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(72) **Inventeurs/Inventors:**
CHANG, Li-Yen Edward, TW;
HWONG, Ching-Long, TW;
LO, Cheng-Kai, TW
(73) **Propriétaire/Owner:**
DEVELOPMENT CENTER FOR BIOTECHNOLOGY,
TW
(74) **Agent:** ROBIC

(54) **Titre :** NOUVELLE LIPASE, GENE L'ENCODANT, COMPOSE CONTENANT CELLE-CI ET PROCESSUS DE
PREPARATION DE LA CATALASE GRACE AU GENIE GENETIQUE
(54) **Title:** NOVEL CATALASE, THE GENE THEREOF AND COMPOSITION COMPRISING THE SAME, AND PROCESS
FOR PREPARING CATALASE USING GENETIC ENGINEERING TECHNOLOGY

(57) **Abrégé/Abstract:**

The invention provides a novel catalase which is cloned from *Bacillus theremoglucosidasius*. The invention further provides a process for preparing catalase in high yield, which comprises constructing expression vectors and transformant cells with catalase genes cloned from microorganisms using genetic engineering technology, and obtaining the expression product. The invention also provides a novel recombinant plasmid and a novel transformant cell constructed in the process. In addition, the invention provides a composition for decomposing hydrogen peroxide contained in the residual disinfectant on contact lenses, which comprising the novel catalase of the invention.



**NOVEL CATALASE, THE GENE THEREOF AND COMPOSITION
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ABSTRACT

The invention provides a novel catalase which is cloned from *Bacillus*
theremoglucosidasius. The invention further provides a process for preparing
catalase in high yield, which comprises constructing expression vectors and
10 transformant cells with catalase genes cloned from microorganisms using
genetic engineering technology, and obtaining the expression product. The
invention also provides a novel recombinant plasmid and a novel transformant
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FIELD OF THE INVENTION

The invention relates to a novel catalase, its use, and a process for preparing the catalase. Specifically, the invention provides a novel catalase cloned from *Bacillus theremoglucosidasius*, a composition comprising said catalase, and a process for preparing catalase in high yield which comprises using genetic engineering technology to clone catalase genes, constructing expression vectors, and transforming host cells.

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BACKGROUND OF THE INVENTION

Hydrogen peroxide, which has germ-killing, cleaning, bleaching and disinfecting activities, is in general utilized in the disinfectant of contact lenses and in the bleaching agent of textile materials, and also recognized as a legal food additive. However, hydrogen peroxide is known to have the tendency to generate free oxygen having high reactivity, the strong oxidizing effect of which will cause proteins to denature. Hence, after the disinfecting operation of contact lenses or the bleaching procedure of textile materials using hydrogen peroxide, the residual hydrogen peroxide must be subsequently decomposed to avoid the undesired injury caused to users.

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For this purpose, skilled artisans often use catalases to decompose hydrogen peroxide residues, which also is generally recognized as the most efficient method. For example, USP 4,585,488, 5,145,644, 5,362,647 and 5,521,091 disclose methods for the destruction of the hydrogen peroxide remaining in the disinfectant of contact lenses in which a catalase is added during the disinfecting operation. In addition, GB 2216149 and JP-A-104781 teach that, after bleached with hydrogen peroxide, textile substrates should be further treated with a catalase to destroy the hydrogen peroxide residues prior to the dyeing step.

Catalases [hydrogen peroxide oxidoreductases (EC 1.11.1.6)] are enzymes which catalyze the conversion of two molecules of hydrogen peroxide (H_2O_2) to one molecule of oxygen (O_2) through oxidation and two molecules of water (H_2O) through reduction. The catalysis reaction is as follows:



Catalases are present in certain animal, plant and microorganism cells, and are the required enzymes for these cells to survive in the aerobic environment. At present almost all the catalases available are obtained by purification from such cells, and among which the catalase derived from bovine livers, in particular, has been the most preferred one for the above mentioned purposes. However, in 1990, a chronic viral disease known as BSE (bovine spongiform encephalopathy) out-broke in European cattle herds. Recent studies have shown that human may be infected with this disease [Dellar and Lacey 1991 Nutr. Health (Bicester) 7:117-134; Dealler and Lacey

1990 Food Microbiol. 7:253-280). It arouses interests in finding non-mammalian-derived catalases, such as those derived from microorganism sources, instead of bovine liver catalases for the industrial applications.

Microorganisms which have been employed to obtain catalase
5 preparations through fermentation include *Aspergillus niger*, *Penicillium notatum*, *Micrococcus luteus*, etc. For instance, USP 3,123,539 discloses catalases derived from fungi such as *Aspergillus niger* and *Penicillium notatum*; USP 2,635,069 and 5,360,732 and WO 93/17721 disclose catalases generated from *Aspergillus niger*; and USP 5,521,091 discloses catalases derived from
10 *Micrococcus luteus* and *Aspergillus niger*. The catalases taught in the above patents are prepared by conventional methods including fermenting microorganisms, breaking cell bodies, and then purifying crude extract.

Nevertheless, the yields of catalases prepared from such fermentation methods are not satisfactory. For example, USP 3,123,539 discloses that the
15 1000g protein product recovered from the cell bodies of *Micrococcus luteus* (wet weight 95 pounds) yields only 5% of catalase; and USP 2,635,069 teaches that each gram crude protein extract obtained from the action of *Aspergillus niger* has only 2.4 units of catalase activity. For the sake of improving catalase yield, USP 5,360,732 tries to produce novel strains of *Aspergillus*
20 *niger*, and the resulted catalytic activity is 14.17 units per milligram crude protein extract. According to the data shown in Table 1 of WO 93/17721, the specific activity of *Aspergillus niger* is about 7.5 units per microgram. It is calculated that the above 14.17 units of activity is performed by 1.9 μ g catalase.

In other words, the crude protein extract obtained in USP 5,360,732 contains less than 0.2% catalases.

Shuichi Furuta and Hiroaki Hayashi disclose in J. Biochem. 107:708-713 (1990) the expression of a recombinant catalase gene using genetic engineering technology. However, the produced catalase was not high in yield, merely 16 mg/4L before further purification.

In view of the above, there continues to be a need for improving the catalase now employed in the art, both in its source and its preparation.

10

SUMMARY OF THE INVENTION

Accordingly, it is an object of the invention to clone a novel catalase.

It is a further object of the invention to provide a process for preparing microorganism-derived catalase using genetic engineering technology. According to the process of the invention, the catalase not only could be obtained in high yield, but also could perform superior activity.

It is an another object of the invention to provide a novel recombinant plasmid and a novel transformant cell constructed during the process of the invention.

It is an additional object of the invention to provide a composition useful in decomposing hydrogen peroxide contained in the residual disinfectant remaining on contact lens, which comprises the novel catalase of the invention.

BRIEF DESCRIPTIONS OF THE DRAWINGS

Figure 1 shows the construction of expression plasmid pET 20b/kat 19.

Figure 2 shows the DNA sequence of catalase kat 19 gene.

5 Figure 3 shows the construction of expression plasmid pET 15b/kat 19.

Figure 4 shows the construction of expression plasmid pET 20b/kat TG.

Figure 5 shows the DNA sequence of catalase kat TG gene.

Figure 6 shows the amino acid sequence of catalase TG.

10 Figure 7 shows the construction of expression plasmid pET 20b/kat
HPH.

Figure 8 shows the DNA sequence of catalase kat HPH gene.

Figure 9 shows the 12% polyacrylamide gel electrophoresis photograph
of the catalases expressed by transformant strains. From left to right are the
electrophoresis results of the catalases which are expressed by *E.coli* BL21
15 (DE3) strains transformed with pET15b, pET15b/kat19, pET 20b, pET
20b/kat19, pET 20b/kat TG and pET 20b/kat HPH, respectively.

Figure 10 shows the 12% polyacrylamide gel electrophoresis photograph
of the catalases expressed by transformant strains after purification. From left
to right are the electrophoresis results of the purified catalases which are
20 expressed by *E.coli* BL21 (DE3) strains transformed with pET 15b/kat 19, pET
20b/kat19, pET 20b/kat TG and pET 20b/kat HPH, respectively.

DETAILED DESCRIPTION OF THE INVENTION

While this specification concludes with claims particularly pointing out and distinctly claiming that which is considered to be the invention, it is
5 believed that the invention can be better understood from a reading of the following detailed description of the invention and the appended examples.

The novel catalase gene of the invention is cloned from *Bacillus thermoglucosdasius*, using proper 5' and 3' primers synthesized from the 5' end and 3' end DNA sequences of the catalase gene of *Bacillus subtilis* (kat 19)
10 disclosed in Bol and Yashin, Gene, 109:31-37 (1991). The resultant gene has been sequenced as listed in Figure 5, with a similarity of 74.4% to kat 19 gene. By inserting this gene into suitable expression vectors, transforming suitable host cells with the vectors and then expressing the gene in the host cells, the novel catalase of the invention can thus be obtained, having the amino acid as
15 listed in Figure 6. The novel catalase concerned by the invention has never been disclosed in any documents.

