Isolation of a cDNA encoding a plant-derived epoxide hydrolase and an expression system of the enzyme in *Escherichia coli* are established, so as to permit the mass-scale production of the plant-derived epoxide hydrolase. The invention provides a cDNA encoding the plant-derived epoxide hydrolase having the amino acid sequence of SQ ID No. 1 in the Sequence Listing, a gene encoding the plant-derived epoxide hydrolase having the amino acid sequence of SQ ID No. 2 in the Sequence Listing, a plasmid vector carrying the cDNA and the transformant (FERM BP-6624) retaining the plasmid vector.
CDNA ENCODING PLANT-DERIVED EPOXIDE HYDROLASE, GENE ENCODING SAME AND TRANSFORMANT

FIELD OF THE INVENTION

[0001] The present invention relates to a cDNA encoding a plant-derived hydrolase, a gene encoding the same, a plasmid vector carrying said cDNA and a transformant.

BACKGROUND OF THE INVENTION

[0002] Epoxide hydrolase is an enzyme involved in the biological protective system functioning for preventing chemical reactions hazardous for living organisms in such a manner that the enzyme hydrolyses hazardous epoxide compounds generated through peroxidation in living organisms, to eliminate the high chemical reactivity of the epoxide compounds.

[0003] It is evidenced that epoxide hydrolase is present in animal cells and plant cells. Particularly, epoxide hydrolase derived from plants is expected to be applicable to agricultural fields and food industries. Because the content thereof is very low, the mass-scale production thereof has been believed to be extremely difficult.

[0004] As to genetic information concerning epoxide hydrolase, currently, reports have been issued about the genetic information thereof from animal cells, such as human liver cell [Beetham, J. K. et al., Arch. Biochem. Biophys., 305, 197-201 (1993)] and liver cells from rats and mice [Knehr, M. et al., J. Biol. Chem., 268, 17623-17627 (1993); Grant, D. F. et al., J. Biol. Chem., 268, 17628-17633 (1993)].

[0005] Meanwhile, the genetic information thereof from plant cells including potato [Stapleton, A. et al., Plant J. 6, 251-258 (1994)] and Arabidopsis [Kiyosue, T. et al., Plant J., 6, 259-269 (1994)] has been reported as well.

[0006] However, no report regarding genetic information about epoxide hydrolase derived from plants has been published yet, except the aforementioned reports. No plant-derived epoxide hydrolase with high activity has been reported yet.

SUMMARY OF THE INVENTION

[0007] The inventors have made attempts to purify a plant-derived epoxide hydrolase and to clone the cDNA and gene of the epoxide hydrolase based on the amino acid sequence thereof. Consequently, the inventors have successfully identified the whole structure of the cDNA of the enzyme and that of the gene thereof and have also achieved successfully the construction of an expression system in Escherichia coli by utilizing the cDNA and the gene. Thus, the invention has been achieved.

[0008] Based on the analysis of the amino acid sequence of the purified epoxide hydrolase, synthetic degenerate oligonucleotides were prepared. By subsequently extracting mRNA from a fully ripened soy seed and synthesizing a double-stranded cDNA using the mRNA, which was then integrated via an adapter into a phage vector, a cDNA library was constructed.

[0009] By labeling the prepared synthetic oligonucleotides and using the labeled synthetic oligonucleotides as probes, cDNA encoding the epoxide hydrolase of its full length was cloned by screening from the aforementioned cDNA library. The analysis of the primary structure of the cDNA indicated the whole amino acid sequence of the epoxide hydrolase including the signal peptide.

[0010] The epoxide hydrolase gene was cloned by using a commercially available gene library prepared by partially digesting nuclear DNA extracted and purified from sprout-developed soy with a restriction endonuclease Mbo I and integrating the digestion product in a phage vector.

[0011] By labeling the preliminarily recovered cDNA of the epoxide hydrolase and using the labeled cDNA as probe for screening from the aforementioned gene library, the epoxide hydrolase gene including the 5' upstream region was cloned.

