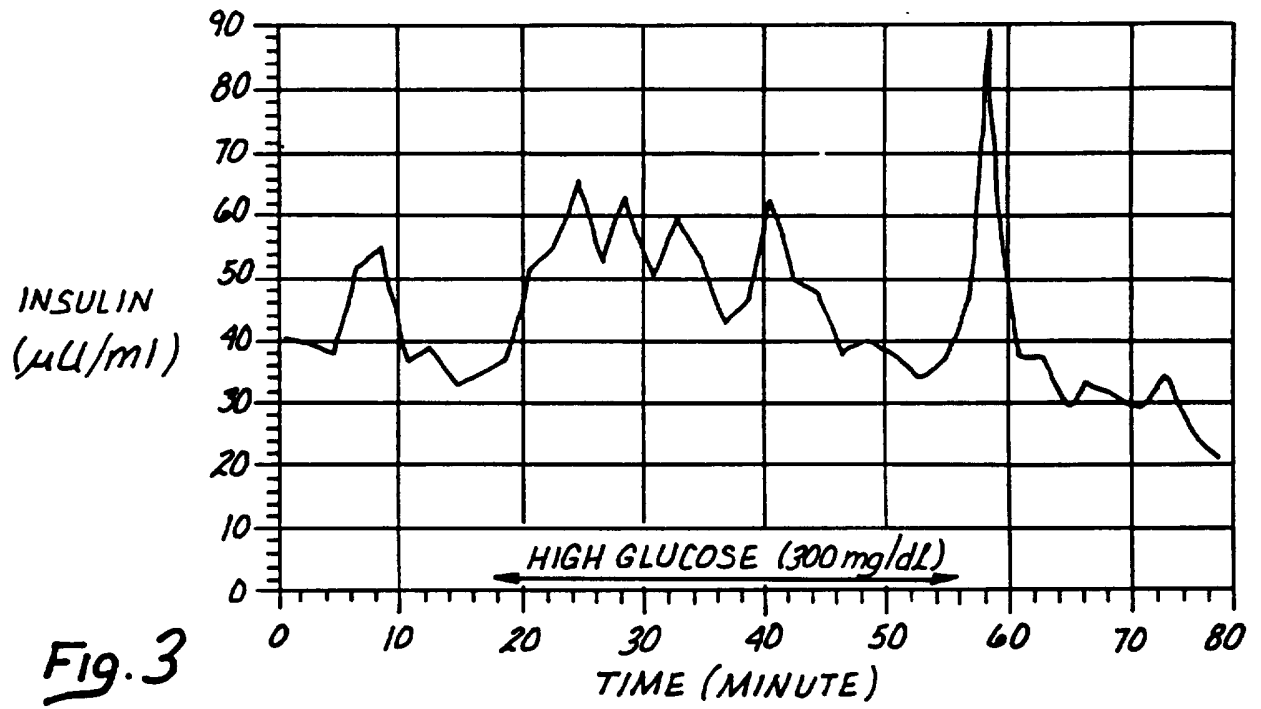
-24-

10. The process of claim 6 wherein said collagenase has an activity of 30 nkat/ml and said chymopapain has an activity of 0.3 nkat/ml.

11. The process of claim 6 wherein the tissue-contacting step is performed by means of perfusion of an organ containing said tissue.

*Fig. 1*

*Fig. 2*



INTERNATIONAL SEARCH REPORT

Inv. No. International Application No.

PC 1/US 96/05725

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N9/52 C12N5/06 A61K38/48

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 C12N A61K

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,A,94 23743 (BAXTER INTERNATIONAL INC.) 27 October 1994 see the whole document ---	1-11
X	WO,A,94 00580 (TRIGEN INC.) 6 January 1994 see page 43-47 ---	1-11
P,X	US,A,5 424 208 (LEE C.T. ET AL.) 13 June 1995 see the whole document -----	1-11

☐ Further documents are listed in the continuation of box C.

Patent family members are listed in 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 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 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

29 July 1996

Date of mailing of the international search report

31.07.96

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Authorized officer

Moreau, J

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/05725

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

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

1. ☒ Claims Nos.: 6-11
because they relate to subject matter not required to be searched by this Authority, namely:
Although these claims (as far their concern in vivo methods) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims 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. ☐ Claims 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:

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

Information on patent family members

International Application No

PC 1/US 96/05725

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9423743	27-10-94	US-A- 5422261	06-06-95
		CA-A- 2160532	27-10-94
		EP-A- 0693935	31-01-96
		US-A- 5424208	13-06-95

WO-A-9400580	06-01-94	AU-B- 4644793	24-01-94
		CA-A- 2138948	06-01-94
		EP-A- 0654084	24-05-95
		JP-T- 8500970	06-02-96

US-A-5424208	13-06-95	US-A- 5422261	06-06-95
		CA-A- 2160532	27-10-94
		EP-A- 0693935	31-01-96
		WO-A- 9423743	27-10-94