The invention also provides a process for preparing catalases using genetic engineering technology, said process comprising:

- (a) constructing a recombinant plasmid by inserting the gene encoding
20 said catalase into an expression vector containing a proper transcription promoter;
- (b) transforming proper host cells with said recombinant plasmid;
- (c) cultivating said transformant cells under the conditions suitable for the transformant cells to express the catalase gene; and

(d) purifying the expressed catalase protein.

The novel process of the invention is not only useful in producing the new catalase mentioned above, but can be adopted to clone the catalases of other microorganism sources. In particular, the process of the invention comprises designing appropriate primers based on the DNA sequences of catalase genes of various microorganisms from the gene library, amplifying the catalase genes existing in the chromosomes of certain microorganisms with these primers, constructing expression vectors and transforming host cells with the vectors, and then expressing the genes so as to obtain the desired catalase.

The microorganisms useful in the invention can be any microorganisms possessing catalase genes, preferably bacteria. For example, the bacteria which may be employed in accordance with the invention to produce the novel catalase of the invention can be *Bacillus subtilis* (e.g. ATCC 6051, 6633), *Escherichia coli* DH5 α , *Pseudomonas aeruginosa* (e.g. ATCC 29260), and *Bacillus thermoglucosdasius* (e.g. ATCC 43742). The microorganisms employed are not limited to any specific one but can be selected by persons skilled in the art according to their own requirements.

The amplification of catalase genes derived from microorganisms can be achieved using any conventional methods, such as polymerase chain reaction (PCR). The polymerase chain reaction has been a broadly-used technique for amplifying certain genes since 1980's (see, for example, USP 4,683,195 and 4,683,202), the protocol of which is as follows: First, the target nucleic acid is isolated from organism samples. Next, the double stranded target gene are denatured and annealed to the primers produced based on the sequences that

flank the target gene. Then the amplification is carried out using polymerase in the presence of deoxynucleotide triphosphates in appropriate amounts. The necessary reaction parameters and reagents are well known to those skilled in the art. Although the polymerase chain reaction described above can be
5 operated step by step, it is usually carried out with a commercial automatic machine (i.e. thermocycler).

The catalase genes obtained from amplification are then inserted into suitable expression vectors using conventional methods; said expression vectors include any vectors useful in bacteria, yeast, mammal or insect cell
10 expression system, such as plasmids of pBR, pUC, pUB or pET series. Persons skilled in the art can adopt what they commonly use or choose a proper one according to their needs.

The expression vectors constructed are subsequently employed to transform proper host cells and then expressed therein. According to the
15 present invention, the host cells which may be employed include, for example, bacterial cells (e.g. *E. coli* or *B. subtilis*), yeast cells (e.g. brewing yeast), mammal cells (e.g. mouse fiberblasts) and insect cells, preferably bacterial cells, and more preferably *E. coli* cells. The transformant cells are cultivated under conditions that facilitate the mass expression of the heterogenetic
20 plasmid, and the desired protein product is then separated. In accordance with the invention, the crude protein extract would contain 15-50% active catalase. After purification by conventional methods such as histidine affinity chromatography or acetone precipitation, a catalase of 95% purity can be obtained.

The advantages of producing catalases according to the present process reside in not only its simple procedures and low cost, but its high production which is superior to that can be made by any conventional methods, such as fermentation. In addition, the produced catalase is surprisingly excellent in catalytic activities; therefore, it is very suitable for use in compositions for destroying hydrogen peroxide, especially compositions for removing the residual hydrogen peroxide remaining on contact lens.

In an aspect of the invention, compositions are provided which comprises the novel catalase of the invention to decompose the residual hydrogen peroxide remaining on contact lens.

The preparation of the compositions comprising the catalase product of the invention, as well as the formulations and application methods thereof, are all well known to skilled artisans. Reference can be referred to include e.g. USP 5,521,091, 5,362,647, 5,145,644 and 4,585,488. Such compositions may be formulated as solutions, or as solid forms including tablets, capsules, etc.

In one embodiment, the composition of the invention comprises an aqueous, substantially isotonic liquid medium containing the catalase of the invention. Such aqueous, substantially isotonic liquid medium preferably includes pH controlling effective amount of a conventional buffer component. More preferably, the buffer component is effective to control the pH of the liquid medium in the range of about 3 to about 10, for example, about 6 to about 8.

The amount of the catalase employed is preferably sufficient to destroy all hydrogen peroxide present in the hydrogen peroxide-containing medium on

disinfected contact lens but not detrimental to lens *per se* and safe and comfortable wear. Typically, the catalase employed is preferably present in an amount of about 10 to about 1000, more preferably about 20 to about 800, International Units of catalase activity per milliliter of liquid medium.

5 In another embodiment of the invention, solid compositions which may be present in the form of tablets, capsules, one or more solid particles, and the like, are provided. Such solid compositions include a coated portion, e.g. a core, and a barrier or release delaying component. The coated portion or core includes the catalase of the invention. The barrier component (which may
10 include water soluble vinyl polymers, such as polyvinylpyrrolidone and polyvinyl alcohol; water soluble proteins; polysaccharides and cellulose derivatives, such as methyl cellulose) acts to delay, after the composition is initially contacted with the hydrogen peroxide-containing liquid medium, the release of the catalase into the medium for a period of time, preferably
15 sufficient to allow the lens to be disinfected by the hydrogen peroxide. The detailed formulations of such compositions can be referred to the documents mentioned above.

The following non-limiting examples further illustrate the contents of the invention more specifically.

EXAMPLE 1

Construction of Expression Plasmid pET 20b/kat19

A. Extraction of chromosomal DNA from *Bacillus subtilis*

Bacto-trypton* (10g), Bacto-yeast* extract (5g), and NaCl (10g) were dissolved in 1L of deionized water. The pH value of the solution was adjusted to 7.5 with 1N NaOH. After the solution was sterilized under high pressure and cooled the medium called Luria-Bertani (LB) medium was obtained. Supplementing the above medium with 2mL of 50mg/mL ampicillin LB/Amp medium was obtained.

Bacillus subtilis strain (ATCC 6051) was cultured with shaking at 37 °C in 3mL of LB broth for 24 hours. The culture was centrifuged (5 krpm/min) for 15 minutes, washed once with water, and then centrifuged again. After the supernatant was removed, 0.75mL of water was added to the samples, and then 0.75mL of phenol was added for extraction. After 30 minutes, the extract was centrifuged (12 krpm) for 15 minutes, and extracted with 0.75 mL of phenol for 30 minutes after the lower layers was removed. The resulted extract was centrifuged (12 krpm) for 15 minutes, extracted with 0.75 mL of chloroform for 15 minutes after removing the phenol layer and centrifuged (12 krpm) again. The lower layer was removed, to the extract two volume of ethanol was added, and an additional centrifugation of 15 minutes was carried out (12 krpm). The DNA was then precipitated, washed once with 75% ethanol, and dissolved with TE buffer containing 10mM Tris-HCl, pH 7.5, and 1mM EDTA.

* (trademarks)

B. Cloning of *Bacillus subtilis* catalase gene (kat-19)

Synthesis of 5' and 3' primers: On the basis of the 5' and 3' end sequences of the catalase gene of *Bacillus subtilis* YB 2003 disclosed in Bol. and Yashin, Gene 109: 31-37 (1991) (kat-19 gene, see Figure 2), a 5' primer, NdeI-kat 19 (+) having the following DNA sequence:

TTCATATGAGTTCAAATAAACTGACAACT

(this sequence possessed the gene for NdeI restriction enzyme), and a 3' primer, kat 19-XhoI (-) having the following DNA sequence:

TTCTCGAGTTAAGAATCTTTTTTAATCGGCAA

(this sequence possessed the gene for XhoI restriction enzyme), were synthesized.

Polymerase chain reaction (PCR): 0.5 µg chromosomal DNA of *Bacillus subtilis* was added into a solution contained 10µL of 2.5mM dNTPs, 10µL of 10xPC2 buffer (containing 50µM Tris-HCl, pH 9.1, 16mM ammonium sulfate, 3.5mM MgCl₂ and 150µg/mL BSA), 1.0µL of 0.2µg/mL 5' primer, 1.0µL of 0.2µg/mL 3' primer, 78µL of water and 1.0µL of 5U/µL Klentaq^{*}(LA Technology Inc., USA), and then 50µL of mineral oil was added. The reaction parameters of the thermocycler (Robocycler,^{*} STRATAGENE, USA) were set as follows: 94 °C for 1 minute, one cycle; 94 °C for 30 seconds, 54 °C for 1 minute, 72 °C for 1.5 minutes, and repeated 30 cycles of these three operations. After the polymerase chain reaction, the resulted product was purified using QIA quick^{*} Spin PCR purifying reagent kit (QIAGEN, Germany), and then eluted with 100µL water. By the analysis based on 0.8% agarose gel electrophoresis and stained with ethidium bromide, the amplified

*** (trademarks)**

product was identified as the desired DNA fragment which has the size previously expected, i.e. about 1.5kb.

