CHONDROITINASE PRODUCTION IN RECOMBINANT *PROTEUS VULGARIS* STRAINS

Field of the Invention

This invention relates to the preparation, identification, and isolation of a plasmid that encodes for the production of large amounts of chondroitinase I and chondroitinase II proteins in *Proteus vulgaris* cells in the absence of natural exogenous chondroitinases I and II inducers such as chondroitin sulfate.

Background of the Invention

Chondroitinases are enzymes of bacterial origin that act on chondroitin sulfate, a component of the proteoglycans that mediate the attachment between the retina and the vitreous body of the human eye. Examples of chondroitinase enzymes are chondroitinase ABC, which is produced by the bacterium *Proteus vulgaris* (*P. vulgaris*), and chondroitinase AC, which is produced by *A. aurescens*. Chondroitinases ABC and AC function by degrading polysaccharide side chains in protein-polysaccharide complexes, without degrading the protein core.

Yamagata et al. (*J. Biol. Chem.* **243**:1523-1535, 1968) describe the purification of the chondroitinase ABC from extracts of *P. vulgaris*. This enzyme selectively degrades the glycosaminoglycans chondroitin-4-sulfate, dermatan sulfate, and chondroitin-6-sulfate (also referred to respectively as chondroitin sulfates A, B, and C which are side chains of proteoglycans) at pH 8 at higher rates

than it degrades chondroitin or hyaluronic acid. The products of the degradation are high molecular weight unsaturated oligosaccharides and an unsaturated disaccharide. However, chondroitinase ABC does not act on keratosulfate, heparin or heparitin sulfate.

Uses of chondroitinases include rapid, specific and non-surgical disruption of the attachment of the vitreous body to the neural retina of the eye, thereby facilitating removal of the vitreous body. See, for example, Hageman, U.S. Patent No. 5,292,509.

Chondroitinase ABC is designated as chondroitinase I in the present invention. *P. vulgaris* chondroitinase I migrates with an apparent molecular mass of about 110 kDa when resolved by SDS-PAGE. The appearance of a doublet in SDS-PAGE resolution of chondroitinase I has been reported (Sato et al., *Agric. Biol. Chem.* 50:4,1057-1059, 1986). However, this doublet represents intact chondroitinase I and a 90 kDa degradation product (U.S. Patent Application Nos. 08/431,068, 08/428,949, 08/428,946, 08/428,948, 08/428,945, and 08/428,947, filed April 24, 1995, (Attorney's Docket Nos. 0646/1B017US1-0646/1B017US6) now pending. Commercial chondroitinase I protein preparations contain variable amounts of this 90 kDa degradation product and an additional 18 kDa degradation product.

Another chondroitinase, chondroitinase II, has also been isolated and purified from *P. vulgaris*. Chondroitinase II is a polypeptide of 990 amino acids with

an apparent molecular mass by SDS-PAGE of about 112 kDa. Its molecular mass as determined by electrospray and laser desorption mass spectrometry is 111,772±27 and 111,725±20 daltons, respectively. Chondroitinase II has an isoelectric point of 8.4-8.45. Its enzymatic activity is distinct from, but complementary to, that of chondroitinase I. Chondroitinase I endolytically cleaves proteoglycans to produce end-product disaccharides, as well as at least two other products which are thought to be tetrasaccharides. Chondroitinase II digests at least one of these tetrasaccharide products of chondroitinase I digestion of proteoglycan.

Native or wild-type *P. vulgaris* bacterial strains typically do not produce significant amounts of chondroitinases I and II under ordinary growth conditions. Wild-type strains of *P. vulgaris* can be induced to produce detectable levels of chondroitinase by providing an inducing substrate, such as chondroitin sulfate, as the sole carbon source. However, chondroitin sulfate, which is obtained from shark cartilage, is expensive and available only in limited quantities. Alternatively, cloned chondroitinase I and II genes in *E. coli* can be expressed using a heterologous expression system with an artificial inducer, which also increases the cost of chondroitinase I and II production.

Thus, there is a need in the art for *P. vulgaris* chondroitinase I and II production that does not require exogenous inducers.

Summary of the Invention

Plasmids pLP²-1531, which encodes chondroitinases I and II, and pLP²-1521, which encodes chondroitinase I, are provided. These plasmids are used to transform wild-type *E. coli* and *P. vulgaris* cells. The transformed cells produce DNA encoding the production of chondroitinases I and II or chondroitinase I in the absence of natural exogenous chondroitinases I and II inducers. Additionally, the transformed *P. vulgaris* cells, in the absence of exogenous chondroitinases I and II inducers, produce the respective enzymes in amounts in excess of those produced by wild-type *P. vulgaris* in the presence of exogenous chondroitinases I and II inducer(s).

Starting plasmid pLP²-751 and intermediate plasmids pLP²-770, pLP²-1512, pLP²-1263, pLP²-1508, pLP²-1510, pLP²-1514, pLP²-1518, and pLP²-1525 are also provided. These intermediate plasmids are typically used in the preparation of pLP²-1531 and pLP²-1521. A BglII/EcoRI fragment of about 3970 bp derived from pLP²-1512, a EcoRI/SmaI fragment of about 2550 bp derived from pLP²-1514, and a BglII/SmaI(AvaI) fragment of about 6520 bp derived from pLP²-1518 are further disclosed.

