Gene encoding enzyme having flavin reducing activity and nitroreductase activity
Für ein Enzym mit flavinreduzierender und Nitroreduktase-Aktivität kodierendes Gen
Gène codant pour un enzyme avec activité réduisant les flavines et activité nitroréductase

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(56) References cited:
G. SPYROU ET AL. ‘Characterization of the flavin reductase gene (fre) of Escherichia coli and construction of a plasmid for overproduction of the enzyme’
G. SPYROU ET AL. ‘Characterization of the flavin reductase gene (fre) of Escherichia coli and construction of a plasmid for overproduction of the enzyme’
Remarks:
The file contains technical information submitted after the application was filed and not included in this specification.
BACKGROUND OF THE INVENTION

(i) Field of the Invention

[0001] The present invention relates to a gene encoding an enzyme having a flavin reducing activity and a nitroreductase activity, the enzyme produced therefrom, a recombinant vector containing the gene and bacteria containing the recombinant vector.

(ii) Description of the Related Art

[0002] A bacterial luciferase derived from luminous bacteria is used to produce oxidized flavin adenine mononucleotide (hereinafter referred to as "oxidized FMN") and a long-chain carboxylic acid in the presence of a long-chain aliphatic aldehyde, oxygen and a reduced flavin adenine mononucleotide (hereinafter referred to as "FMNH₂") as a luminescent substrate, and in this case, the bacterial luciferase catalyzes a reaction in which blue light is emitted. FMNH₂ which is a substrate can be obtained from a reduced nicotinamide adenine dinucleotide:flavin mononucleotide (NADH:FMN) reductase and a reduced nicotinamide adenine dinucleotide phosphate:flavin mononucleotide (NADPH:FMN) reductase, and the long-chain aldehyde can be obtained from a fatty acid reductase complex.

[0003] Lavi et. al. (Journal of Bioluminescence and Chemiluminescence Vol. 5, pages 187-192 (1990)) disclose a modified purification method for bacterial luciferases and NAD(P)H:FMN oxido-reductases, which uses FMN-Sepharose alone or coupled to DEAE ion exchange chromatography for the simultaneous purification of luciferase and the various oxidoreductases from Vibrio harveyi, a bright mutant of Vibrio fischeri, Vibrio fischeri, and Photobacterium phosphoreum.


[0005] Around us, there are many substances (mutagens) which damage chromosomal DNA, and during our lives we are exposed to these substances. Nitroarenes are members of one group of environmental mutagens, and they are contained in the exhaust gas of automobiles, the smoke of incinerators, the atmosphere of cities, the bottoms of rivers, the air in rooms where stoves are lighted, and the burnt portions of grilled chickens. Of nitroarenes having mutability and carcinogenicity, 2-nitrofluorene is well known.

[0006] A nitroarene itself does not react directly with DNA to damage the same, but a metabolite of the nitroarene gives rise to a mutation in DNA to damage the DNA. For example, it can be presumed that nitrofluorene is reduced to an N-hydroxy form in the cell of a microorganism by a nitroreductase and then activated by an o-acetyl transferase, to thus finally produce nitrenium ions which attack the DNA. Therefore, it can be considered that the reaction of the nitroreductase with 2-nitrofluorene is a rate determining step in the mutagenesis of DNA by 2-nitrofluorene.


[0008] As understood from the foregoing, the FMN reductase is essential to utilize the luminescent reaction of bacterial luciferase to the utmost. Therefore, the isolation of the FMN reductase gene permits preparing the enzyme in large quantities, and thus, an important object is the isolation of the gene encoding this enzyme.

[0009] Furthermore, the nitroreductase gene is useful to improve the detection sensitivity of the above-mentioned mutagen or carcinogen.

[0010] However, with regard to the isolation of the FMN reductase gene of luminous bacteria and the nitroreductase gene as well as the expression of them in Escherichia coli, no report has been made so far.

SUMMARY OF THE INVENTION

[0011] In view of the above-mentioned technical situation, an object of the present invention is to provide a gene encoding an enzyme having an FMN reducing activity of luminous bacteria and a nitroreductase activity and the enzyme therefor. Another object of the present invention is to provide a recombinant vector containing this gene and bacteria containing the recombinant vector.

[0012] As a result of intensive research, the present inventors have succeeded in isolating a gene encoding an enzyme having the FMN reducing activity and the nitroreductase activity from the luminous bacteria Vibrio fischeri (ATCC 7744), and in elucidating its primary structure. In addition, they have succeeded in cultivating Escherichia coli transformed with a vector containing the gene which can express the protein in large quantities. As a result, the present
The present invention has now been completed.

[0013] The present invention has the following parts (1) to (8).

(1) A gene containing a nucleotide sequence shown in Fig. 1 and encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

(2) A gene containing a nucleotide sequence shown in Fig. 2 and encoding an enzyme having the flavin reducing activity and the nitroreductase activity described in the previous paragraph (1).

(3) A gene containing a nucleotide sequence shown in Fig. 3 and encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

(4) An enzyme containing an amino acid sequence shown in Fig. 4 and having a flavin reducing activity and a nitroreductase activity.

(5) A recombinant vector containing a DNA whose nucleotide sequence is shown in Fig. 1.

(6) The recombinant vector described in the previous paragraph (5) in which the gene having the nucleotide sequence shown in Fig. 1 is inserted into a plasmid vector.