[0012] Using the cloned epoxide hydrolase cDNA to construct an Escherichia coli expression system by utilizing a promoter T5, the present enzyme was successfully expressed while a simple and rapid purification method thereof was also established.

[0013] More specifically, a first aspect of the invention relates to a cDNA encoding the plant-derived epoxide hydrolase having the amino acid sequence of SQ ID No. 1 in the Sequence Listing.

[0014] A second aspect of the invention relates to a gene encoding the plant-derived epoxide hydrolase having the amino acid sequence of SQ ID No. 2 in the Sequence Listing.

[0015] A third aspect of the invention relates to a plasmid vector carrying the cDNA encoding the plant-derived epoxide hydrolase in the first aspect of the invention.

[0016] A fourth aspect of the invention relates to a transformant (FERM BP-6624) retaining the plasmid vector in the third aspect of the invention.

DETAILED DESCRIPTION OF THE INVENTION

[0017] The invention will now be described in more detail hereinafter.

[0018] As described in the column of “Description of the Related Art”, the epoxide hydrolase of the invention is an enzyme with an action hydrolyzing epoxide hazardous to living organisms to generate diol and the like.

[0019] The first aspect of the invention is now described.

[0020] The first aspect of the invention relates to the cDNA encoding the plant-derived epoxide hydrolase having the amino acid sequence of SQ ID No. 1 in the Sequence Listing.

[0021] The inventors have succeeded to obtain the cDNA of the first aspect of the invention in the following manner.

1. Preparation of cDNA Library

[0022] 1.1 Extraction and Purification of Epoxide Hydrolase in Plants

[0023] Epoxide hydrolase is present in cells of various plants and animals. In the first aspect of the invention, epoxide hydrolase derived from the cells of a plant is specifically used.
The cells of a plant are preferably cells of any seed with abundance of epoxide hydrolase, but are not limited thereto.

For extracting epoxide hydrolase from soy seed, use is preferably made of soy between early growing stage and ripe stage, preferably soy between early growing stage and later growing stage.

So as to obtain epoxide hydrolase, a raw material plant should necessarily be purified at a high level. The raw material plant includes for example soy, Arabidopsis, and potato.

The method for purifying the enzyme is illustrated in a case using soy as the raw material. Grinding the plant seed between early growing stage and ripe stage, and adding an appropriate buffer solution to the ground seed to extract the soluble fraction, the fraction is subjected to dialysis to separate the liquid fraction from the solid fraction. The resulting crude enzyme solution is applied to hydrophobic chromatography or gel filtration, whereby almost pure epoxide hydrolase can be recovered.

Analysis of Inner Amino Acid Sequence of Purified Epoxide Hydrolase

The purified epoxide hydrolase obtained in 1.1 cannot be analyzed as such of its inner amino acid sequence because the N terminus thereof is blocked. Therefore, the following procedures should be carried out.

Enzymatically degrading the epoxide hydrolase into short peptides and fractionating samples of the individual peptides by high performance liquid chromatography, the amino acid sequence of each of the samples is determined. The amino acid sequence is conveniently determined by Edman degradation using an automatic amino acid sequencer.

The individual samples are examined of their amino acid sequences; oligonucleotides are synthesized by using selected regions with less degeneracy among them; and the resulting oligonucleotides are used as the following probes.

Preparation of Poly(A)* RNA

RNA is then extracted from the plant seed, to prepare poly(A)* RNA from the extracted total RNA.

The poly(A)* RNA can be prepared from the plant by using the SDS-phenol method according to the method by Fukazawa, C. et al., Journal of Biological Chemistry, 200, 6234-6239 (1985).

Construction of cDNA Library

Synthetically preparing a double-stranded cDNA by using the resulting mRNA and integrating the cDNA via an adapter in a phage vector, a cDNA library is constructed.


The Gubler-Hoffman method is now described below.