Also contemplated is the use of transformed *P. vulgaris* cells to produce chondroitinase I and/or II. The *P. vulgaris* cells above are cultured in a bacterial culture medium and in the absence of an exogenous chondroitinase I and II inducer. The cells are then harvested from the culture, and the chondroitinase I and/or II enzymes are

recovered. Preferably, chondroitinases I and II are recovered by

(a) preparing a clarified homogenate of harvested *P. vulgaris*, the homogenate having a pH of 5.8 to 7.4;

(b) loading the homogenate onto a negatively charged cation exchange resin chromatographic support so that any positively charged proteins comprising chondroitinase I and chondroitinase II in the homogenate form a non-covalent bond with the negatively charged support;

(c) affinity-eluting, in pools, the chondroitinase proteins from the support with an aqueous solution of chondroitin sulfate at a pH 7.0-9.5;

(d) loading the affinity eluted protein pools onto an anion exchange resin chromatographic support to yield an unbound eluate; and

(e) recovering the chondroitinase I and chondroitinase II proteins in the unbound eluate.

The proteins can be further purified by metal chelating chromatography.

Brief Description of the Drawings

Figure 1 is a schematic illustration of the preparation of pLP^2-1518 from pLP^2-751.

Figure 2 is a schematic illustration of the preparation of pLP^{2}1531 from pACYC184 and pLP^2-1518.

Detailed Description of the Invention

The present invention encompasses constructs and methods for producing large quantities of chondroitinase I and II proteins in *Proteus vulgaris*.

Chondroitinase I and Chondroitinase II

Chondroitinase I and chondroitinase II are enzymes produced by *P. vulgaris* that catalyze the breakdown of chondroitin sulfate, including that present in proteoglycans. The physical and enzymatic characteristics of each enzyme are summarized in Table 1.

TABLE 1

Chondroitinase I Protein and Chondroitinase II Protein

	110 kDa	112 kDa
SDS-PAGE molecular weight	~110,000 daltons	~112,000 daltons
Electrospray mass spectrometry	112,527 ± 25 daltons	111,772 ± 27 daltons
Laser desorption mass spectrometry	112,508 ± 20 daltons	111,725 ± 20 daltons
Isoelectric point/s	pH 8.35 and pH 8.45	pH 8.45
Amino acid composition	absence of cysteines, rich in serine	otherwise similar to 110 kDa
Release of di and oligosaccharides from chondroitin sulfate	+++	–
Digestion of tetrasaccharides not digested by chondroitinase I	–	+++

Wild-Type *P. Vulgaris* Strains

Wild-type strains of *P. vulgaris* accumulate easily detectable levels of enzymatically active chondroitinases I and II only when grown in a culture containing

an exogenous chondroitinase I and II inducer, such as chondroitin sulfate, as the sole carbon source. Growth of wild-type strains of *P. vulgaris* in media without such an inducer, such as, for example, in a rich medium containing many carbon sources or in a minimal medium containing, for example, glucose as a sole carbon source, results in insignificant or no detectable accumulation of chondroitinase I or II activity.

Constructs

The genes encoding both chondroitinase I protein and chondroitinase II protein are present on a *P. vulgaris* genomic DNA sequence of about 30 kb in length flanked by EcoRI sites, which is contained within a cosmid clone designated LP^2-751 (see Example 1 below). According to the present invention, DNA encoding *P. vulgaris* chondroitinase I and/or chondroitinase II protein(s) is cloned into a plasmid that replicates in *P. vulgaris* cells. Preferably, the plasmid is engineered so that the chondroitinase I and II genes are placed immediately 3' to a sequence that serves as a constitutive promoter. A promoter is a DNA sequence that stimulates transcription of the gene sequences that are located immediately downstream of, i.e., 3' to, the promoter sequence. A constitutive promoter is a DNA sequence that stimulates transcription of downstream sequences (for example, chondroitinase I and/or II-encoding sequences) independent of the presence of exogenous transcriptional inducers.

The promoters or constitutive promoters of the present invention may be derived from the native chondroitinase I promoter typically present upstream of the chondroitinase I gene in wild-type *P. vulgaris* cells, if the promoter sequence is engineered so that regulatory sequences involved in induction of expression by

exogenous inducers, such as, for example, chondroitin sulfate, are absent or are not functional. Alternatively, the promoter to which the chondroitinase I and II genes are linked may comprise another known constitutive promoter that stimulates transcription in *P. vulgaris*, such as the promoter region of other *P. vulgaris* genes, or the promoter region of *E. coli* genes that, for example, confer resistance to antibiotics such as, for example, chloramphenicol, tetracycline, and ampicillin in *P. vulgaris*. The sequence of both the homologous chondroitinase promoter or that of heterologous promoters may be altered by deletions, insertions, and substitutions, using recombinant DNA methods that are well-known in the art. (See, for example, Maniatis et al., *Molecular Cloning*, Cold Spring Harbor Laboratory, 1982).

In addition to promoter-linked chondroitinase I- and/or chondroitinase II- coding sequences as described above, plasmids suitable for use in the present invention include sequences that allow the plasmid to replicate to high copy number in *P. vulgaris*, as well as sequences that encode a selectable marker. Selectable markers may comprise gene products that confer resistance to tetracycline, ampicillin, chloramphenicol, kanamycin, or streptomycin. Preferably, the chondroitinase I and/or II-encoding plasmid includes a gene encoding a chloramphenicol-resistance trait, such as, for example, chloramphenicol acetyl transferase. Examples of suitable plasmids include without limitation pBR322, pNEB193, pUC19, and derivatives therefrom. (New England Biolabs, Beverly, MA).

A plasmid encoding chondroitinase I and/or chondroitinase II protein under the control of a constitutive promoter is introduced into *P. vulgaris* cells. Suitable host cells include any strain of *P. vulgaris*, including without limitation wild-type *P. vulgaris* (for example, ATCC strain 6896), mutant *P. vulgaris* strains such as

those described in U.S. Patent Application No. 08/476,261 filed June 7, 1995 (attorney docket # 0646/0B123). Introduction of the plasmid may be achieved by any DNA transformation method well-known in the art, such as, for example, electroporation or calcium-mediated permeabilization of cells. Preferably, electroporation is used to achieve effective uptake of DNA into *P. vulgaris* host cells.