(7) Bacteria containing a recombinant vector containing a DNA whose nucleotide sequence is shown in Fig. 1.

(8) A method for preparing an enzyme containing an amino acid sequence shown in Fig. 4 which comprises the step of cultivating bacteria transformed with a recombinant vector containing a DNA whose nucleotide sequence is shown in Fig. 1.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Fig. 1 shows a nucleotide sequence of a gene encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

Sequence length: 657
Sequence type: Nucleic acid
Strandedness: 1
Topology: Linear
Molecular type: Genomic DNA

Feature of sequence description:
Feature key defined in Gene Bank Authorin Reference Manual Release 1.1 (hereinafter referred to simply as "feature key"): CDS
Procedure for determining the feature:
Prediction from an amino acid sequence of Fig. 4 based on genetic code table.

[0015] (On the left and right sides of each triplet, a 5' terminal and a 3' terminal are present, respectively. This triplet represents a purine base (Pu) and a pyrimidine base (Py) constituting a nucleotide sequence.

A: adenine,
G: guanine,
C: cytosine,
J: A or G,
K: T or C,
L: A, T, C or G,
M: A, C or T,
T: thymine,
X: when Y is A or G, X is T or C, or when Y is C or T, X is C,
Y: when X is C, Y is A, G, C or T, or when X is T, Y is A or G,
W: when Z is C or T, W is C or A, or when Z is C or T, W is C,
Z: when W is G, Z is A, G, C or T, or when W is A, W is A or G,
QR: when S is A, G, C or T, QR is TC, and

*** represents TAA, TAG or TGA.)

[0016] Under each triplet codon of the nucleotide sequence, the amino acid encoded thereby is represented.

[0017] Fig. 2 shows a typical nucleotide sequence of the gene encoding the enzyme having a flavin reducing activity and a nitroreductase activity.

Sequence length: 657
Fig. 3 shows a nucleotide sequence of a gene encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

Sequence length: 929
Sequence type: Nucleic acid
Strandedness: 1
Topology: Linear
Molecular type: Genomic DNA
Original source:

Organism: Vibrio fischeri
Strain: ATCC 7744

Feature of sequence description:
Feature key: CDS
Site having the feature: 109-762
Procedure for determining the feature:
Experimental procedure.

Fig. 4 shows an amino acid sequence of an enzyme having a flavin reducing activity and a nitroreductase activity.

Sequence length: 218
Sequence type: Amino acid
Molecular type: Protein.

Fig. 5 shows a N-terminal amino acid sequence of a NAD(P)H:FMN reductase and synthetic oligonucleotide probes (FR1 and FR2).

Fig. 6 shows a restriction map of the gene of the present invention and a sequencing strategy. Arrows denote directions for the determination of the nucleotide sequences. The portion indicated by a box corresponds to the gene.

Fig. 7 shows a process of constructing a recombinant vector (an expression vector pFR7) containing a gene encoding an enzyme having an FNM reducing activity and a nitroreductase activity of luminous bacteria according to the present invention.

Fig. 8 shows the confirmation of the expressed protein by SDS-polyacrylamide gel electrophoresis. Lane 1 is a pUC8/D1210 strain, lane 2 is a pFR7/D1210 strain, lane 3 is a pFR5/D1210 strain, and lane 4 is a Boehringer Mannheim NAD(P)H:FMN reductase.

The symbols used in the drawings have the following meanings.

- lacP lactose promoter
- Amp' Ampicillin resistant gene
- pUC8 plasmid vector
- pFR3, pFR5, pFR7 recombinant vector
- pFR7 recombinant vector (expression vector)
AILLED DESCRIPTION OF PREFERRED EMBODIMENTS

...sequence of Fig. 1 can be predicted from an amino acid sequence shown in Fig. 4 as mentioned later.

A preferable sequence contains a nucleotide sequence as shown in Fig. 2.

A typical nucleotide sequence is a DNA having a sequence length of 929 bases as shown in Fig. 3.

The basic nucleotide sequence of the present invention is derived from Genomic DNA isolated from luminous bacteria Vibrio fischeri (ATCC 7744). This sequence is characterized by encoding a protein having a molecular weight of 24562 and comprising 218 amino acids, corresponding to nucleotides numbered 109 to 762.

The gene of the present invention encodes a protein having a flavin reducing activity and a nitroreductase activity, for example, an FMN reducing activity and a nitrofurazone reducing activity.

An enzyme of the present invention is a protein having an amino acid sequence shown in Fig. 4 which can be predicted from the nucleotide sequence in Fig. 3. This protein comprises 218 amino acids and has a molecular weight of 24562 and the two activities of luminous bacteria, i.e., the flavin reducing activity and the nitroreductase activity.

A recombinant vector of the present invention contains a DNA whose nucleotide sequence is shown in Fig. 1. That is, the recombinant vector of the present invention contains a nucleotide sequence which is the same or is functionally equal to the DNA having the nucleotide sequence shown in Fig. 3. A "functionally equal nucleotide sequence" means any DNA fragment which can be used in accordance with a substantially similar method to the present invention so as to obtain the substantially identical results, i.e. the production of an enzyme having the FMN reducing activity and the nitroreductase activity of luminous bacteria in a suitable host.