First, a double-stranded cDNA is synthetically prepared by using the poly(A)* RNA. To the cDNA is ligated an adapter carrying digestion sites with restriction endonucleases such as Eco RI, Not I and Bam HI. By polyacrylamide gel electrophoresis, cDNAs of 500 bp or more are scissored out and collected from the gel, while excessive such adapter is removed.

Subsequently, a phosphate group is inserted at the 5' termini of the resulting nucleotide sequences, which are then restriction digested and ligated to the dephosphorylated λgt10 phage vector arm (manufactured by TaKaRa Brewery, Co.). The resulting nucleotide sequences are packaged in a λ phage. In such manner, a cDNA library is prepared.

2. Cloning of Plant-Derived Epoxide Hydrolase cDNA of Full Length from cDNA Library and Determination of Nucleotide Sequence Thereof

Labeling the oligonucleotides obtained above in 1.2 and using the resulting oligonucleotides as probes, the cloning of the plant-derived epoxide hydrolase cDNA of full length is accomplished by screening from the cDNA library described in 1.4.

The screening from the cDNA library is successfully executed by separating plaques positive at hybridization with plaques of about 1,000,000 in number, using, as probes, the radio-labeled synthetic oligonucleotides [Fukazawa, C. et al., Journal of Biological Chemistry, 200, 6234-6239 (1985)].

Purifying the resulting positive plaques and allowing Escherichia coli to be infected with the plaques to proliferate the phage, the phage particle is purified to obtain phage DNA. The phage DNA can be purified by ultrafiltration method on a CsCl step-wise density gradient.

2.2 Structure Determination of Recovered Phage DNA

From the purified phage DNA is cleaved the insert with a restriction enzyme, which is then purified. The insert is sub-cloned in a plasmid vector, for DNA sequencing. Consequently, the cDNA encoding the amino acid sequence of SQ ID No. 1 in the Sequence Listing was obtained. The analysis of the cDNA suggested that the cDNA of the positive plaque cloned encoded the full-length epoxide hydrolase. More specifically, the cDNA is according to the first aspect of the invention.

The cDNA composed of 1,332 bp in its full length encodes 341 amino acids from the starting methionine.

The second aspect of the invention is now described.

The second aspect of the invention relates to the gene encoding the plant-derived epoxide hydrolase having the amino acid sequence of SQ ID No. 2 in the Sequence Listing.

The gene of the second aspect of the invention can be obtained on the basis of the cDNA of the first aspect of the invention. Continuously subsequent to the identification
of the cDNA sequence in the first aspect of the invention, the inventors have selected the following steps.

3. Cloning of Plant-Derived Epoxyde Hydrolase Gene of Full Length and Determination of Nucleotide Sequence Thereof

[0051] Through screening from a commercially available soy gene library (Stratagene Ltd.) using the labeled cDNA of the first aspect of the invention as probe, the cloning of the plant-derived epoxyde hydrolase gene of its full length is accomplished. The cloning is successfully achieved through the aforementioned plaque hybridization, to subsequently select positive phages.

[0052] The soy gene library purchased from the commercial source has been prepared by extracting and purifying nuclear DNA from sprout-developed soy and partially digesting the nuclear DNA with a restriction endonuclease Mbo I, and thereafter integrating the digestion product in a phage vector.

[0053] Purifying the resulting positive phages and allowing the phages to be subcloned in a plasmid vector, the DNA was sequenced by the same method as described above in 2.2. Consequently, the nucleotide sequence of the plant-derived epoxyde hydrolase gene and the amino acid sequence encoded thereby can be recovered in accordance with the second aspect of the invention (see SQ ID No. 2 in the Sequence Listing).

[0054] The sequence is composed of 1,933 bp in its full length (see SQ ID No. 2 in the Sequence Listing), wherein two introns divide the sequence into three exons.

[0055] The individual introns, namely first intron and second intron, are composed of 168 bp and 148 bp, respectively, in nucleotide number. (See SQ ID No. 2 in the Sequence Listing.)