C. Construction of expression plasmid pET 20b/kat-19 (Figure 1)

The PCR product was digested with restriction enzymes NdeI and XhoI, reacted at 37 °C for 3 hours, then separated by agarose gel electrophoresis, and a fragment of about 1.5kb was obtained. The DNA fragment was eluted with electrophoresis, and subsequently purified using QIA quick Spin PCR purification kit. By the reaction with T4 DNA ligase at 16 °C for 16 hours, the purified DNA was inserted into the expression vector pET-20b* (Novogen, USA), which was pre-digested with NdeI and XhoI restriction enzymes. After ligation, the reaction mixture was employed to transform DH5α competent cells, and the recombinant plasmid having a size larger than pET 20b was then screened out. By the analyses with restriction enzymes and DNA sequencing technique based on Sanger's method using Sequence* Version 2.0 DNA sequencing kit (United States Biochemical) and T7 promoter sequence (ATTAATACGACTCACTATAGG) as a primer, it was confirmed that the cloned DNA fragment of 1.5kb was the kat-19 gene and the construction of expression plasmid pET 20b*/kat19 had thus been accomplished.

20

EXAMPLE 2

Construction of Expression Plasmid pET 15b/kat19 (Figure 3)

The kat-19 gene was digested with restriction enzymes NdeI and XhoI, then separated by agarose gel electrophoresis, and a fragment of about 1.5kb was obtained. The DNA fragment was eluted with electrophoresis, and

* (trademarks)

purified using QIA quick Spin PCR purification kit. By the reaction with T4 DNA ligase at 16 °C for 16 hours, the purified DNA was ligated to the expression vector pET-15b* that was pre-digested with NdeI and XhoI restriction enzymes, whereby the construction of the PET 15b*/kat19 expression plasmid was accomplished. Using T7 promoter sequence as a primer to determine the 5' end DNA sequence, it was confirmed that the cloning of expression plasmid pET 15b*/kat19 had been achieved.

EXAMPLE 3

Construction of Expression Plasmid pET 20b/katTG (Figure 4)

The cells of *Bacillus thermoglucosdasius* (ATCC 43742) were cultivated at 55 °C, and the chromosomal DNA was extracted from these bacterial cultures according to the method described in Example 1. To amplify the catalase gene of *Bacillus thermoglucosdasins*, the procedure is similar to Example 1 except the sample was *Bacillus thermoglucosdasins* chromosomal DNA.

After polymerase chain reaction, a DNA fragment of 1.5kb was obtained. The DNA fragment was purified, digested with XhoI restriction enzyme at 37 °C for 2 hours, purified again, and then stored at 4 °C.

The expression vector pET 20b* was digested with NdeI restriction enzyme at 37 °C for 2 hours, and treated with Klenow enzyme after purification. The vector was purified again and digested with XhoI restriction enzyme for 2 hours at 37 °C. The DNA was separated using 0.8% agarose gel and a fragment of 3.7kb was obtained, which was then eluted by electrophoresis and purified with QIA quick* Spin PCR purification kit so as to

* (trademarks)

produce the pET 20b vector useful in the following steps (in this vector, the NdeI site had been filled in and the XhoI site had been cut open). This vector was subsequently ligated to the above fragment of 1.5kb, using T4 DNA ligase and incubated for 16 hours at 16 °C, and the resulted vector was employed to transform DH5α competent cells. After screening procedures, an expression vector, pET 20b/kat TG, was obtained. Using the DNA sequence analysis based on Sanger's method with Sequence Version 2.0 DNA sequencing kit, it was found that the cloned DNA fragment (which DNA sequence is listed in Figure 5) had a similarity to kat 19 gene of 74.4%. In addition, the protein product from translation of the DNA fragment (the amino acid sequence of which is listed in Figure 6) was found to have a similarity of 81.0% to the vegetative catalase of *Bacillus subtilis*.

EXAMPLE 4

Construction of Expression Plasmid pET 20b/kat HP11 (Figure 7)

The chromosomal DNA of *E. coli* DH5α (GIBCO BRL, USA) was extracted according to the method described in Example 1. On the basis of the DNA sequence of *E. coli* HP11 gene disclosed in J. Bacteriol 173: 514-520 (1991) (see Figure 8), a 5' primer, NdeI-HP11 (+) having the following DNA sequence:

TCCCATATGTCGCAACATAACGAAAAGAAC

(this sequence possessed the gene for the restriction enzyme NdeI), and a 3' primer, HP11-XhoI (-) having the following DNA sequence:

TTTCTCGAGGGCAGGAATTTTGTCAATCTTAGG

(this sequence possessed the gene for the restriction enzyme XhoI), were synthesized. Using the chromosomal DNA of *E. coli* DH5 α as the template, the HP11 gene was amplified under the same PCR conditions set forth in Example 1 (except that the time for primer extension was 2 minutes). As the
5 result, a DNA fragment of 2.0kb (the expected size) was obtained.

The construction of expression plasmid pET 20b/kat HP11 was carried out in the accordance with the method illustrated in Example 1. By the analysis using restriction enzymes and DNA sequencing techniques, it was confirmed that the cloned DNA fragment of 2.0 kb was HP11 gene.

10 The above plasmids pET20b/kat19, pET15b/kat19, pET20b/HP11 and pET20b/katTG were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, USA, on 18 November 1997, under the Budapest Treaty, with the Accession Nos. ATCC 209467, 209469, 209470 and 209468 respectively.

15 EXAMPLE 5

Expression of Catalase

The expression vectors pET 20b, pET 15b, pET 20b/kat 19, pET 20b/kat TG and pET 20b/kat HP11 were employed to transform the cells of *E. coli* strain BL 21 (DE3), and the bacteria were cultivated with shaking at 37 °C in
20 20mL of LB/Amp medium until the O.D. 600 value had reached 2.0. To the cultures 0.1mM isopropyl thio- β -galactoside (IPTG) was added to induce the expression of catalase. After 24 hours, the bacteria were collected. Centrifugation of 1.5mL of each of the harvested bacterial suspensions was done and the supernatant was then removed. To the suspensions, 150 μ L of

50mM sodium phosphate buffer (pH 6.0) was first added and then 150 μ L of 2x electrophoresis protein dyes containing 0.1M dithiothreitol, 2% SDS, 0.08M Tris-Cl, 15% glycerol and 0.06% bromphenol blue followed. The mixtures were heated at 95 °C for 5 minutes and then centrifuged for 5 minutes (12krpm). The 12% SDS-PAGE analysis of 20 μ L of each of the supernants obtained was subsequently carried out to analyze all the proteins present in the bacteria. The results are shown in Figure 9; from left to right are the electrophoresis results of the catalases expressed by *E.coli* BL21 (DE3) strains transformed with pET 15b, pET 15b/kat 19, pET 20b, pET 20b/kat19, pET 20b/kat TG and pET 20b/kat HP11, respectively. According to Figure 9, in the bacteria, there are approximately 15-50% of the total protein contained catalases. In other words, such host cells carrying catalase-expressing vectors could all produce catalases in very high yield.

EXAMPLE 6

Purification of Catalase

The cells of BL 21 (DE3) (Novogen, USA) strain transformed with expression vectors pET 20b/kat 19 were inoculated on 100mL of LB/Amp medium, and cultivated with shaking at 37 °C till O.D. 600 =2.0. After induction with 0.1M IPTG, the cultures were centrifuged and the medium were removed. The bacterial cells were resuspended in 10mL of 50mM sodium phosphate buffer (pH 6.4) and disrupted by sonication. After centrifugation for 15 minutes (12krpm), the bacterial debris was precipitated and the supernatant was collected. The supernatant was mixed with an equivalent amount of acetone (added at 4 °C) for 30 minutes, centrifuged and the

supernatant was removed. The precipitate obtained was dissolved in 10mL of 50mM sodium phosphate buffer, pH 6.4, stored at 4 °C for 12 hours, and subjected to centrifugation for 5 minutes (5krpm). The supernatant obtained from the centrifugation was collected so as to complete the purification procedure.

On the other hand, the cells of BL 21 (DE3) strain carrying expression vectors pET 15b*/kat 19, pET 20b*/kat TG and pET 20b*/kat HP11 were individually inoculated on 100mL of LB/Amp medium, and cultivated with shaking at 37 °C. When the O.D. 600 value had reached 2.0, 0.1M IPTG was added and the cultivation continued for additional 24 hours. The cultures were subjected to centrifugation followed by removing the medium, and then 20 mL of IMAC-5 containing 20mM Tris-HCl (pH 7.9), 0.5M NaCl, 10% glycerol and 5mM imidazole was added to resuspend the bacteria. The bacterial cells were then disrupted by sonication. After centrifugation, the bacterial debris was precipitated and the supernatant obtained was collected. The purification of catalases was done using 2.5 mL of histidine affinity chromatographic column (His-Bind* resin, Novogen), according to the protocol suggested by Novogen (pET His. Tog^{TG} System Protocols, Novogen).