That is, the "functionally equal nucleotide sequence" means any DNA fragment which can encode a protein having the same amino acid sequence, even if the nucleotide sequence is different, or a DNA fragment which can code a protein having the FMN reducing activity and the nitroreductase activity, even if there is a slight difference in the amino acid sequence attributed to a slight difference in the nucleotide sequence. Typical examples are the nucleotide sequence of Fig. 3 and the nucleotide sequence of Fig. 1 into which a site-specific mutation may be introduced.

The nucleotide sequence in Fig. 1 will be described as follows:

The trinucleotides, termed codons, are presented as DNA trinucleotides, as they exist in the genetic material of a living organism. Expression of these codons in protein synthesis requires intermediate formation of messenger RNA (mRNA). The mRNA codons have the same sequences as the DNA codons, except that uracil is found in place of thymine. Complementary trinucleotide DNA sequences having opposite strand polarity are functionally equivalent to the codons, as is understood in the art. An important and well known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet may be employed. Therefore, a number of different nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they can result in the production of the same amino acid sequence in all organisms, although certain strains may translate some sequences more efficiently than they do others. Occasionally, a methylated variant of a purine or pyrimidine may be found in a given nucleotide sequence. Such meth-
ylations do not affect the coding relationship in any way.

The typical example is a plasmid vector into which the DNA fragment having the nucleotide sequence is introduced. As this kind of vector, there can be used pUC [C. Yanisch-Perron, J. Vieira and J. Messing, Gene, 33, p. 110-115 (1985)] and pIN III [Y. Masui, J. Coleman, M. Inouye, Experimental Manipulation of Gene Expression (ed. M. Inouye), Academic Press, p. 15 (1983)].

Fig. 7 shows a construction process of this recombinant vector (the expression vector).

That is, a vector pFR3 having a reductase gene is cleaved with restriction enzymes HincII and StuI to obtain a fragment including a coding region, and this fragment is then inserted into an SmaI site of a pUC8 plasmid DNA [Hanna Z., Fregeau C., Prefontaine G. and Brousseau R., Gene, p. 30247 (1984)] to construct a recombinant vector pFR5. Furthermore, this vector pFR5 is cleaved with a restriction enzyme EcoRI and then subjected to a Klenow treatment in the presence of dNTP. Afterward, the vector is recirculized using a T4 DNA ligase to construct a recombinant vector pFR7 (an expression vector). For the orientation of the thus constructed product, a restriction enzyme cleavage site is shown in an ampicillin resistant gene (Amp').

Bacteria of the present invention contain a recombinant vector DNA having the nucleotide sequence shown in Fig. 1. The bacteria of the present invention are characterized by producing a protein having the flavin reducing activity and the nitroreductase activity.

A method for preparing the enzyme of the present invention comprises the steps of cultivating bacteria modified with a recombinant vector (an expression vector) containing a DNA whose nucleotide sequence is shown in Fig. 3, and then producing a protein containing an amino acid sequence shown in Fig. 4. Examples of the bacteria include Escherichia coli and Bacillus subtilis, and examples of a culture medium to be used include an LB culture medium and a YT culture medium.

A gene of the present invention is that which has been isolated for the first time encoding an enzyme having an FMN reducing activity and a nitroreductase activity. This gene can be used to produce a highly sensitive strain of bacteria to a mutagen or a carcinogen by the use of a suitable host such as Escherichia coli. Additionally, from this Escherichia coli, a reductase protein can also be prepared in large quantities.

By inserting this expression vector into a suitable host such as Escherichia coli, organisms or bacteria can be produced which express an enzyme having the FMN reducing activity and the nitroreductase activity of luminous bacteria. Furthermore, the reductase can also be prepared in large quantities by extraction from the organisms into which the gene is introduced. The organisms or microorganisms into which the gene is introduced have a high sensitivity to a mutagen or a carcinogen owing to the above-mentioned function, and thus they are useful as an indicator for detecting the mutagen or the carcinogen.

The reductase amplifies a luminous reaction of bacterial luciferase owing to the above-mentioned function. Thus, the reductase can be applied to many measuring methods and it is useful, for example, as a diagnosis drug or an inspection drug.

EXAMPLES

Now, the isolation and identification of a gene which is important to the present invention will be described in reference to examples.

Example 1

[Identification of NAD(P)H:FMN reductase and determination of N-terminal amino acid sequence]

An NAD(P)H:FMN reductase sample (available from Boehringer Mannheim) was introduced into a "Sparose 12" gel filtration column (made by Pharmacya Co., Ltd.) to fractionate the sample. For each fraction, NADH and NADPH:FMN reducing activities were measured by a procedure described in Jablonski E. and DeLuca M., Biochemistry, 16, p. 2932 (1977), and analysis was then made in accordance with a procedure described in Laemmli, U.K., Nature, 277, p. 680 (1970) by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

As a result, it was clarified that the FMN reducing activity is directly proportional to the amount of a protein of 26 kDa (which is denoted by an arrow in Fig. 8).