[0056] The third aspect and fourth aspect of the invention will now be described collectively.

[0057] The third aspect of the invention relates to a plasmid vector carrying the cDNA encoding the plant-derived epoxyde hydrolase in the first aspect of the invention.

[0058] The fourth aspect of the invention relates to a transformant retaining the plasmid vector in the third aspect of the invention.

[0059] In other words, the third and fourth aspects of the invention provide an expression system of the plant-derived epoxyde hydrolase.

[0060] The plasmid vector and transformant described in the third and fourth aspects, respectively, of the invention are prepared by the following procedures.

4. Preparation of Plasmid Vector and Transformant

[0061] 4.1 PCR Amplification of cDNA Nucleotide Sequence in the First Aspect of the Invention

[0062] Based on the cDNA nucleotide sequence in the first aspect of the invention (see SQ ID No. 1 in the Sequence Listing) and the ion-spray mass spectrometry of the purified epoxyde hydrolase, a 32-mer oligonucleotide primer containing a restriction Nde I site at the 5' terminus (N terminus) and a 34-mer oligonucleotide primer containing a restriction Eco RI site at the 5' terminus (C terminus), are synthesized (see SQ ID Nos. 4 and 5 in the Sequence Listing). Restriction enzymes suitable for the expression vectors described below can satisfactorily be used, so the enzymes are not limited to the restriction enzymes described above.

[0063] PCR was conducted by using these primers and using the plant-derived epoxyde hydrolase cDNA of the first aspect of the invention as template. PCR can be performed, for example, under the conditions described in Example 3, with no specific limitation.

[0064] 4.2 Preparation of Plasmid Vector and Transformant

[0065] The band recovered in 4.1 was sub-cloned in a TA vector [pCR2.1] (manufactured by Invitrogen Co.) and was then subjected to DNA sequencing by the same method as described in 2.2, whereby it was confirmed that no mutation occurred in the expression cDNA amplified by PCR.

[0066] Additionally, the insert was cleaved out of the cloned TA vector by using restriction endonucleases Nde I and Eco RI contained in the aforementioned individual PCR primers, which was then purified; the resulting purified insert was sub-cloned in between the Nde I site and Eco RI site of the pRSET vector (manufactured by Invitrogen Co.) as expression plasmid. The resulting plasmid is a plasmid of the third aspect of the invention.

[0067] The plasmid of the third aspect of the invention is used for transformation of expression Escherichia coli, for example Escherichia coli BL21 (DE3) (manufactured by Novagen), to recover a transformant of the fourth aspect of the invention.


[0069] The transformed Escherichia coli is deposited as Accession No. FERM BP-6624 at National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukubashi, Ibaraki-ken, Japan).

[0070] By culturing the transformant Escherichia coli of the fourth aspect of the invention and identifying the amino acid sequence carried in the Escherichia coli or assaying the specific activity of the enzyme, the expression of the gene of the second aspect of the invention can be confirmed.

[0071] Not any plant-derived epoxyde hydrolase with a high activity has been demonstrated insofar. The system for expressing such highly active plant-derived epoxyde hydrolase at a mass scale is very useful for assessing the function of the enzyme gene in a simple manner.

[0072] According to the first and second aspects of the invention, the cDNA encoding the plant-derived epoxyde hydrolase and the gene encoding the plant-derived epoxyde hydrolase are provided. By using the cDNA and the gene, an epoxyde hydrolase gene with significant functions for protecting biological organisms can readily be developed and may be applicable to plants for foods.
According to the third and fourth aspect of the invention, the plasmid vector carrying the cDNA encoding the plant-derived epoxide hydrolase and the transformant retaining the plasmid vector are provided. The transformant can be utilized effectively as an expression system in microorganisms for assessing the functions of the gene encoding the epoxide hydrolase simply and easily.