The amount and the activity of the purified protein were measured as follows: The protein amount was determined by the reagents and procedures for protein quantitative analysis from Bio-RAD Co., using bovine serum albumin as a standard. The catalase activity was determined by measurement of OD value, under the UV light of 240nm wavelength, the decomposed rate of hydrogen peroxide present in 50mM sodium phosphate buffer (pH 7.0, 25 °C)

* (trademarks)

from the reaction of the catalase obtained above. The initial concentration of hydrogen peroxide contained in the buffer was 20mM, and the measurement interval was 20 seconds. Each unit (U) of catalase activity is defined as 1 μ mol of hydrogen peroxide decomposed in 1 minute.

5 The measurement of the amounts and the activities of various catalases was performed according to the methods described above. In addition, the purity of the catalases (using 5 μ g for each catalase) was determined by 12% polyacrylamide gel electrophoresis analysis. The results are shown in Figure 10. From left to right are the protein standard, the catalases expressed by
10 expression vectors pET 15b/kat 19, pET 20b/kat19, pET 20b/kat TG and pET 20b/kat HP11, respectively. The electrophoresis results indicate that all proteins have a purity higher than 95%. The specific activities of such catalases are listed as follows:

Expression vectors	Specific activity (U/ μ g)
pET 15b/kat 19	18-22
pET 20b/kat 19	18-22
pET 20b/kat TG	30-40
pET 20b/kat HP11	10-14

15

From the above table, it is recognized that all the catalases prepared according to the invention have superior catalytic activities. In addition, the specific activities of these catalases are all better than commercialized catalases

derived from *Aspergillus niger* (about 5U/ μ g according to the Merck catalog, and about 4-8U/ μ g according to the Sigma catalog).

EXAMPLE 7

Decomposition Effect on Hydrogen Peroxide

5 Present in Contact Lens Disinfectant

A commercialized contact lense disinfectant typically contains about 3%(w/v) hydrogen peroxide. The disinfection procedures are as follows: The contact lens to be disinfected were placed in 10mL of 3% (w/v) aqueous solution of hydrogen peroxide for approximately 20 minutes. Afterward, the
10 disinfected contact lens were removed from the hydrogen peroxide solution and placed in 10mL of an aqueous solution containing a catalase, or alternatively, a catalase is added directly into the hydrogen peroxide solution. After about 10-20 minutes, the disinfected contact lens were removed from the solution, rinsed in saline solution and placed into the wearer's eye. To test the
15 performance of the catalase of the invention in decomposing the hydrogen peroxide contained in the disinfectant for contact lens, a quantity, 50 μ g, of each of the catalases prepared according to the example 6 were added into a 3% aqueous solution of hydrogen peroxide. It was found that the concerned catalases took only 5 minutes to destroy the hydrogen peroxide effectively,
20 causing its concentration lower than 0.02% (w/v). Apparently, the efficacy of the catalase of the invention is superior to that of the commercialized product available.

EXAMPLE 8**Solid composition**

A layered tablet which has a core surrounded by a delayed release layer is prepared. The layered tablet has the following composition:

5 Core

Sodium Chloride	89.4 mg
Dibasic Sodium Phosphate (Anhydrous)	12.5 mg
Monobasic Sodium Phosphate	
Monohydrate (Anhydrous)	0.87mg
Polyethylene Glycol	
(M.W.3350)	1.05 mg
Lyophilized Catalase of the Invention	2500 International Units

Coating Layer

Hydroxypropylmethylcellulose	8 mg
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EXAMPLE 9**Solution composition**

10 A two unit dose (10mL) formulation is prepared and has the following composition:

Sodium Chloride	0.85%
Dibasic Sodium Phosphate heptahydrate	0.402%
Monobasic Sodium Phosphate	
Monohydrate	0.091%
Disodium Edetate	0.100%

Liquid Catalase of The Invention⁽¹⁾

260 International Units/mL

Purified Water

QS-ad

⁽¹⁾The liquid catalase include 35-40 %wt glycerol and 10 %wt ethanol.

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: DEVELOPMENT CENTER FOR BIOTECHNOLOGY
 - (ii) TITLE OF INVENTION: NOVEL CATALASE, THE GENE
THEREOF AND COMPOSITION COMPRISING THE
SAME, AND PROCESS FOR PREPARING CATALASE
USING GENETIC ENGINEERING TECHNOLOGY
 - (iii) NUMBER OF SEQUENCES: 2
 - (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: DEVELOPMENT CENTER FOR BIOTECHNOLOGY
 - (B) STREET: 81, CHANG-HSING STREET
 - (C) CITY: TAIPEI
 - (E) COUNTRY: The Republic of China (Taiwan)
 - (F) ZIP:
 - (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: TXT ASCII
 - (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME:
 - (B) REGISTRATION NUMBER:
 - (C) REFERENCE/DOCKET NUMBER:
 - (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE:
 - (B) TELEFAX:
- (2) INFORMATION FOR SEQ ID NO: 1:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1449 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ATGAGTTCAA  ATAAACTGAC  AACTAGCTGG  GGAGCACCTG  TTGGCGATAA  CCAAAACTCG  60
ATAACGGCCG  GCAATCCTGG  CCCGACATTA  ATCCAAGACG  TACATCTTAT  CGAAAAATTA  120
GCACACTTCA  ATAGAGAACG  TGTCCCAGAA  CGTGTTGTCC  ATGCGAAAGG  CGCTGGTGCG  180

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CACGGCTATT	TCGAAGTAAC	AAACGATATG	TCGAAATACA	CAAAAGCGAA	AGTGTTTAAC	240
GGTGTTGGCA	AACGCACGCC	TGTATTCGTC	CGCTTCTCTA	CTGTCGCCGG	TGAATTGGGA	300
TCTGCGGATA	CAGTCCGCGA	CCCGCGCGGT	TTTGCCGTCA	AATTTTATAC	CGAAGAAGGA	360
AACTATGACA	TCGTTGGCAA	CAACACACCG	ATTTTCTTCA	TTCGTGATGC	GATTAAATTC	420
TCGGATTTTA	TCCATACACA	AAAACGCGAC	CCGCGCACCC	ATTTGATTTA	TCCGACAGCA	480
ATGTGGGATT	TCTTGTCTTT	ATCTCCGGAA	TCTTTGCACC	AAGTCACTTA	TTTATTCGGG	540
GATCGCGGCA	TCCCATTGAC	ATACCGCCAT	ATGAACGGAT	ACGGAAGCCA	TACATTCAAA	600
TGGGTGAATG	AAAAAGGCGA	AGCGGTATGG	GTAAAATACC	ACTTTAAAAC	AAACCAAGGC	660
GTGAAAAACA	TGGATCCGGA	ACTAGCGGTT	AAAATCGCCG	GAGAAAATCC	GGATTACCAT	720
ACGGAAGATT	TATATAACGC	CATCGAAAAA	GGCGACTATC	CATCTTGGAC	ATTATATGTG	780
CAAATTATGC	CGTTAGAAGA	CGCAAAAACA	TACCGTTTCA	ATCCATTTGA	TGTCACAAAA	840
GTTTGGTCAC	ATAAAGATTA	TCCGTTAATT	GAAGTCGGCC	GTATGGTATT	AAACCGCAAT	900
CCAGAAAATT	ATTTTGCCGA	AGTCGAACAA	GCGACATTCT	CTCCTGGAAA	CCTTG TTCCT	960
GGCGTTGAAC	CATCGCCGGA	TAAAATCTTG	CAAGCCCGTT	TGTTCGCTTA	TGCGGATGCG	1020
CACCGTTACC	GCGTCGGCGT	GAACCATAAC	TTGCTTCCGA	TCAACCGCCC	GCGCGTGGAA	1080
GTAAACAATT	ATCAACGTGA	CGGCTTCATG	CGCTTTGACA	ATAATGGCGG	CGGTTCGGTC	1140
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TTCCCGGTAT	CCGGCGTGGC	AGAAAGCGTG	CCATATGACG	ACGATGATCA	TTATACGCAA	1260
GCAGGCGACT	TATACCGTCT	CATGAGCGAA	GAAGAAAAAG	CGCGCCTTGT	GAAAAACATT	1320
GTCGAATCAT	TGAAACAAGT	AACAAAAGAA	GAAATTAAAC	TTCGCCAAAT	CCGCCACTTC	1380
TACAAAGCAG	ACCCTGACTA	CGGCCGCCGC	GTTGCCGAAG	GTCTTGGATT	GCCGATTAAA	1440
AAAGATTCT						1449

(2) INFORMATION FOR SEQ ID NO: 2:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 483 amino acids