After an SDS-PAGE analysis of this protein, it was transferred into a nylon membrane, and its amino acid sequence was determined in a usual manner by the use of a protein sequencer [made by Applied Biosystems Inc. (ABI)]. The results are set forth in Fig. 5. From these results, an N-terminal amino acid sequence having sequence numbers of 1 to 24 was confirmed.
Example 2

[Preparation of luminescent bacteria genomic library]

[0048] A photobacterium medium containing luminescent bacteria Vibrio fischeri (ATCC7744) was shaken at 26°C overnight to cultivate the bacteria. The bacteria were collected by means of centrifugal separation at 10000 rpm, and the resultant cell pellets were then dispersed in a Tris-HCl EDTA buffer solution (hereinafter referred to as “a TE buffer”). After a lysozyme treatment at 37°C for 1 hour, sodium dodecyl sulfate (hereinafter abbreviated to “SDS”) was added, followed by a proteinase K treatment at 50°C for 3 hours. Afterward, a phenol treatment was carried out three times, followed by ethanol precipitation. After drying, the dried material was dissolved in the TE buffer, and then subjected to the proteinase K treatment again. Afterward, the three cycles of the phenol treatment and then the ethanol precipitation were carried out to recover the genomic DNA. 10 units of a restriction enzyme Sau3AI were reacted with 50 μm of this genomic DNA at 37°C. Some parts of the reaction mixture were taken out at reaction times of 5, 10, 20, 30, 45, 60, 90 and 120 minutes, and afterward, EDTA (ethylenediaminetetraacetic acid) was added to the reaction system to bring the reaction to an end. Each part of the DNA was subjected to agarose gel electrophoresis to confirm the degree of partial decomposition of the genomic DNA. The reaction solutions at the respective times were combined into one, followed by the ethanol precipitation, to recover the DNA. Next, this DNA was dissolved in a small amount of the TE buffer, and then subjected to agarose gel electrophoresis to recover a fraction of 4 to 6 Kb by the use of a DE81 paper. The DNA fraction of 4 to 6 Kb was dissolved out of the DE81 paper with 1 M NaCl, subjected to the phenol treatment three times, and then precipitated with ethanol. The sample was dissolved in the TE buffer so as to be about 200 ng/μl. Afterward, the DNA fraction of 4 to 6 Kb was reacted with a pUC18 plasmid DNA (a plasmid vector), which was previously cleaved with a restriction enzyme BamH I and then treated with an alkaline phosphatase (an enzyme for catalyzing dephosphorization at the 5' terminal of the DNA), at 16°C overnight in the presence of a T4 DNA ligase (an enzyme for ligating DNA chains to each other or ligating the DNA and the 3'OH of an RNA or the 5'P terminal by a phosphodiester bond), whereby the DNA fraction was ligated to the plasmid. The resultant ligation reaction solution was transferred to JM109 Escherichia coli so as to perform transformation, and the thus obtained transductant represented a gene library.

[Preparation of synthetic oligonucleotide probe]

[0049] On the basis of the information of an amino acid sequence shown in Fig. 5, two probes of an oligonucleotide probe (FR-1) and an oligonucleotide probe (FR-2) were synthesized by means of a DNA synthesizer (made by ABI). Each synthetic probe was purified by the use of an OPC cartridge (made by ABI).

[Cloning of NAD(P)H:FMN reductase gene and analysis of its structure]

[0050] The gene library of Example 2 was screened in accordance with a colony hybridization method by the use of the FR-1 probe and the FR-2 probe. The FR-1 probe and the FR-2 probe were labelled at the 5' terminal with [γ-32P]ATP and then used as labelled probes. After the titer of the gene library was measured, this gene library was scattered on a nitrocellulose filter so as to be 200 colonies per plate. Cultivation was made at 37°C overnight, and two replicas were taken per filter. Each pair of two replicas was cultivated at 37°C and then used for hybridization. The filter was air-dried and then irradiated with ultraviolet rays (UV) to fix the DNA. Afterward, the filter was put in a hybridization solution [20 ml of a 6xSET buffer [20xSET buffer:3 M of NaCl, 0.6 M of Tris-HCl (pH 8.0) and 0.04 M of EDTA], a 10xDehhardt's solution [(a solution containing 0.2% of each of serum albumin, polyvinylpyrrolidone and Ficoll), a 0.1% SDS and a salmon sperm DNA (thermally denatured, 50 μm/ml)], and it was then maintained at 68°C for 1 hour. Furthermore, the solution was replaced with a new one and then maintained for 1 hour, and a 32P-leveling probe was added, followed by hybridization at room temperature overnight. The solution was thrown away, and the filter was then washed with the 6xSET buffer, followed by shaking at 37°C for 20 minutes. After this operation was repeated twice, the filter was air-dried and then subjected to autoradiography. The filter was superposed upon a developed X-ray film, and the position of an ink marker was photographed on the film. Identification was made by aligning signals which were coincident with each other on the two films of the probe FR-1 and the probe FR-2 made from the one plate, and thus, five identified colonies (clones) were obtained.

[Preparation of recombinant vector]

[0051] For these five clones, a restriction analysis was carried out (Fig. 6), and as a result, it was apparent that three of these five clones were the same clones, which meant that three kinds of positive clones were prepared. Above all, a recombinant vector pFR3 (Fig. 7) having the smallest inserted DNA was used for the subsequent analysis.
A Southern blotting analysis was made by the use of the FR-1 probe, and the region of an FMN reductase gene was determined in accordance with a dideoxynucleotide-enzyme method [Hattori M. and Sakaki Y., Anal. Biochem., 152, p. 232 (1986)], whereby a primary structure shown in Fig. 3 was elucidated. As a result, it was understood that the FMN reductase gene encoded a polypeptide of 24562 Da comprising 218 amino acids shown in Fig. 4, and this gene was about 30% homologous with a nitroreductase gene of Salmonella [Watanabe M., Ishidate M. Jr and Nohmi T., Nucleic Acid Res., 18, p. 1059 (1990)].