Selection of useful and preferred chondroitinase I and/or II-encoding plasmids, including selection of useful and preferred constitutive promoters, is achieved by transforming individual *P. vulgaris* cultures with each plasmid, selecting transformants, and analyzing the transformants for their production of chondroitinase I and/or chondroitinase II. For preliminary assessment of relative chondroitinase production (as when, for example, a nested set of promoter deletions is analyzed), colony screening methods can be used.

In one embodiment, the native chondroitinase I upstream region (including the presumptive promoter) is subjected to exonucleolytic cleavage with, for example, Bal 31 to generate a nested set of 5' deletion mutants. The mixture of mutants is used to transform *P. vulgaris*. The library of clones is spread and subjected to screening for chondroitinase production in the absence of an exogenous inducer.

Suitable screening methods include, but are not limited to, those that detect colonies that either bind chondroitinase-specific antibodies or that catalyze the breakdown of chondroitin sulfate or proteoglycan. Colony immunoblotting assays use well-known methods such as those disclosed in, for example, Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory (1988). Detection assays that depend on chondroitinase enzymatic activity include, without limitation, the chondroitin depletion method described below and colorimetric assays that detect

chondroitin or chondroitin hydrolysis. Either screening method (immunoblotting and chondroitin depletion) can be used at any of the stages in initial identification and subsequent colony purification of constitutive mutant cells.

In one embodiment, an antibody-based screening method is used to identify *P. vulgaris* colonies that produce chondroitinases I and II even when an exogenous inducer such as, for example, chondroitin sulfate is absent from the culture medium as follows:

(1) *P. vulgaris* cultures are seeded onto filters that are placed onto agar plates containing either rich or casamino acid-supplemented minimal medium lacking chondroitin sulfate and/or N-acetylgalactosamine. The filters may comprise nylon, paper, nitrocellulose, or polyvinylidiene difluoride, preferably NYTRAN (Schleicher & Schuell, Keene, NH). The optimum colony density for this step is about 1000 per plate.

(2) The filters are then transferred from the plates to a solution that permeabilizes and lyses the cells on the filter, so that chondroitinase polypeptides are released from the cells and become fixed to the filter.

(3) The filters are then incubated with specific antibodies directed against chondroitinase I and/or chondroitinase II, such as , for example, goat anti-chondroitinase I antibody and/or rabbit anti-chondroitinase II antibody.

(4) Finally, specifically bound antibody is detected using any enzymatic, fluorescent, radioactive, or other detection means well known in the art.

Goat anti-chondroitinase I antibodies are prepared by (a) purifying *P. vulgaris* chondroitinase I from an *E. coli* strain that expresses chondroitinase I from

an overexpression plasmid (as disclosed in U.S. patent application No. 08/233,008, filed April 22, 1994 hereinafter the '008 application) using purification methods described in U.S. patent application No. 08/231,534, filed April 22, 1994; and (b) mixing the purified chondroitinase I with Freund's adjuvant and inoculating the resulting emulsion into goats. Rabbit anti-chondroitinase II antibodies are prepared by (a) purifying *P. vulgaris* chondroitinase II from an *E. coli* strain that expresses chondroitinase II from an over expressing plasmid (as disclosed in the '008 application) and (b) mixing the purified chondroitinase II with Freund's adjuvant and inoculating the resulting emulsion into rabbits. Procedures for the purification and analysis of antibodies are those well-known in the art.

In a preferred embodiment of the antibody screen, *P. vulgaris* colonies are grown on Nytran filters that are placed onto agar plates containing "20-10-5" medium, which includes 20 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl. After spraying the bacterial colony-containing filters with a solution of bovine serum albumin to block background sites, the filters are floated on liquid chloroform for several hours or placed on chloroform-saturated paper, releasing chondroitinases and binding them to the filter in the immediate vicinity of chondroitinase-producing colonies. The washed filters are then incubated sequentially with (1) goat anti-chondroitinase I antibodies, (2) peroxidase-conjugated rabbit anti-goat antibody (BioRad), and (3) color reagents to visualize filter-bound peroxidase (BioRad). In parallel, colony-purified untransformed wild-type *P. vulgaris* cells are grown on medium lacking and containing chondroitin sulfate to serve as negative and positive controls, respectively, for the screening procedure.

Colonies that display detectable amounts of chondroitinase I and/or II using this assay are picked, diluted, re-inoculated on plates, and the entire detection procedure is repeated. Several cycles of colony purification are performed in this manner, until a pure culture of each individual strain is obtained.

In another embodiment, a chondroitin depletion assay is used to identify chondroitinase I and/or II-producing colonies. In this procedure, mutagenized *P. vulgaris* cultures are seeded onto filters as described above, and the filters are placed onto agar plates containing either rich or casamino acid-supplemented minimal medium lacking chondroitin sulfate. After overnight growth, the filters are transferred to plates containing agar supplemented with 5 mg of chondroitin sulfate/ml and a protein synthesis inhibitor at a concentration effective to inhibit protein synthesis on the filter-bound bacterial colonies, preferably 100 μg/ml tetracycline. After incubation for about 4 to about 8 hours, the filters are removed, and the plates are flooded with about 10 ml each of 0.5% cetyl pyridinium chloride (Sigma Chemical Co., St. Louis, MO). This treatment causes chondroitin sulfate to form a cloudy precipitate within the agar. Colonies that elaborate chondroitinase I and II produce an obvious clear zone surrounding the colony that is easily visualized by eye.