(B) TYPE: amino acid
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Met Ser Ser Asn Lys Leu Thr Thr Ser Trp GLN Ala Pro Val Gly Asp
 1          5          10          15
Asn Gln Asn Ser Ile Thr Ala Gly Asn Pro Gly Pro Thr Leu Ile Gln
 20          25          30
Asp Val His Leu Ile Glu Lys Leu Ala His Phe Asn Arg Glu Arg Val
 35          40          45
Pro Glu Arg Val Val His Ala Lys Gly Ala Gly Ala His Gly Tyr Phe
 50          55          60
Glu Val Thr Asn Asp Met Ser Lys Tyr Thr Lys Ala Lys Val Phe Asn
 65          70          75          80
Gly Val Gly Lys Arg Thr Pro Val Phe Val Arg Phe Ser Thr Val Ala
 85          90          95
Gly Glu Leu Gly Ser Ala Asp Thr Val Arg Asp Pro Arg Gly Phe Ala
100          105          110
Val Lys Phe Tyr Thr Glu Glu Gly Asn Tyr Asp Ile Val Gly Asn Asn
115          120          125
Thr Pro Ile Phe Phe Ile Arg Asp Ala Ile Lys Phe Ser Asp Phe Ile
130          135          140
His Thr Gln Lys Arg Asp Pro Arg Thr His Leu Ile Tyr Pro Thr Ala
145          150          155          160
Met Trp Asp Phe Leu Ser Leu Ser Pro Glu Ser Leu His Gln Val Thr
165          170          175
Tyr Leu Phe Gly Asp Arg Gly Ile Pro Leu Thr Tyr Arg His Met Asn
180          185          190
Gly Tyr Gly Ser His Thr Phe Lys Trp Val Asn Glu Lys Gly Glu Ala
195          200          205
Val Trp Val Lys Tyr His Phe Lys Thr Asn Gln Gly Val Lys Asn Met
210          215          220
Asp Pro Glu Leu Ala Val Lys Ile Ala Gly Glu Asn Pro Asp Tyr His
225          230          235          240

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Thr Glu Asp Leu Tyr Asn Ala Ile Glu Lys Gly Asp Tyr Pro Ser Trp
 245 250 255
 Thr Leu Tyr Val Gln Ile Met Pro Leu Glu Asp Ala Lys Thr Tyr Arg
 260 265 270
 Phe Asn Pro Glu Asp Val Thr Lys Val Trp Ser His Lys Asp Tyr Pro
 275 280 285
 Leu Ile Glu Val Gly Arg Met Val Leu Asn Arg Asn Pro Glu Asn Tyr
 290 295 300
 Phe Ala Glu Val Glu Gln Ala Thr Phe Ser Pro Gly Asn Leu Val Pro
 305 310 315 320
 Gly Val Glu Pro Ser Pro Asp Lys Ile Leu Gln Ala Arg Leu Phe Ala
 325 330 335
 Tyr Ala Asp Ala His Arg Tyr Arg Val Gly Val Asn His Asn Leu Leu
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 Pro Ile Asn Arg Pro Arg Val Glu Val Asn Asn Tyr Gln Arg Asp Gly
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 Phe Met Arg Phe Asp Asn Asn Gly Gly Gly Ser Val Asn Tyr Glu Pro
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 Asn Ser Phe Gly Gly Pro Thr Glu Val Pro Glu His Lys Thr Thr Pro
 385 390 395 400
 Phe Pro Val Ser Gly Val Ala Glu Ser Val Pro Tyr Asp Asp Asp Asp
 405 410 415
 His Tyr Thr Gln Ala Gly Asp Leu Tyr Arg Leu Met Ser Glu Glu Glu
 420 425 430
 Lys Ala Arg Leu Val Lys Asn Ile Val Glu Ser Leu Lys Gln Val Thr
 435 440 445
 Lys Glu Glu Ile Lys Leu Arg Gln Ile Arg His Phe Tyr Lys Ala Asp
 450 455 460
 Pro Asp Tyr Gly Arg Arg Val Ala Glu Gly Leu Gly Leu Pro Ile Lys
 465 470 475 480
 Lys Asp Ser

CLAIMS

1. A catalase gene, which comprises the nucleotide sequence of SEQ. ID.NO: 1; and the degenerate sequences thereof.

2. The gene according to claim 1, which is derived from *Bacillus thermoglucosdasius*.

3. A catalase, which comprises the amino acid sequence of SEQ ID. NO: 2; and the functional fragments thereof encoding a protein exhibiting substantially the same biological activity set forth in SEQ ID NO:2.

4. A process for preparing *Baccillus theremoglucosidasius*-derived catalase using genetic technology, which comprises:

a) constructing a recombinant plasmid by inserting the gene encoding said catalase into an expression vector containing a proper transcription promoter;

b) transforming proper host cells with said recombinant plasmid;

20 c) culturing said transformant cells under the conditions suitable for the transformant cells to express the catalase gene; and

d) purifying catalase protein expressed.

5. The process according to claim 4, wherein the gene encoding said catalase is according to claim 2.

6. The process according to claim 4, wherein said host cell is an *E. coli* cell.

7. A recombinant plasmid for expressing a *Baccillus theremoglucosidasius*-derived catalase, which is constructed by inserting the gene encoding said *Baccillus theremoglucosidasius*-derived catalase into an expression vector.

8. The recombinant plasmid according to claim 7, wherein the gene encoding said *Baccillus theremoglucosidasius*-derived catalase is according to claim 3.

10 9. The recombinant plasmid according to claim 7, wherein said expression vector is the expression vector useful in *E. coli* expression system.

10. The recombinant plasmid according to claim 7, which is selected from the group consisting of ATCC 209467, 209468, 209469 and 209470.

11. A transformant cell for expressing a *Baccillus theremoglucosidasius*-derived catalase, which is a host cell transformed with the recombinant plasmid according to any of claims 7 to 10.

20 12. The transformant cell according to claim 8, wherein said host cell is an *E. coli* cell.

13. A composition for use in destroying hydrogen peroxide present in a residual disinfectant remaining on contact lens, which comprises an effective amount of the catalase according to claim 3 and a physiologically acceptable carrier or excipient.

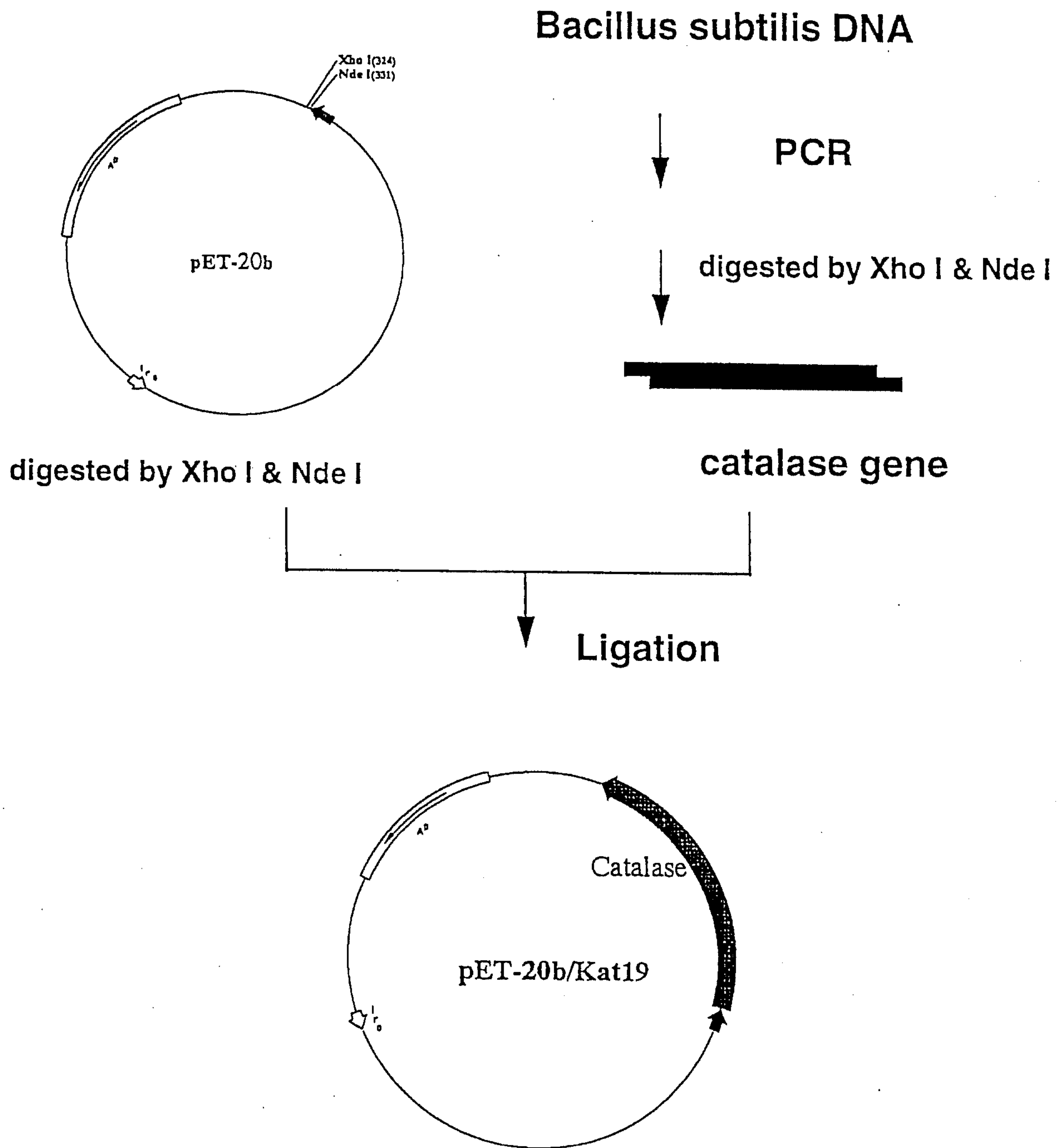


Fig. 1

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201 aaacgacgta acaaaataca cgaaagccgc tttcctttct gaagtcggca