EXAMPLES

Example 1
Preparation of cDNA of Plant-Derived Epoxide Hydrolase and Determination of Nucleotide Sequence Thereof

1. Preparation of cDNA Library

From a soy seed on day 18 after blooming was extracted total RNA by the SDS-phenol method according to the method by Fukazawa, C. et al., Journal of Biological Chemistry, 200, 6234-6239 (1985), to prepare poly(A)* RNA.

A double-stranded DNA was synthetically prepared by using 2.5 μg of the poly(A)* RNA and a cDNA synthesis kit based on the principle of the Gubler-Hoffmann method (manufactured by Amersham-Pharmera Co., Ltd.) while adding [α-32P]-dCTP for monitoring the synthesis thereof.

1 nmol of the Eco RI-Not I-Bam HI adapter (manufactured by TaKaRa, Co. Ltd.) was ligated to the resulting cDNA by using a ligation pack manufactured by Nippon Gene Co. Said cDNAs of 500 bp or longer were cleaved and collected out of the gel by polyacrylamide gel electrophoresis according to the method by Fukazawa, C. et al., Journal of Biological Chemistry, 200, 6234-6239 (1985), while an excess of the adapter was removed.

Because no phosphate group was present at the 5′ terminus of the adapter, a phosphate group was introduced therein by using a T4 nucleotide kinase (manufactured by Nippon Gene Co.).

Subsequently, the resulting cDNAs were digested with Eco RI and ligated to the dephosphorylated Agt10 phage vector arm (manufactured by TaKaRa, Co. Ltd.) in the same manner as described above by using the ligation pack (manufactured by Nippon Gene Co.), for packaging into λ phage by using an in vitro packaging kit (manufactured by Amersham-Pharmera Co.). In such manner, a cDNA library of ripened soy seed on day 18 after blooming was prepared.

2. Amino Acid Sequencing of Plant-Derived Epoxide Hydrolase and Probe Preparation

A soy seed on day 18 after blooming was ground, to which was added an acetate buffer. Then, a soluble fraction containing epoxide hydrolase was extracted and dialyzed; the resulting fraction was subjected to hydrophobic chromatography (column: Butyl-Toyo-pearl of 5 cm×30 cm; manufactured by Tosoh, Co. Ltd.) and subsequent gel filtration on a column Sephacryl S-200 of a size of 2.6 cm×180 cm; manufactured by Amersham-Pharmera Co.), to recover nearly purified epoxide hydrolase.

The plant-derived epoxide hydrolase had the blocked N terminus. So as to determine the inner amino acid sequence, therefore, the epoxide hydrolase was degraded with V8 protease (manufactured by TaKaRa, Co. Ltd.) and lysyl endopeptidase (manufactured by Wako Pure Chemicals, Co.) according to the method by Arahira M. and Fukazawa C., Plant Molecular Biology, 25, 597-605 (1994).

After degradation, individual samples were fractionated by an HPLC system (LC-6AD manufactured by Shimadzu, Co. Ltd.) in connection to a reverse-phase column (Silica ODS 120T of 4.6 mm×50 mm manufactured by Tosoh, Co. Ltd.) with an eluent on a linear 0.1% TFA-0.1% TFA/60% acetonitrile density gradient.

The amino acid sequences of the resulting peptides were assayed by a gas-phase amino acid sequencer (Type 477A manufactured by Perkin-Elmer Japan), which demonstrates the inner amino acid sequence of the epoxide hydrolase.

Among the amino acid sequences, a sequence suitable as probe was examined. A 23-mer degenerate oligonucleotide was synthesized (see SQ ID No. 3 in the Sequence Listing).

3. Cloning of Plant-Derived Epoxide Hydrolase cDNA

The oligonucleotide recovered above in 2 was labeled with [γ-32P]-ATP according to the method by Fukawaza, C. et al., Journal of Biological Chemistry, 200, 6234-6239 (1985).