Chondroitinase-encoding plasmids that appear by the above screening assays to provide detectable production of chondroitinases I and II in the absence of the exogenous inducers chondroitin sulfate or N-acetylgalactosamine are analyzed for chondroitinase production in a quantititative manner. Individual colonies are inoculated into small-scale (3-5 ml) fermentation cultures and incubated at 30-37°C with shaking. Samples are removed at different times after initiation of growth, cells are disrupted using a French pressure cell, and the amount of chondroitinase protein

and enzymatic activity is measured in the cell homogenates. Useful plasmids are those that result in the production of at least 0.2 chondroitinase activity units per A_{600} unit of bacterial culture. Preferably, expression of chondroitinase I and/or chondroitinase II using the plasmids of the present invention results in the production of at least 0.5 chondroitinase activity units per A_{600} unit of bacterial culture.

Purification

The chondroitinase I and II enzymes produced by a transformed *P. vulgaris* strain may be co-purified to homogeneity (i.e., to obtain a pure mixture of chondroitinase I and II) and the co-purified enzymes are suitable for use in, for example, vitreal disinsertion.

Although a variety of methods can be used to isolate and purify native chondroitinases I and II, a preferred affinity chromatography includes:

(a) preparing a clarified homogenate of mutant *P. vulgaris* cells, the homogenate having a pH of 5.8 to 7.4;

(b) loading the homogenate onto a negatively charged cation exchange resin chromatographic support so that any positively charged proteins comprising chondroitinase I and chondroitinase II in the homogenate form a non-covalent bond with the negatively charged support;

(c) affinity-eluting, in pools, the chondroitinase proteins from the support with an aqueous solution of chondroitin sulfate at a pH 7.0-9.5;

(d) loading the affinity eluted protein pools onto an anion exchange resin chromatographic support to yield an unbound eluate; and

(e) recovering the chondroitinase I and chondroitinase II proteins in the unbound eluate.

The proteins can be further purified by metal chelating chromatography by

(1) contacting the unbound eluate with a metal chelating affinity chromatography support to bind further the chondroitinase proteins;

(2) eluting with an appropriate solvent; and

(3) recovering the chondroitinase proteins.

If desired, the copurified proteins can be separated from each other by additional process steps involving further cation exchange chromatography. The individually purified proteins can be used in ratios other than those obtained by the copurification procedure.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following examples illustrate the present invention without limitation. All cloning steps are in *E. coli* unless otherwise indicated.

Example 1: Construction of A Plasmid for the Expression of Chondroitinases I and II in P. Vulgaris

A. ***Assembly of a Chondroitinase I and II Protein-Encoding DNA Fragment***

The overall strategy was to create a DNA fragment containing the chondroitinase I and II genes but lacking most of the upstream regulatory region. It was desired that the final fragment be flanked by BglII-SmaI sites so that it could be inserted into a plasmid vector containing appropriately placed BglII and SmaI sites.

The chondroitinase region was initially engineered in halves and then was reassembled to achieve this result.

The steps used in construction of the BglII-SmaI fragment are illustrated in Figure 1. In this figure, chondroitinase I-encoding DNA is designated "110 K" and chondroitinase II-encoding DNA is designated "112 K".

The starting plasmid was a cosmid clone designated pLP^2-751, which contains a ~30kb *P. vulgaris* genomic DNA insert flanked by EcoRI sites. Since the insert contains an internal EcoRI site, EcoRI digestion results in recovery of the inserted DNA as two EcoRI fragments of 20kb and 10kb, which are arbitrarily designated as left and right, respectively. The entire chondroitinase I gene is located on the extreme right side of the 20kb DNA fragment. The chondroitinase II gene is located immediately downstream to the right of the chondroitinase I gene. The proximal portion of this gene is at the rightmost end of the 20kb EcoRI fragment, while the remaining, distal, portion of the gene is on the 10kb EcoRI fragment. The coding sequence for the chondroitinase II gene extends rightward and terminates about 2300 bp to the right of the EcoRI site. Approximately 240bp further downstream is a unique SmaI site.

pLP^2-751 also contains a 10kb NsiI fragment that contains the entire chondroitinase I gene and the proximal portion of the chondroitinase II gene. This fragment maps to the right side of the pLP^2-751 20kb EcoRI fragment, but extends 400 bp further to the right into the adjoining 10kb EcoRI fragment. Therefore the 10kb NsiI fragment contains the internal EcoRI site described above.

The overlapping 10kb NsiI and 10kb EcoRI fragments from pLP^2-751 were subcloned as follows. pLP^2-751 was digested with NsiI, and the 10kb NsiI

fragment was isolated and cloned into a pIBI24 derivative (International Biotechnologies, Inc., New Haven, CT) containing an NsiI linker. This step yielded a plasmid designated LP²-770 (Figure 1). pLP²-770 was then digested with EcoRV to remove about 3700 bp of unsequenced upstream DNA that presumably contains the regulatory gene for the chondroitinase operon and possibly all or part of the regulatory binding site for theses proteins at the 5'-end of the chondroitinase I gene. The EcoRV ends were dephosphorylated, after which BglII linkers were ligated to the dephosphorylated ends. The resulting plasmid was designated LP²-1512. Immediately downstream of the BglII site are the presumed chondroitinase promoter -35 and -10 elements beginning at nucleotides 40 and 62. The start codon for chondroitinase I is at nucleotide 119. The chondroitinase I termination codon is at nucleotide 3182, and the start codon for chondroitinase II is at position 3238. The DNA contains an EcoRI site at position 3974, corresponding to that contained in plasmid LP²-1514 (see below), and extends to the NsiI site at position 4363. Accordingly, digestion of pLP²-1512 with BglII and EcoRI produced an approximately 3970 bp fragment containing the entire chondroitinase I gene and the 5' proximal portion of the chondroitinase II gene.

In parallel, plasmid LP²-751 was digested with EcoRI, and the 10 kb EcoRI fragment described above was cloned into the EcoRI site of a pNEB193 derivative (New England Biolabs, Beverly, MA) to yield a plasmid designated LP²-1263. This plasmid was digested with AvaI and self-ligated, to form plasmid LP²-1510. This step removed about 7450 bp of DNA downstream of the chondroitinase II coding sequence. pLP²-1510 was then digested at a sole vector-derived AatII site, filled in with the Klenow fragment of DNA polymerase I, dephosphorylated, and a

BglII linker was ligated at that site. The resulting plasmid, designated LP^2-1514, contains an approximately 2550 bp EcoRI/SmaI(AvaI) insert containing the 3' distal portion of the chondroitinase II gene.