251 aacgcacacc gttgttcac cgtttctcaa cagttgccgg tgaacttggc
301 tctgctgaca cagttcgcga cccgcgcgga tttgctgtta aattttatac
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401 tccgcgatgc gattaagttc cctgatttca tccatacaca aaaaagagat
451 ccaaaaacac acctgaaaaa ccctacggct gtatgggatt tctggtcact

501 ttcaccagag tcactgcacc aagtgacaat cctgatgtct gaccgcggaa
551 ttctgcgac acttcgccac atgcacggct tcggaagcca tacattcaaa
601 tggacaaatg ccgaaccgga aggcgtatgg attaaatata actttaaaac
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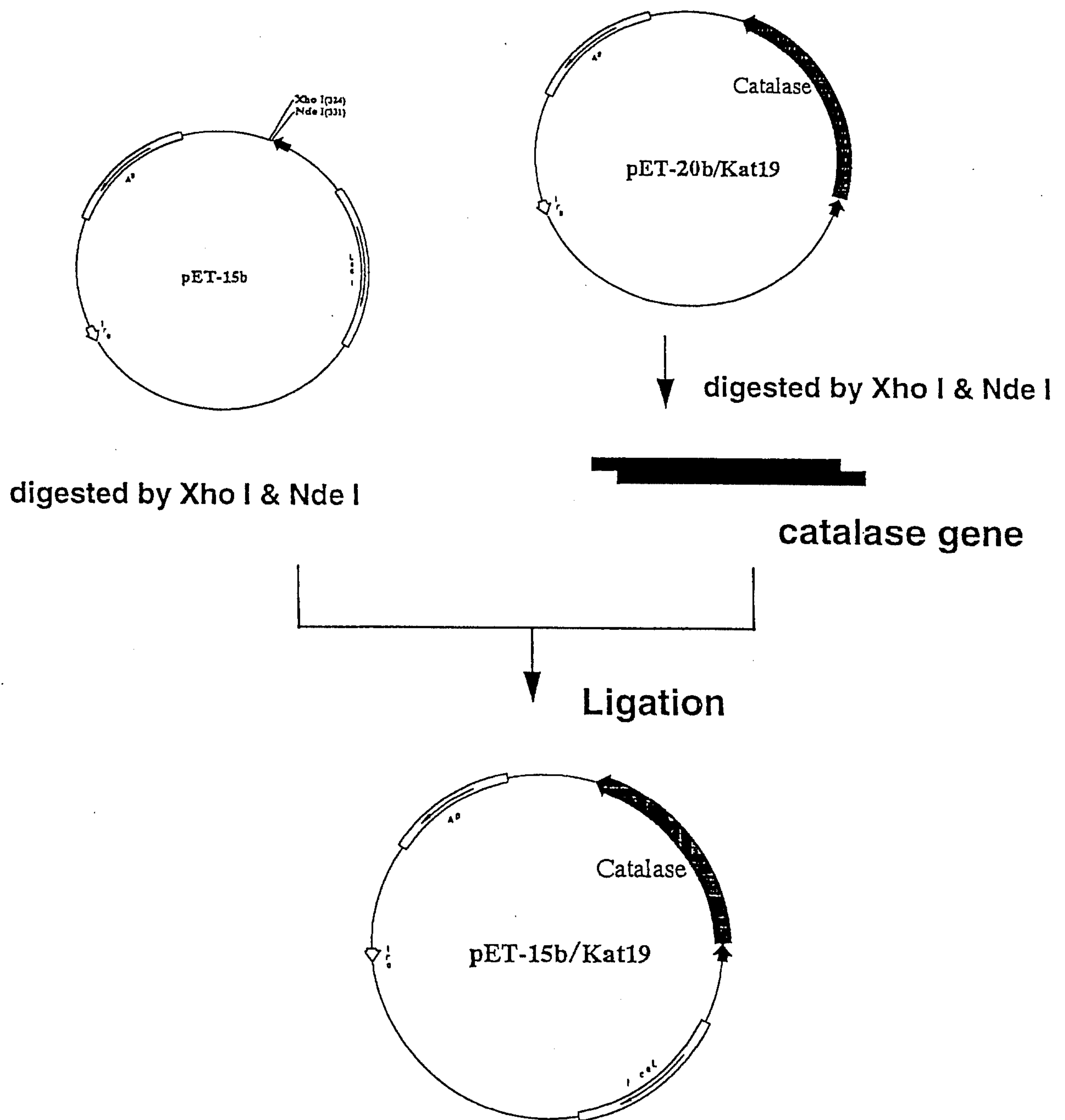
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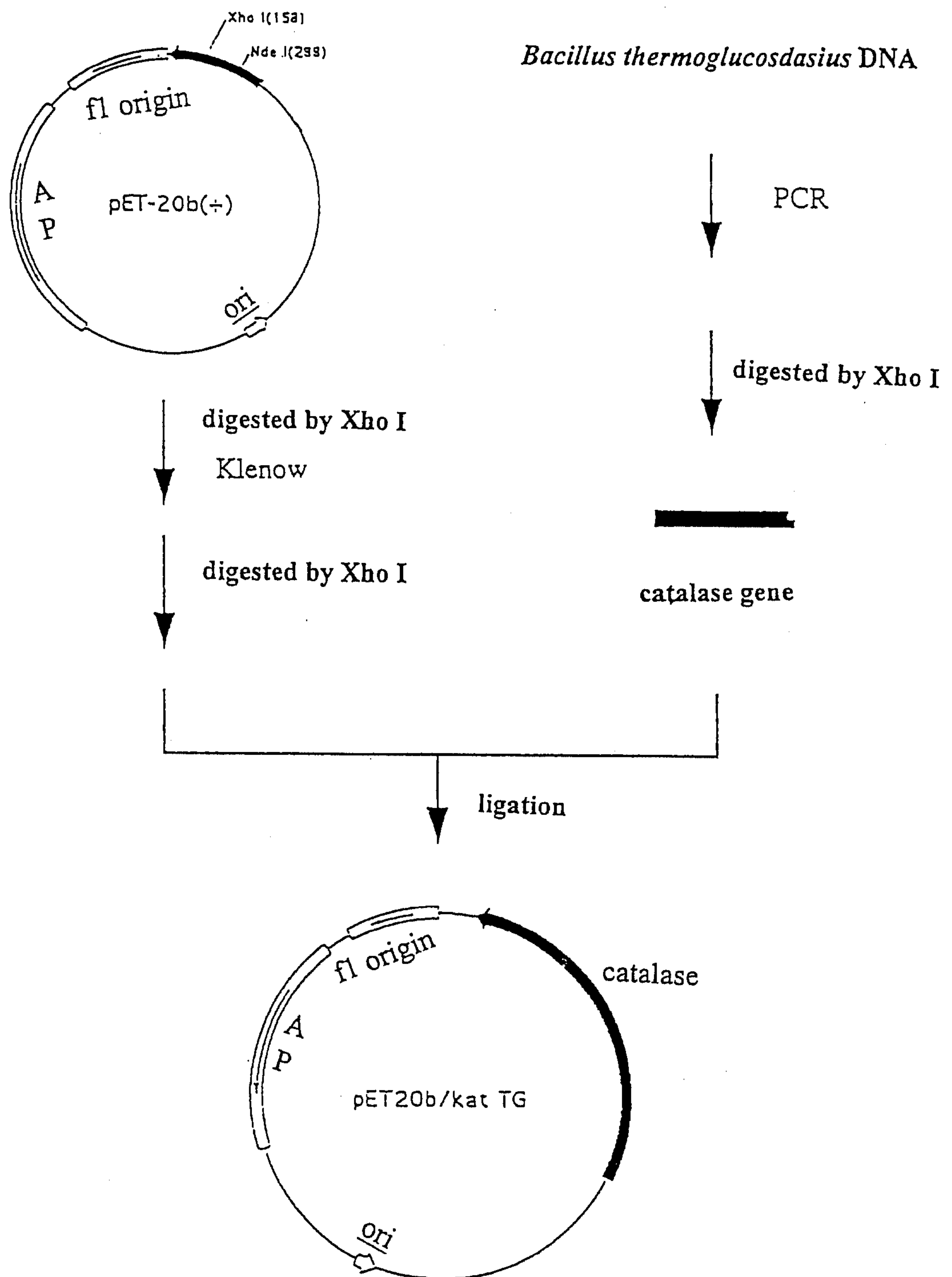
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1351 gaaatcaagc tgcgccaaat cgagcacttc tacaaagcgg atcctgaata
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1451 aa