Example 3

Recombinant vector of NAD(P)H:FMN reductase gene, and construction of expression vector

A recombinant vector pFR3 plasmid DNA was cleaved with a restriction enzyme Hinc II/Stu I and then treated at -80°C for 10 minutes. The thus treated DNA was then subjected to agarose gel electrophoresis to separate and recover a DNA fragment of about 1 Kb by the use of a DE81 paper. The DNA was dissolved out of the DE81 paper with 1 M NaCl, subjected to the phenol treatment three times, and then precipitated with ethanol. Next, the sample was dissolved in the TE buffer so as to be about 200 ng/µl. The above-mentioned DNA was reacted, at 16°C overnight in the presence of a T4DNA ligase, with a pUC8 plasmid DNA (a plasmid vector) which was previously cleaved with a restriction enzyme Sma I and then treated with an alkaline phosphatase, whereby the DNA was ligated to the plasmid. The resultant ligation reaction solution was transferred to JM109 Escherichia coli to perform transformation, and the Escherichia coli was selected and then cultivated overnight in a culture medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) to form a white colony. This white colony was a transductant containing the plasmid into which the heterologous DNA was inserted.

A plasmid DNA was prepared from these transductants, and a restriction analysis was then carried out to obtain a strain containing a transformed vector pFR5. A plasmid DNA of the recombinant vector pFR5 was prepared, cleaved with EcoR I, subjected to a Klenow treatment, ligated with a T4DNA ligase, and then was transferred to D1210 Escherichia coli to perform transformation. Of the transductants, one in which an EcoR I cleavage site disappeared was selected. This was a recombinant vector (an expression vector) pFR7.

The recombinant vector pFR5 was constructed so as to express a peptide derived from a N-terminal β-galactosidase gene (lacZ) and a fused protein of the FMN reductase enzyme. The expression vector pFR7 was constructed so as to express lacZ and frameshift FMN reductase singly.

Example 4

Preparation of Escherichia coli incorporated with NAD(P)H:FMN reductase gene

Expression vectors pFR5 and pFR7 and a pUC8 plasmid DNA were transferred to D1210 Escherichia coli to perform transformation.

Preparation of enzyme

These transductants were incubated overnight, and 0.25 ml of the resultant incubation solution was transferred to an LB liquid (10 ml) culture medium containing ampicillin. After the culture medium was shaken at 37°C for 2 hours to cultivate the transductants, isopropyl-β-D(-)-thiolactopyranoside (hereinafter abbreviated to “IPTG”) was added thereto so that a final concentration might be 1 mM, and the transductants were further cultivated for 3 hours. For the bacteria, an SDS-PAGE analysis was carried out to confirm the expression of a protein (this protein corresponds to the enzyme of the present invention).

The results are set forth in Fig. 4, but in the cases of the recombinant vectors pFR5 and pFR7, new bands appeared at 26 kDa which was the same size as in a commercial crude enzyme sample. In addition, in the case of the recombinant vector pFR5, a band appeared even at 29 kDa, and this vector was considered to be derived from a fused protein with lac Z.

1.5 ml of the incubation solution which was subjected to an IPTG induction treatment was centrifugally separated at 10000 rpm to remove a supernatant. The bacteria were dispersed in 0.5 ml of a 50 mM potassium phosphate-1 mM dithiothreitol buffer, and then sonically disrupted by ultrasound. Centrifugal separation was further carried out at 4°C for 30 minutes at 12000 rpm, and the resultant supernatant was a cell extract.

For this cell extract, the following enzyme reducing activity was measured. The results are set forth in Tables 1, 2 and 3.
(1) Flavin reducing activity: This was measured in accordance with a procedure described in Jablonski E and Deluca M., Biochemistry, 16, p. 2932 (1977).
(2) Iron reducing activity: This was measured in accordance with a procedure described in Fontecave M., Eliasson R. and Reichard P., J. Biol. Chem., 262, p. 12325-12331 (1987).
(3) Nitroreductase activity: This was measured in accordance with a procedure described in Watanabe M., Ishidate M. Jr. and Nohmi T., Mutation Research, 216, p. 211-220 (1989).

[0061] Protein amounts in the respective tables were determined in accordance with a Bradford method by the use of a protein assay kit made by Bio-RAD [Bradford M. M., Anal. Biochem., 72, p. 248-254 (1976)].
<table>
<thead>
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<th>Strain (IPTG)</th>
<th>Flavin Reducing Activity (nmol/min/mg protein)</th>
<th>Riboflavin (+)</th>
<th>(+)</th>
<th>(-)</th>
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<tr>
<td>PFR5/D1210</td>
<td>18330</td>
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Table 1
Table 2

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<tr>
<th>Strain (IPTG)</th>
<th>FMN (+)</th>
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<th>FAD (+)</th>
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<th>Riboflavin (+)</th>
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<td>0.6</td>
<td>0.3</td>
<td>0.1</td>
<td>0.0</td>
<td>0.4</td>
<td>0.0</td>
</tr>
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</table>
Comparing the activities in these tables, the activities of pFR5 and pFR7 are about 1 to 3 orders higher than those of pUC13 which is a negative control. This gene could therefore be identified as a gene encoding an enzyme protein having a flavin reducing activity and a nitroreductase activity.