In a final step, the 3970bp BglII/EcoRI fragment isolated from plasmid LP^2-1512 as described above was cloned into pLP^2-1514 that had been digested with BglII and EcoRI. The resulting plasmid, designated LP^2-1518, has an approximately 6520 bp BglII-SmaI (AvaI) insert that contains the chondroitinase I and II gene fragment.

B. ***Construction of a P. vulgaris Expression Vector:***

Plasmid pACYC184 (ATCC #37033) was engineered to serve as a receptor for the BglII-SmaI (AvaI) chondroitinase fragment described above. The steps of this construction are shown in Figure 2.

pACYC184 was digested with AvaI, after which the ends were filled in using the Klenow fragment of DNA polymerase, dephosphorylated, and ligated to a synthetic BglII linker to form plasmid LP^2-1508. This plasmid was then digested with HindIII, filled in, dephosphorylated, and ligated to synthetic SmaI linkers to form plasmid LP^2-1525.

In a final step, pLP^2-1525 was digested with BglII and AvaI and was dephosphorylated. The approximately 2850 bp fragment (including the origin of replication and chloramphenicol resistance gene) was then ligated to the BglII/AvaI insert containing the chondroitinase I and II fragment to yield plasmid LP^2-1531. The *E. coli* strain carrying this plasmid is designated LL4136.

C. ***Construction of an expression plasmid for chondroitinase I:***

A chondroitinase I only construct was also prepared by inserting a BglII to SphI (position 3414) fragment from pLP^2-1512 into a BglII-SphI digested derivative of pACYC184 which contains a BglII linker at the pACYC184 BclI site; the SphI site is internal to the pACYC184 tetracycline-resistance gene. The resulting plasmid is designated LP^2-1521 and is contained in *E. coli* strain LL4093.

Example 2: Transformation of *Proteus vulgaris* with a Chondroitinase I and II Expression Vector

Proteus vulgaris strains LL2480 (wild-type) and LL2492 (exhibiting constitutive expression of chondroitinases) were transformed with plasmids using the following procedure.

An overnight culture of each strain was inoculated into 20-10-5 medium and incubated at 37°C until the cultures reached an A_{600} of 0.5. The cells were collected by centrifugation and repeatedly washed in cold distilled water; they were then concentrated 150-fold in cold 20% glycerol and stored frozen at -70°C.

Electroporation was performed using a BioRad Gene Pulser. 200μl of washed cells were mixed with 1-10 μl of DNA (corresponding to 0.1-2.0 μg DNA) in a 0.2 cm cuvette and pulsed at 2.4 kilovolts using a 25μ Farad capacitor with a 200 Ohm resistor. The electroporated cells were then inoculated into 2ml of 20-10-5 medium, and the cultures were incubated at 37°C for about 75 min, after which they were plated on 20-10-5 agar containing 25 μg/ml chloramphenicol. After overnight incubation at 37°C, chloramphenicol-resistant colonies were observed. At least one colony of each transformant was streaked onto chloramphenicol agar. Individual colonies were then inoculated into 20-10-5 liquid medium containing 25 μg/ml chloramphenicol and grown overnight at 37°C. Glycerol was then added and the strains were stored at -70°C.

The plasmids transformed were pACYC184, pLP^2-1521 and pLP^2-1531. Strain LL2480 transformed with plasmids pACYC184, pLP^2-1521 and pLP^2-1531 were designated strains LL4202, LL4198 and LL4192, respectively. Strain LL2492 transformed with pACYC184, pLP^2-1521, and pLP^2-1531 were designated strains LL4119, LL4107, and LL4142, respectively.

Example 3: Analysis of Chondroitinases I and II Production

The plasmid-transformed *P. vulgaris* strains prepared as described in Example 2 above were analyzed for their production of chondroitinases I and II. The growth medium was casamino acid-supplemented minimal medium containing 25 μg/ml chloramphenicol. Incubations were performed at 30°C and samples were taken at 7 and 24 hours after initiation of growth. The starting cell densitites were about 10^6-10^7 cells/ml. In each case, cells were collected by centrifugation, disrupted in a French pressure cell, and subjected to chondroitinase enzymatic activity assays. The results, expressed as activity units/A_{600} units of the culture, are shown in Table 2.

TABLE 2

CHONDROITINASES I AND II PRODUCTION

[illegible]	[illegible]	[illegible]	7 HOUR SAMPLE		24 HOUR SAMPLE	
			U/OD CHON I	U/OD CHON II	U/OD CHON I	U/OD CHON II
LL2480	LL2480	NONE	0	0	0	0.01*
LL4202	LL2480	pACYC184	0	0	0.02*	0.02*
LL4198	LL2480	LP²-1521	0.8	0	1.9	0.02*
LL4192	LL2480	LP²-1531	2.08	0.45	2.85	1.43
LL2492	LL2492	NONE	0.4	0.2	0.55	0.44
LL4119	LL2492	pACYC184	0.41	0.13	0.6	0.46
LL4107	LL2492	LP²-1521	1.14	0.05	2.59	0.32
LL4142	LL2492	LP²-1531	1.39	0.72	3.96	1.93

* at or below limit of detection.

pLP²-1531 confers increased production of chondroitinases I and II in both constitutive and regulated *Proteus* host strains. Plasmid LP²-1521 confers increased production of chondroitinase I only. Thus, in a regulated strain background high

levels of chondroitinase I are seen, but no chondroitinase II is observed. In a constitutive strain, pLP^2-1521 confers high levels of chondroitinase I and low levels of chondroitinase II. The production of chondroitinase II in this strain is attributable to expression from the chromosomal copy of this gene.