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Fig. 2

**Fig. 3**

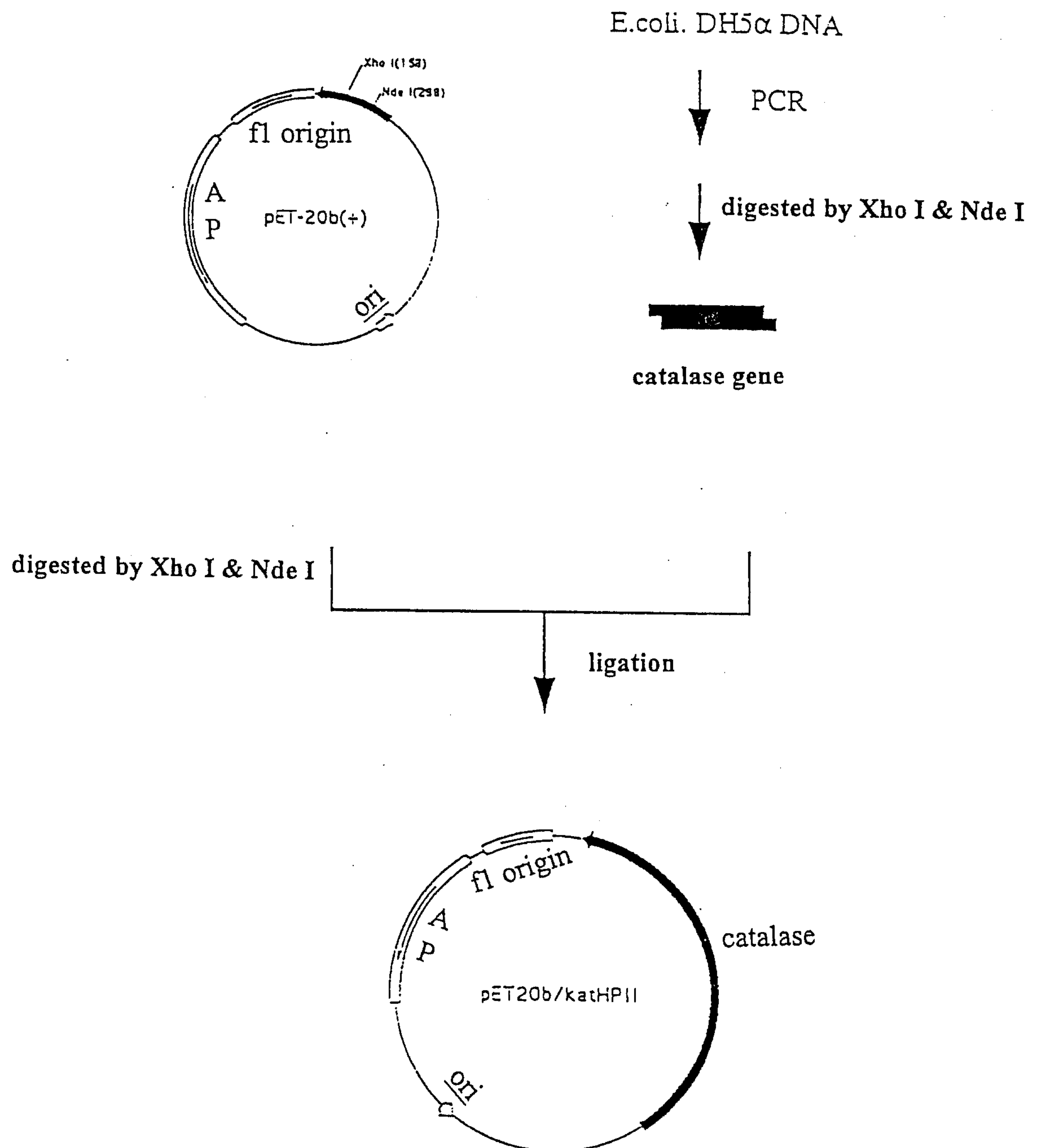
*Fig. 4*

10	20	30	40	50	60
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70	80	90	100	110	120
ATAACGGCCG	GCAATCCTGG	CCCGACATTA	ATCCAAGACG	TACATCTTAT	CGAAAAATTA
130	140	150	160	170	180
GCACACTTCA	ATAGAGAACG	TGTCCCAGAA	CGTGTGTGTC	ATGCGAAAGG	CGCTGGTGCG
190	200	210	220	230	240
CACGGCTATT	TCGAAGTAAC	AAACGATATG	TCGAAATACA	CAAAAGCGAA	AGTGTTTAAC
250	260	270	280	290	300
GGTGTGGCA	AACGCACGCC	TGTATTCTGC	CGCTTCTCTA	CTGTCCGCCG	TGAATTGGGA
310	320	330	340	350	360
TCTGCGGATA	CAGTCCGCGA	CCCGCGCGGT	TTTGCCGTCA	AATTTTATAC	CGAAGAAGGA
370	380	390	400	410	420
AACTATGACA	TCGTTCGGCA	CAACACACCG	ATTTTCTTCA	TTCGTGATGC	GATTAAATTC
430	440	450	460	470	480
TCGGATTTTA	TCCATACACA	AAAACGCGAC	CCGCGCACCC	ATTTGATTTA	TCCGACAGCA
490	500	510	520	530	540
ATGTGGGATT	TCTTGTCTTT	ATCTCCGGAA	TCTTTGCACC	AAGTCACTTA	TTTATTCGGG
550	560	570	580	590	600
GATCGCGGCA	TCCCATTGAC	ATACCGCCAT	ATGAACGGAT	ACGGAAGCCA	TACATTCAAA
610	620	630	640	650	660
TGGGTGAATG	AAAAAGGCGA	AGCGGTATGG	GTAAATATACC	ACTTTAAAC	AAACCAAGGC
670	680	690	700	710	720
GTGAAAAACA	TGGATCCGGA	ACTAGCGGTT	AAAATCGCCG	GAGAAAATCC	GGATTACCAT
730	740	750	760	770	780
ACGGAAGATT	TATATAACGC	CATCGAAAAA	GGCGACTATC	CATCTTGGAC	ATTATATGTG
790	800	810	820	830	840
CAAATTATGC	CGTTAGAAGA	CGCAAAAACA	TACCGTTTCA	ATCCATTTGA	TGTCACAAAA
850	860	870	880	890	900
GTTTGGTCAC	ATAAAGATTA	TCCGTTAATT	GAAGTCGGCC	GTATGGTATT	AAACCGCAAT
910	920	930	940	950	960
CCAGAAAATT	ATTTTGCCGA	AGTCGAACAA	GCGACATTCT	CTCCTGGAAA	CCTTGTTCCT
970	980	990	1000	1010	1020
GGCGTTGAAC	CATCGCCGGA	TAAAATCTTG	CAAGCCCGTT	TGTTGCTTA	TCCGGAATGCG
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CACCGTTACC	GCGTCGGCGT	GAACCATAAC	TTGCTTCCGA	TCAACCGCCC	GCGCGTGGAA
1090	1100	1110	1120	1130	1140
GTAAACAATT	ATCAACGTGA	CGGCTTCATG	CGCTTTGACA	ATAATGGCGG	CGGTTCCGTC
1150	1160	1170	1180	1190	1200
AACTACGAAC	CAAACAGCTT	CGGCGGACCG	ACAGAAGTGC	CAGAACATAA	AACGACCCCA
1210	1220	1230	1240	1250	1260
TTCCCGGTAT	CCGGCGTGGC	AGAAAGCGTG	CCATATGACG	ACGATGATCA	TTATACGCAA
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GCAGGCGACT	TATACCGTCT	CATGAGCGAA	GAAGAAAAG	CGCGCCTTGT	GAAAAACATT
1330	1340	1350	1360	1370	1380
GTCGAATCAT	TGAAACAAGT	AACAAAAGAA	GAAATTAAAC	TTGCCCAAAT	CCGCCACTTC
1390	1400	1410	1420	1430	1440
TACAAAGCAG	ACCCTGACTA	CGGCCGCCGC	GTGCCGAAG	GTCTTGATT	GCCGATTAAA
1450	1460	1470	1480	1490	1500
AAAGATTCT.					

Fig. 5

10	20	30	40	50	60
MSSNKLTTSW	GAPVGDNQNS	ITAGNPGPTL	IQDVHLIEKL	AHFNRRERVPE	RVVHAKGAGA
70	80	90	100	110	120
HGYFEVTNDM	SKYTKAKVFN	GVGKRTPVFN	RFSTVAGELG	SADTVRDPRG	FAVKFYTEEG
130	140	150	160	170	180
NYDIVGNNTF	IFFIRDAIKF	SDFIHTQKRD	PRTHLIYPTA	MWDFLSLSPE	SLHQVTYLFQ
190	200	210	220	230	240
DRGIPLTYRH	MNGYGSHTFK	WVNEKGEAVW	VKYHFKNQOG	VKNMDPELAV	KIAGENPDYH
250	260	270	280	290	300
TEDLYNAIEK	GDYPSWTLVY	QIMPLEDAKT	YRFNPFQVTK	VWSHKDYPLI	EVGRMVLNRN
310	320	330	340	350	360
PENYFAEVEQ	ATFSPGNLVP	GVEPSPKIL	QARLFAYADA	HRYRVGVNHN	LLPINRPRVE
370	380	390	400	410	420
VNNYQRDGM	RFDNNGGGSV	NYEPNSFGGP	TEVPEHKTTP	FPVSGVAESV	PYDDDDHYTQ
430	440	450	460	470	480
AGDLYRLMSE	EEKARLVKNI	VESLKQVTKE	EIKLRQIRHF	YKADPDYGRR	VAEGLGLPIK
490	500	510	520	530	540
KDS.....					