Using the oligonucleotide as probe and the library of a ripened soy seed on day 18 after blooming as prepared above in 1, plaque hybridization was carried out according to the method by Arahira M. and Fukazawa C., Plant Molecular Biology, 25, 597-605 (1994). Consequently, a single positive clone was isolated from 1,000,000 plaques.

Escherichia coli was infected with the positive plaque purified, to proliferate the phage; subsequently, the phage particle was purified by ultra-centrifugation on a CsCl step-wise density gradient, to recover phage DNA.

From the purified phage DNA was cleaved the insert with Bam HI; the insert was purified from the agarose gel and subcloned in the Bam HI site of a plasmid vector pUC19. From the cloned Escherichia coli was prepared the plasmid DNA by a routine method [Maniatis, T. et al., “Molecular Cloning”, Cold Spring Harbor Labo. (1982)].

The prepared plasmid was subjected to fluorescence auto-sequencing using a DNA sequencer DSO1000 manufactured by Shimadzu. The results of the analysis indicate that the cDNA of the positive cloned plaque contained the full-length epoxide hydrolase and was of the nucleotide sequence and amino acid sequence of SQ ID No. 1 in the Sequence Listing.

The full-length cDNA was composed of 1,332 bp, where 22 bp composed poly A. The number of the total amino acids encoded by the DNA was 341 from the starting methionine; based on the molecular weight (36171 Da) analysis by ion-spray mass spectrometry, it was estimated that the amino acid sequence was composed of 315 amino acid residues.
Example 2

Cloning of Plant-Derived Epoxide Hydrolase Gene

1. Cloning of Epoxide Hydrolase Gene

Using [α-32P]dCTP, the cDNA recovered in Example 1 was labeled according to the method by Arabira M. and Fukazawa C., Plant Molecular Biology, 25, 597-605 (1994), which was used as probe.

By the same method as described in Example 1, Section 3, the epoxide hydrolase gene was screened from the commercially available soy gene library (manufactured by Stratagene Ltd.) with the use of said probe to recover a single positive plaque from about 200,000 plaques, to purify phage DNA.

The library purchased from the commercial manufacturer had been prepared by extracting and purifying nuclear DNA from a sprouted soy plant, which had been digested partially with a restriction endonuclease Mbo I to subsequently be integrated into a phage vector.

The positive phage DNA contained an insert of about 12 kbp. The insert was cleaved out with a restriction endonuclease Sal I and was then purified on agarose gel; the resulting insert was subcloned in a plasmid vector pUC19. Subsequently, DNA sequencing was carried out by the method described in Example 1, Section 3. The results of the sequencing indicate that the cloned gene was of the nucleotide sequence and amino acid sequence in SQ ID No. 2 in the Sequence Listing.

The full-length sequence was composed of 1,933 bp, which was divided by two introns into three exons. The numbers of nucleotides in the first and second introns were 168 bp and 148 bp, respectively (see SQ ID No. 2 in the Sequence Listing).

The nucleotide sequence of the gene of the resulting soy epoxide hydrolase was analyzed with reference to database. The results indicate that the nucleotide sequence was different from any nucleotide sequence of cDNAs elucidated for Arabidopsis and potato. Thus, the nucleotide sequence was a novel genetic sequence in plants.

Example 3

Expression of Plant-Derived Epoxide Hydrolase in Escherichia coli

1. PCR Amplification of Epoxide Hydrolase cDNA

Preparing first individual primers containing restriction sites required for constructing an expression plasmid in Escherichia coli at the 5' terminus (see SQ ID Nos. 4 and 5 in the Sequence Listing) respectively for the N-terminus side of a protein speculated on the basis of the molecular weight of the epoxide hydrolase as determined by ion-spray mass spectrometry and for the side of the stop codon of the epoxide hydrolase cDNA (C-terminus side of the epoxide hydrolase protein). PCR was carried out, using as template the plant-derived epoxide hydrolase cDNA cloned in Example 1.

PCR conditions are as follows.