The above mentioned patents, applications, test methods, and publications are hereby incorporated by reference in their entirety.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed description. All such obvious variations are within the full intended scope of the appended claims.

What is claimed:

1. The plasmid pLP^2-751.

1. The plasmid pLP^2-770.

1. The plasmid pLP^2-1512.

1. A DNA fragment comprising an about 3970 base pair
2 BglII/EcoRI fragment derived from a plasmid as defined in claim 3.

1. The plasmid pLP^2-1263.

1. The plasmid pLP^2-1508.

1. The plasmid pLP^2-1510.

1. The plasmid pLP^2-1514.

1. A DNA fragment comprising an about 2550 base pair
2 EcoRI/SmaI fragment derived from a plasmid as defined in claim 8.

1. The plasmid pLP^2-1518.

11. A DNA fragment comprising an about 6520 base pair BglII-SmaI (AvaI) fragment derived from a plasmid as defined in claim 10.

12. The plasmid pLP^2-1525.

13. The plasmid pLP^2-1531.

14. An *E. coli* cell transformed with a plasmid as defined in claim 13.

15. An *E. coli* cell as defined in claim 14 having ATCC accession number 66839 (LL4136).

16. A wild-type *P. vulgaris* cell transformed with a plasmid as defined in claim 13.

17. A *P. vulgaris* cell as defined in claim 16, wherein said cell produces chondroitinase I protein and chondroitinase II protein in the absence of an exogenous chondroitinase I and II inducer.

18. A *P. vulgaris* cell as defined in claim 17, wherein said inducer is selected from the group consisting of chondroitinase sulfate, N-acetylgalactosamine, and a combination thereof.

19. A *P. vulgaris* cell as defined in claim 18, wherein said inducer comprises chondroitin sulfate.

20. The plasmid pLP^2-1521.

21. A DNA fragment comprising an about 3420 base pair BglII/SphI fragment derived from a plasmid as defined in claim 20.

22. An *E. coli* cell transformed with a plasmid as defined in claim 20.

23. A wild-type *P. vulgaris* cell transformed with a plasmid as defined in claim 20.

24. A *P. vulgaris* cell as defined in claim 23, wherein said cell produces chondroitinase I protein in the absence of an exogenous chondroitinase I inducer.

25. A *P. vulgaris* cell as defined in claim 24, wherein said inducer is selected from the group consisting of chondroitinase sulfate, N-acetylgalactosamine, and a combination thereof.

26. A *P. vulgaris* cell as defined in claim 25, wherein said inducer comprises chondroitin sulfate.

27. A *P. vulgaris* cell as defined in claim 16, wherein said cell produces at least about 2 to about 5 times as much chondroitinase I and chondroitinase II proteins in the absence of said inducer than does an untransformed wild-type *P. vulgaris* cell in the presence of said inducer.

28. A method for producing chondroitinase I protein and chondroitinase II protein, said method comprising:

(a) culturing a cell as defined in claim 16 in a bacterial culture medium and in the absence of an exogenous chondroitinase I and II inducer;

(b) harvesting said cells from said culture; and

(c) recovering said chondroitinase I and chondroitinase II from said harvested cells.

29. A method as defined in claim 28, wherein said culturing comprises growing said cell in a medium selected from the group consisting of rich medium and minimal medium containing glucose as a sole carbon source.

30. A method as defined in claim 28, wherein said recovery comprises

(i) preparing a clarified homogenate of said harvested cells, the homogenate having a pH of 5.8 to 7.4;

(ii) loading the homogenate onto a negatively charged cation exchange resin chromatographic support so that any positively charged proteins

comprising chondroitinase I and chondroitinase II in the homogenate form a non-covalent bond with the negatively charged support;

(iii) affinity-eluting, in pools, the chondroitinase proteins from the support with an aqueous solution of chondroitin sulfate at a pH 7.0-9.5;

(iv) loading said affinity eluted protein pools from step (iii) onto an anion exchange resin chromatographic support to yield an unbound eluate; and

(v) recovering the chondroitinase I and chondroitinase II proteins in the unbound eluate.

31. A method as defined in claim 30, wherein step (v) comprises:

(1) contacting said unbound eluate with a metal chelating affinity chromatography support to bind further said chondroitinase proteins;

(2) eluting with an appropriate solvent;

(3) recovering said chondroitinase proteins.

32. A method for producing a *P. vulgaris* cell that produces chondroitinases I and II in the absence of an exogenous chondroitinase I and II inducer, said method comprising transforming a wild-type *P. vulgaris* cell with a plasmid as defined in claim 13.

33. A method for producing a *P. vulgaris* cell that produces chondroitinase I in the absence of an exogenous chondroitinase I and II inducer, said method comprising transforming a wild-type *P. vulgaris* cell with a plasmid as defined in claim 20.