Fig. 6

*Fig. 7*

```

1  ctgcagcctt tctttaaaaag agtcgaaagc caggcttttta atattttaaat
51  caccataatt actctgtatt aagtttgtag aaaacatctc ccgcctcata
101 ttgttaacaa aattattatc tcattttaaat ctaagtcatt tacaatataa
151 gtttaagagc gacgccacag gatgaactat caaaaatagc tcatcatgat
201 tagcaaaaact taaccattttt aaaataaata aacaattaaa gaaaaaagat

251 cacttatttta tagcaataga tcgtcaaagg cagcttttttg ttacagggtgg
301 tttgaatgaa tgtagcaacg aaatacagaa tttcagggtca tgtaactccc
351 ggcaaaccgg gaggtatgta atccttactc agtcacttcc ccttcctggc
401 ggatctgatt tgcccaacgt tgggcagatt caggcacagt aaacgccggt
451 gagcgcagaa atgactctcc catcagtaca aacgcaacat atttgccacg

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751 ggtctatagt tagagagttt tttgaccaa acagcggccc tttcagtaat
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Fig. 8(to be continued)

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```

Fig. 8(continued)

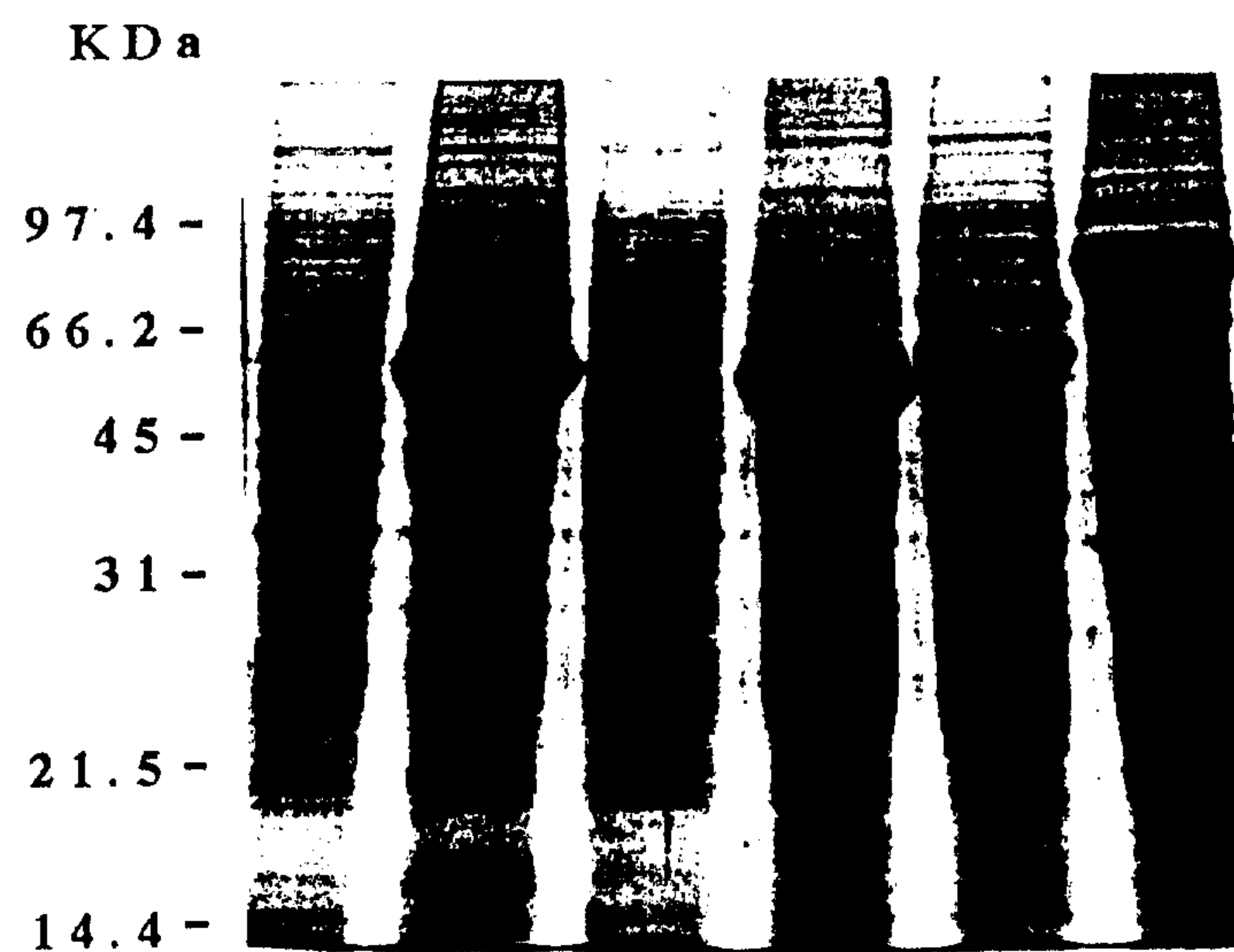


Fig. 9

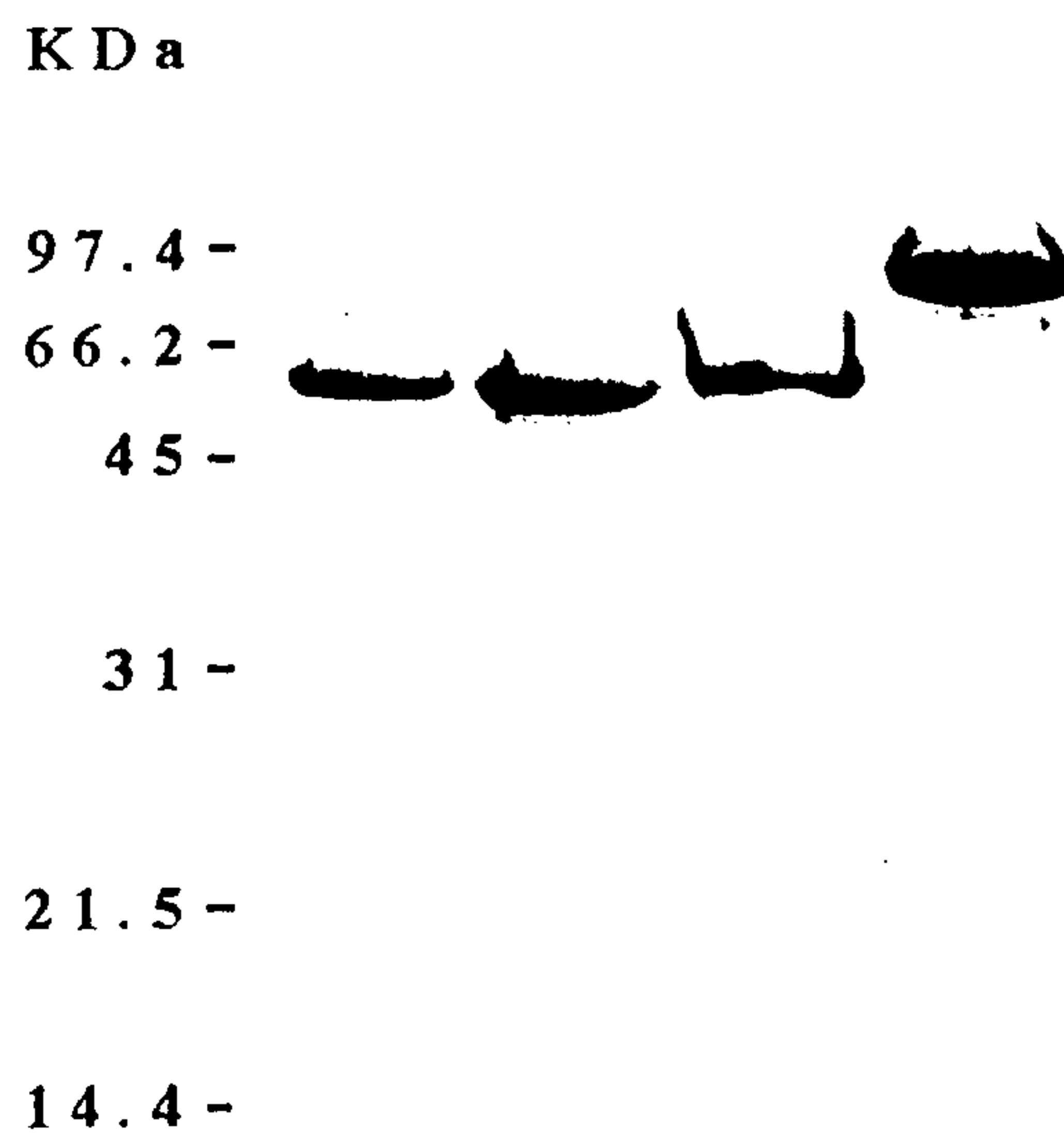


Fig. 10