**Claims**

1. An isolated and purified gene containing the nucleotide sequence of Fig. 1 and encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

2. An isolated and purified gene containing the nucleotide sequence of Fig. 2 and encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

3. An isolated and purified gene containing the nucleotide sequence of Fig. 3 and encoding an enzyme having a flavin reducing activity and a nitroreductase activity.

4. An enzyme containing the amino acid sequence of Fig. 4 and having a flavin reducing activity and a nitroreductase activity.

5. A recombinant vector containing the DNA whose nucleotide sequence is that of Fig. 1.

6. The recombinant vector according to Claim 5 wherein the nucleotide sequence of Fig. 1 is inserted into a plasmid vector.

7. A bacterial host containing a recombinant vector according to claim 5 or 6.

8. A method for preparing an enzyme containing the amino acid sequence of Fig. 4 which comprises a step of culti-vating bacteria modified with a recombinant vector according to claim 5 or 6.

**Patentansprüche**

1. Isoliertes und gereinigtes Gen, das die Nucleotidsequenz von Fig. 1 enthält und ein Enzym mit einer flavinreduzierenden Aktivität und einer Nitroreductase-Aktivität kodiert.

2. Isoliertes und gereinigtes Gen, das die Nucleotidsequenz von Fig. 2 enthält und ein Enzym mit einer flavinreduzierenden Aktivität und einer Nitroreductase-Aktivität kodiert.

3. Isoliertes und gereinigtes Gen, das die Nucleotidsequenz von Fig. 3 enthält und ein Enzym mit einer flavinreduzierenden Aktivität und einer Nitroreduktase-Aktivität kodiert.

4. Enzym, das die Aminosäuresequenz von Fig. 4 enthält und eine flavinreduzierende Aktivität und einer Nitroreduktase-Aktivität hat.

5. Rekombinanter Vektor, der die DNA, deren Nucleotidsequenz diejenige von Fig. 1 ist, enthält.

6. Rekombinanter Vektor nach Anspruch 5, wobei die Nucleotidsequenz von Fig. 1 in einen Plasmidvektor eingefügt

8. Verfahren zum Herstellen eines Enzyms, das die Aminosäuresequenz von Fig. 4 enthält, das eine Stufe des Kultivierens von mit einem rekombinanten Vektor nach Anspruch 5 oder 6 modifizierten Bakterien umfaßt.

**Revendications**

1. Gène isolé et purifié contenant la séquence nucléotidique de la figure 1 et codant un enzyme ayant une activité réduisant les flavines et une activité de nitroréductase.

2. Gène isolé et purifié contenant la séquence des nucléotides de la figure 2 et codant un enzyme ayant une activité réduisant les flavines et une activité de nitroréductase.

3. Gène isolé et purifié contenant la séquence des nucléotides de la figure 3 et codant un enzyme ayant une activité réduisant les flavines et une activité de nitroréductase.

4. Enzyme contenant la séquence d'acides aminés de la figure 4 et ayant une activité réduisant les flavines et une activité de nitroréductase.

5. Vecteur de recombinaison contenant le DNA dont la séquence nucléotidique est la séquence de la figure 1.

6. Vecteur de recombinaison selon la revendication 5 dans lequel la séquence nucléotidique de la figure 1 est insérée dans un vecteur plasmidique.


8. Procédé de préparation d'un enzyme contenant la séquence d'acides aminés de la figure 4, comprenant une étape de culture de bactéries modifiées par un vecteur de recombinaison selon les revendications 5 ou 6.
Fig. 1