First, 2.5 U of Taq polymerase TaKaRa ExTaq (manufactured by TaKaRa Brewery, Co.) was used per one reaction. Furthermore, the primers were used at a final concentration of 0.4 μM; dNTP mix was at 0.2 mM; and the template epoxide hydrolase cDNA fragment was used at about 10 ng.

TaKaRa PCR thermal cycler 480 (manufactured by TaKaRa Brewery, Co.) was used as a gene amplification system for carrying out 35 cycles of amplification using a step program as follows;

95℃ C. for 30 seconds for denaturation step, 56℃ C. for 30 seconds for annealing step and 72℃ C. for 1.5 minutes for extension step.

The resulting band was subcloned in a TA vector [PCR2.1] (manufactured by Invitrogen Co.). Subsequently, DNA sequencing was carried out by the method described in Example 1, Section 3. The results of the analysis indicate no observed mutation due to PCR.

2. Preparation of Plasmid Vector and Transformant

Clearing the insert out of the cloned TA vector with the restriction endonucleases Nde I and Eco RI contained in the individual primers, then, the resulting DNA fragment was purified on agarose gel and subcloned between the Nde I site and the Eco RI site of an expression plasmid pRSET vector (manufactured by Invitrogen Co.).

So as to examine whether or not the DNA sequence at the part connecting the insert epoxide hydrolase cDNA fragment and the PSET vector was accurate, the subcloned DNA fragment was used for transformation of an Escherichia coli strain JM109 with no protein expression ability although the strain carried an expression plasmid. This is because if an expressed protein might be toxic to Escherichia coli, said Escherichia coli could be damaged by a slight expression of the expression plasmid resulting in hindering the DNA extraction, etc.

By the method described in Example 1, [3], DNA sequencing was carried out. The results of the analysis indicate that the connection part was accurately sequenced.

An expression Escherichia coli strain BL21 (DE3) was transformed with the expression plasmid. The Escherichia coli strain thus transformed is deposited at National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan), under Accession No. FERM BP-6624.

Example 4

Expression of Epoxide Hydrolase from Transformant

Then, the expression Escherichia coli strain BL21 (DE3) transformed with the expression plasmid was allowed to express the epoxide hydrolase. A single colony was first inoculated on a culture medium of 2 ml LB/ampicillin (at a concentration of 50 μg/ml), which was then cultured under agitation at 37℃ C. and 200 rpm overnight, which was used as pre-culture.

One liter of the culture medium was placed in a 5-liter Erlenmeyer flask, into which 1 ml of the pre-cultured bacteria solution was inoculated and cultured at 37℃ C. and 200 rpm, until the OD at 600 nm reached about 0.6. Just
when the OD at 600 nm reached about 0.6, isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM, for culturing for additional 3 hours under the aforementioned conditions.

[0114] After termination of the culturing, the culture was centrifuged to harvest the bacteria; the bacteria were washed with 200 mM acetate buffer, pH 5.0 containing 100 mM NaCl and 1 mM EDTA and was then suspended in the same buffer; then, the bacteria in the resulting suspension were disrupted by ultrasonic treatment, to recover the supernatant.

[0115] The resulting supernatant was therally treated at 55°C for 10 minutes, to denature most of the protein derived from Escherichia coli, which was then separated by centrifugation.

[0116] Most of the resulting supernatant protein was epoxide hydrolase expressed, which was further purified.

[0117] The supernatant was subjected to and fractionated by gel filtration on a Sephacryl S-200 column (2.6×90 cm) (manufactured by Amersham-Pharmacia Co.). The resulting fraction was at a single band on SDS-PAGE. The N-terminal sequence of the protein was determined by a gas-phase amino acid sequencer (Type 477A; manufactured by Perkin-Elmer) in the same manner as in Example 1, Section 2. It was confirmed that the protein was epoxide hydrolase.

[0118] The resulting soy-derived epoxide hydrolase was of a molecular weight of 35 kDa (as a single band) on SDS-PAGE and with a specific activity of 1.36 U/mg (1 U=1 μmol/min) to styrene oxide.