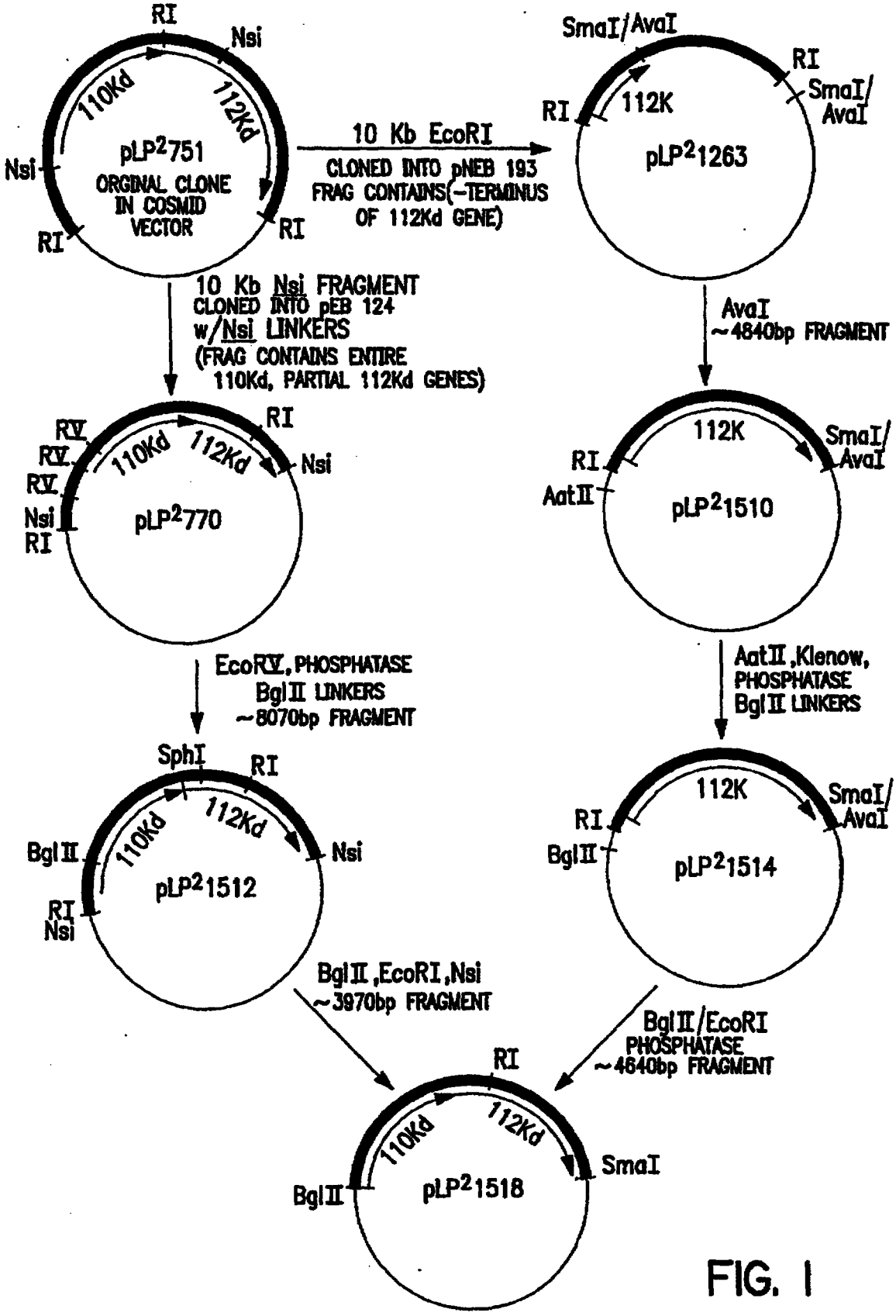

RI
NsiI
110Kd
112Kd
pLP2751
ORGINAL CLONE IN COSMID VECTOR
NsiI
RI
RI
10 Kb EcoRI
CLONED INTO pNEB 193
FRAG CONTAINS(-TERMINUS OF 112Kd GENE)
SmaI/AvaI
RI
112K
RI
SmaI/AvaI
pLP21263
10 Kb NsiI FRAGMENT
CLONED INTO pEB 124
w/NsiI LINKERS
(FRAG CONTAINS ENTIRE 110Kd, PARTIAL 112Kd GENES)
AvaI
~4840bp FRAGMENT
RV
RV
RV
NsiI
RI
110Kd
112Kd
RI
NsiI
pLP2770
112K
SmaI/AvaI
RI
AatII
pLP21510
EcoRV, PHOSPHATASE
BglII LINKERS
~8070bp FRAGMENT
AatII, Klenow,
PHOSPHATASE
BglII LINKERS
SphI
RI
BglII
110Kd
112Kd
NsiI
RI
NsiI
pLP21512
112K
SmaI/AvaI
RI
BglII
pLP21514
BglII, EcoRI, NsiI
~3970bp FRAGMENT
BglII/EcoRI
PHOSPHATASE
~4640bp FRAGMENT
RI
110Kd
112Kd
SmaI
BglII
pLP21518