ATG ACL CAK CCL ATM ATM CAK GAK XTY GAJ AAK WQZ TAK ACL QRS AAJ 48
Met Thr His Pro Ile Ile His Asp Leu Glu Asn Arg Tyr Thr Ser Lys
1 5 10 15
AAJ TAK GAK CCL QRS AAJ AAJ GTL QRS CAJ GAK XTY GCL GTL XTY 96
Lys Tyr Asp Pro Ser Lys Val Ser Glu Glu Leu Ala Val Leu
20 25 30
XTY GAJ GCL XTY WQZ XTY QRS GCL QRS QRS ATM AAK QRS CAJ CCL TGG 144
Leu Glu Ala Leu Arg Leu Ser Ala Ser Ser Ile Asn Ser Ser Glu Pro Trp
35 40 45
AAJ TTK ATM GTL ATM GAJ QRS GAK GCA GCL AAJ CAJ GGL ATG CAK GAK 192
Lys Phe Ile Val Ile Glu Ser Asp Ala Ala Lys Gln Gly Met His Asp
50 55 60
QRS TTK GCL AAK ATG CAJ CAJ TTK AAK CAJ CCL CAK ATM AAJ GCL TGK 240
Ser Phe Ala Asn Met His Gln Phe Asp Glu His Pro His Ile Lys Ala Cys
65 70 75 80
QRS CAK GTG ATM XTY TTK GCL AAK AAJ XTY QRS TAK ACL WQZ GAK 288
Ser His Val Ile Leu Phe Ala Asn Lys Leu Ser Tyr Thr Arg Asp Asp
85 90 95
TAK GAK GTG GTL XTY QRS AAJ GCL GTL GCL GAK AAJ WQZ ATM ACL GAJ 336
Tyr Asp Val Val Leu Ser Lys Ala Val Ala Asp Lys Arg Ile Thr Glu
100 105 110
GAJ CAJ AAJ GAJ GCL TTK GCL QRS TTK AAJ TTK GTL GAJ TTG AAK 384
Glu Gln Lys Glu Ala Ala Phe Ala Ser Phe Lys Phe Val Glu Leu Asn
115 120 125
TGK GAK GAJ AAK GGL GAJ CAJ AAJ GCL TGG ACL AAJ CCL CAJ GCL TAK 432
Cys Asp Glu Asn Gly Glu His Lys Ala Trp Thr Lys Pro Glu Gin Ala Tyr
130 135 140
XTY GCL XTY GGL AAK GCL XTY CAK ACL XTY GCL WQZ XTY AAJ ATM GAK 480
Leu Ala Leu Gly Asn Ala Leu His Thr Leu Ala Arg Leu Asn Ile Asp
145 150 155 160
QRS ACL ACL ATG GAJ GGL ATM GAK CCL GAJ XTY TTK QRS GAJ ATM TTK 528
Ser Thr Thr Met Glu Ile Asp Pro Glu Leu Leu Ser Glu Ile Phe
160 170 175
GCL GAJ XTY AAJ GGL TAK GAJ TGK CAK GTL GCL XTY GCL ATM GGL 576
Ala Asp Glu Leu Lys Gly Tyr Glu Cys His Val Ala Leu Ala Ile Gly
180 185 190
TAK CAK CCL QRS GAJ GAJ TAK AAK GCL QRS TTG CCL AAJ QRS WQZ 624
Tyr His His Pro Ser Glu Asp Tyr Asn Ala Ser Leu Pro Lys Ser Arg
195 200 205
AAJ GCL TTK GAJ GCL GTL ATM ACL ATM XTY TJJ 657
Lys Ala Phe Glu Ala Val Ile Thr Ile Leu ***
210 215
Fig. 2

ATG ACG CAT CCA ATT ATT CAT GAT CTT GAA AAT CGT TAT ACA TCA AAA
Met Thr His Pro Ile Ile His Asp Leu Glu Asn Arg Tyr Thr Ser Lys
1 5 10 15
AAA TAT GAC CCA TCA AAG AAA GTA TCT CAA GAA GAT TTA CCG GTT TTG
Lys Tyr Asp Pro Ser Lys Val Ser Gin Glu Asp Leu Ala Val Leu
20 25 30
CTT GAG GCT CTG CTG TTA TCT GCT TCT TCA ATT TAC CAG CCT TGG
Leu Glu Ala Leu Arg Leu Ser Ala Ser Ile Asn Ser Gin Pro Trp
35 40 45
AAA TTC ATT GTT ATT GAA TCC GAT GCA GGC AAG CAA GGT ATG CAT GAT
Lys Phe Ile Val Ile Glu Ser Asp Ala Ala Lys Gin Gly Met His Asp
50 55 60
TCG TTT GCA AAT ATG CAT CAG TTT AAT CAA CCT CAC ATC AAA GCG TGT
Ser Phe Ala Asn Met His Gin Phe Asn Gin Pro His Ile Lys Ala Cys
65 70 75 80
TCT CAT GTG ATT TTA TCT GCA AAT AAC GTT TCG TAT ACA CGA GAT GAT
Ser His Val Ile Leu Phe Ala Asn Lys Leu Ser Tyr Thr Arg Asp Asp
85 90 95
TAT GAT GTG GTC GTC AAA GCG GTT GCT GAC AAG CGT ATT ACT GAA
Tyr Asp Val Val Leu Ser Lys Ala Val Ala Asp Lys Arg Ile Thr Glu
100 105 110
GAG CAA AAA GAA GCT GCT TTT GCT TCG TTT AAG TTT GTA GAA TTG AAC
Glu Gin Lys Glu Ala Ala Phe Ala Ser Phe Lys Phe Val Glu Leu Asn
115 120 125
TGT GAT GAA AAT GGT GAG CAT AAA GCA TGG ACT AAG CCT CAA GCT TAT
Cys Asp Glu Asn Gly Glu His Lys Ala Trp Thr Thr Lys Pro Gin Ala Tyr
130 135 140
TTA GCT CTT GGT AAT GCT CTG CAT ACA TTA GCT AGA CTG AAC ATT GAC
Leu Ala Leu Gly Asn Ala Leu His Thr Leu Ala Arg Leu Asn Ile Asp
145 150 155 160
TCA ACA ATA ATG GAA GCC ATT GAT CCT GAA TTA TTG AGT GAA ATT TTT
Ser Thr Thr Met Glu Gin Ile Asp Pro Glu Leu Leu Ser Glu Ile Phe
160 165 170 175
GCT GAT GAA TTA AAA GGG TAT GAA TGT CAT GGT GCT TTA GCC ATT GGT
Ala Asp Glu Leu Lys Gly Tyr Glu Cys His Val Ala Leu Ala Ile Gly
180 185 190
TAT CAT CAT CCA AGC GAA GAT TAT AAT GCC TCT TGT CCT AAG TCT CTT
Tyr His His Pro Ser Glu Tyr Asp Ala Ser Leu Pro Lys Ser Arg
195 200 205
AAG GCA TTT GAA GCA GTA ATT ACC ATC CTT TAG
Lys Ala Phe Glu Ala Val Ile Thr Ile Leu ***
210 215

16
Fig. 3

TGTACATAT GGCAATTAA ATATTGAGTA TGCCCTTGCTTT GTCGACATCA TAAGTTGTGC 60
AGACAAGAT GTCTGTTGGAT TAAATTTCA CAAAGTACGT TTAATTTA ATG ACG CAT 117
Wet Thr His

1

CCA ATT ATT CAT GAT CTT GAA AAT CUG TAT ACA TCA AAA AAA TAT GAC 165
Pro Ile Ile Asp Leu Glu Asn Arg Thr Ser Lys Tyr Thr Asp
5 10 15

CCA TCA AAG AAA GGA GAA GAT TTA GCC GTC TTT GAC GCT 213
Pro Ser Lys Val Ser Gin Glu Asp Leu Ala Val Leu Leu Glu Ala
20 25 30 35

GTC CTG TTA CTC TCT GCT CAA ATT AAT TCA CAG CTT TGG AAA TAC ATT 261
Leu Arg Leu Ser Ala Ser Ser Ile Asn Ser Gin Pro Trp Lys Phe Ile
40 45 50

GTT ATT GAA GCC GCA GCG AAG CAA GAT GAT TCG TTT GCA 309
Val Ile Glu Ser Ala Ala Lys Gin Gly Wet His Asp Ser Phe Ala
55 60 65

AAT ATG CAT CAG TTT AAT CAA CCT CAC ATC AAA GCG TGT TCT CAT GTG 357
Asn Met His Glu Phe Asn Gin Pro His Ile Lys Ala Cys Ser His Val
70 75 80

ATT TTA TTT GCA AAT AAG CTT TCG TAT ACA CAA GAT GAT TAT GCG 405
Ile Leu Phe Ala Asn Lys Leu Ser Tyr Thr Arg Asp Tyr Asp Val
85 90 95

GTT TTA TCT AAA GCG GTC CTG GAC AAG CAG ATT ACT GAA GAG CAA AAA 453
Val Leu Ser Lys Ala Val Ala Asp Lys Arg Ile Thr Glu Glu Gin Lys
100 105 110 115

GAA GCT GCT TTT GTA GAA TTA TGG AAC TGT GAA 501
Glu Ala Ala Phe Ala Ser Phe Lys Phe Val Glu Leu Asn Cys Asp Glu
120 125 130

AAT GTG GAG CAT AAA GCA TGG ACT AAG CTA CAC GCT TAT TTA GCT CTT 549
Asn Gly Glu His Lys Ala Thr Thr Lys Pro Gin Tyr Thr Leu Ala Leu
135 140 145

GCT AAT GCT CTG CAT ACA TTA GCT AGA CTC AAC ATT GAC TCA ACA ACA 597
Gly Asn Ala Leu His Thr Leu Ala Arg Leu Asn Ile Ser Thr Thr
150 155 160

ATG GAA GGC ATT GAT CCT GAA TTA TTG AGT GAA ATT TTT GCT GAT GAA 645
Met Glu Gly Ile Pro Glu Leu Ser Glu Ser Gin Phe Ala Asp Glu
160 170 175

TTA AAA GGG TAT GAA TTG CAT TTG GCT TTA GCC ATT GGG TAT CAT CAT 693
Leu Lys Tyr Glu Cys His Val Ala Leu Ala Ile Gly Tyr His His
180 185 190 195

CCA AGC GAA GAT TAT GCC TCT TGG CCT AAG TCT CTT TAA GCC TTT 741
Pro Ser Glu Asp Tyr Asn Ala Ser Leu Pro Lys Ser Arg Lys Ala Phe
200 205 210

GAA GCA GTA ATT ACC ATC CTT 762
GlUa Val Ile Thr Ile Leu
215

TAGATTTA ATGTGGAGA TGAAGAAGAAG CACGGCAATTT AGCTGTTGGT TGTGGTGCA 822
***

AAAATGGTCC TTAAGGCGGA TTACTACGGT AGGAAGTCTA TTTAAAGTTTT CTTTTACTCT 882
TTGGTATGAC TGTCGATA ATTATCAATC GCMCGGAAATC ATTTACAC TAGGGCT 929
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<td>Lys Ala Phe Glu Ala Val Ile Thr Ile Leu</td>
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<td>210   215</td>
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**Fig. 5**

Met-Thr-His-Pro-Ile-Ile-His-Asp-Leu-Glu-Asn-Arg-Tyr

FR1: 5'ATG-ACT-CAT-CCT-AT 3'

A     A
C     C
G     G

15
-Thr-Ser-Lys-Lys-Tyr-Asp-Pro-Ser-Lys-Lys-Val- ...

FR2: 5'AAA-AAA-TAT-GAT-CC 3'

G     G
G     C
C     C

**Fig. 6**

(EcoR I) (Hind III)

EcoRI Hinc II Hind II Hind III Stu I Nsp V Hind III Hind III EcoRI

1kb

19
Fig. 7

pFR3

Sau3A I

Sau3A I

Hinc II

Stu I

Hinc II Cleavage

Stu I

(Separate DNA fragment)

Sma I

Sma I Cleavage

T4DNA ligase

EcoR I

Sma I/Hinc II

Sma I/Stu I

EcoR I Cleavage

Klenow treatment

Sma I/Hinc I

Sma I/Stu I