FIG. 1

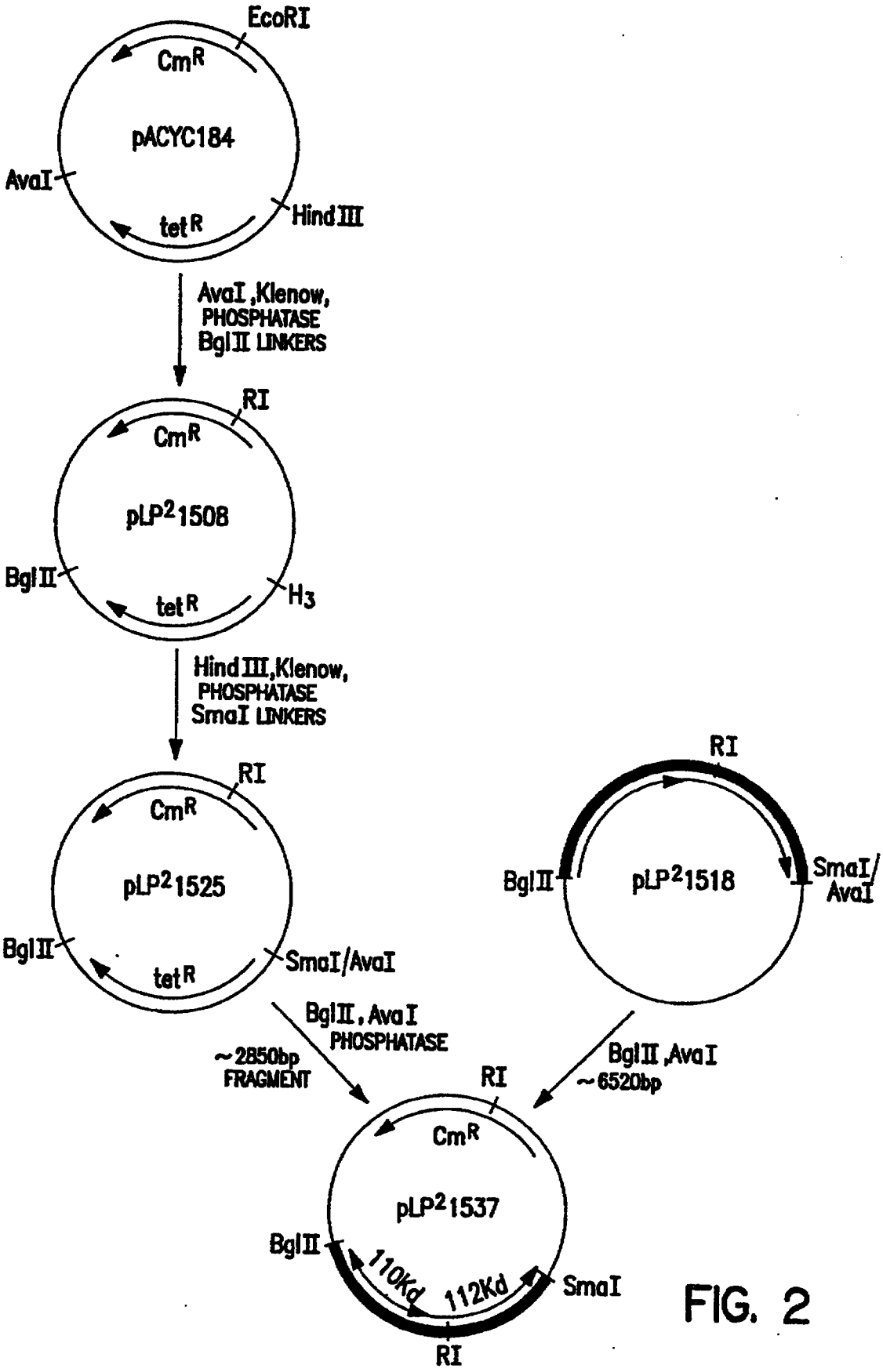

EcoRI
Cm^R
pACYC184
AvaI
tet^R
Hind III
AvaI, Klenow,
PHOSPHATASE
BglII LINKERS
RI
Cm^R
pLP^2 1508
BglII
tet^R
H3
Hind III, Klenow,
PHOSPHATASE
SmaI LINKERS
RI
Cm^R
pLP^2 1525
BglII
tet^R
SmaI/AvaI
RI
pLP^2 1518
BglII
SmaI/
AvaI
BglII, AvaI
PHOSPHATASE
~2850bp
FRAGMENT
BglII, AvaI
~6520bp
RI
Cm^R
pLP^2 1537
BglII
110Kd
112Kd
SmaI
RI

FIG. 2

RTMP copy

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/55, 9/16, 1/21 // (C12N 1/21, C12R 1:19, 1:37) — A1

(11) International Publication Number: WO 96/40938

(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/US96/08509

(22) International Filing Date: 4 June 1996 (04.06.96)

(30) Priority Data:
08/481,179 7 June 1995 (07.06.95) US

(71) Applicant: AMERICAN CYANAMID COMPANY [US/US]; Five Giralda Farms, Madison, NJ 07940 (US).

(72) Inventors: LOTVIN, Jason, Arnold; 985 Roosevelt Avenue, Union, NJ 07083 (US). KHANDKE, Kiran, Manohar; 120 East Allison Avenue, Nanuet, NY 10954 (US). RUPPEN, Mark, Edward; 6 Lea Court, Garnerville, NY 10923 (US).

(74) Agents: MATTHEWS, Gale, F.; American Home Products Corporation, Five Giralda Farms, Madison, NJ 07940 (US) et al.

(81) Designated States: AL, AM, AU, BB, BG, BR, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KG, KP, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, PT, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: CHONDROITINASE PRODUCTION IN RECOMBINANT PROTEUS VULGARIS STRAINS

(57) Abstract

Plasmids pLP^2-1531, which encodes chondroitinases I and II, and pLP^2-1521, which encodes chondroitinase I, are provided. These plasmids are used to transform wild-type *E. coli* and *P. vulgaris* cells. The transformed cells produce DNA encoding the production of chondroitinases I and II or chondroitinase I in the absence of natural exogenous chondroitinases I and II inducers. Additionally, the transformed *P. vulgaris* cells in the absence of exogenous chondroitinases I and II inducers, produce the respective enzymes in amounts in excess of those produced by wild-type *P. vulgaris* in the presence of exogenous chondroitinases I and II inducer(s). Starting plasmid pLP^2-751 and intermediate plasmids pLP^2-770, pLP^2-1512, pLP^2-1263, pLP^2-1508, pLP^2-1510, pLP^2-1514, pLP^2-1518, and pLP^2-1525 are also provided. These intermediate plasmids are typically used in the preparation of pLP^2-1531 and pLP^2-1521. A BglII/EcoRI fragment of about 3970 bp derived from pLP^2-1512, a EcoRI/SmaI fragment of about 2550 bp derived from pLP^2-1514, and a BglII/SmaI(AvaI) fragment of about 6520 bp derived from pLP^2-1518 are further disclosed. Also contemplated is the use of transformed *P. vulgaris* cells to produce chondroitinase I and/or II. The *P. vulgaris* cells above are cultured in a bacterial culture medium and in the absence of an exogenous chondroitinase I and II inducer. The cells are then harvested from the culture, and the chondroitinase I and/or II enzymes are recovered.