[0119] As has been described above, it is shown that the purified enzyme had a high enzyme activity and exerts an excellent ability to hydrolyze hazardous epoxide to modify the epoxide into non-hazardous materials.

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SEQUENCE LISTING

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Met Cys Tyr Ile Trp Val Arg Thr Glu Arg Ile Val Glu Phe Aam
1 15

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Leu Ser Cys Tyr Ile Trp Val Arg Thr Glu Arg Ile Val Glu Phe Aam
10 15 20

gag atg gag cca ata aag cac aca gtg gas tgt gag atc ggc ata aac
Glu Met Glu Gln Ile Lys His Arg Thr Val Glu Val Aam Gly Ile Lys
25 30 35 40

atg cct ggt gcc gaa gga gga ggt gcc gct gtt tgt ttc ctc ccc
Met His Gly Ala Glu Gly Gly Gly Pro Val Leu Val Leu Phe Leu His
45 50 55

ggc tcc ctc gac ctc tgg tcc agc cat ceg att ctc tct ttc
Gly Phe Pro Glu Leu Trp Tyr Ser Trp Arg His Glu Ile Leu Ser Leu
60 65 70

agc tcc ctc gcc tac gcc gtc gct gcc gc gct cac ata
Ser Leu Gly Tyr Arg Ala Val Ala Pro Aam Arg Arg Gly Trp Gly
75 80 85

gac acc gaa gcc cca cct taa atc agc tac aac tgc ttc cac ata
Aam Thr Ala Pro Pro Ser Ile Ser Ser Tyr Aam Cys Phe His Ile
90 95 100

gtg gtg gtt gcc gtt acc att gcc ttc tcc gtg gtt gcc cac cca gty
Val Gly Asp Leu Val Leu Ile Asp Ser Leu Gly Gly Glu Val Val
105 110 115 120

ltc tgc gtt gct gac tgg gga gcc gcc atc ata tgg tat ctc gcc
Phe Leu Val Ala His Asp Ile Ile Gly Trp Tyr Leu Cys
125 130 135

atg ttc ccc tgc gac aas gtt aag goc tat gtc ctc aag gtc ccr Met Phe Arg Pro Asp Ile Lys Ala Tyr Val Cys Leu Ser Pro
140 145 150

ccc tcc cgc aag gac cca aac atc aca aag aag gct gat gcc atg att goc
Leu Leu Arg Arg Asp Pro Aam Ile Arg Thr Val Aam Gly Met Arg Ala
155 160 165

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Leu Tyr Gly Asp Tyr Tyr Val Cys Arg Phe Glu
170 175 180

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cctttgacgg aatatattt gttgtttgya aatggggtcg gaaggtttga tgaacccagtgy 940

cgatatgta tttttaaaatgtgctggttgattc aactgcag aaaa cca ggg gaa atg gag
Lys Pro Gly Glu Met Glu
185

gct cag atg gtt gaa gtg gcc act gaq tat gtt ctc gaa aac atc ctt
Ala Gln Met Ala Glu Val Gly Thr Glu Tyr Leu Val Leu Aam Ile Leu
190 195 200

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aatgtgatgg gtccacgacgcc gcaaccttccac atg tgc gag cac ttc ctc gtc tca 294

Met Cys Tyr Ile Trp Val Arg Thr Glu Arg Ile Val Glu Phe Aam
1 15

ctg ttc tgc tat att tgg gtg aag aca ceg aeg ata tgt gag ttc aac
Leu Ser Cys Tyr Ile Trp Val Arg Thr Glu Arg Ile Val Glu Phe Aam
10 15 20

ctg ttc tgc tat att tgg gtg aag aca ceg aeg ata tgt gag ttc aac
Leu Ser Cys Tyr Ile Trp Val Arg Thr Glu Arg Ile Val Glu Phe Aam
10 15 20
ccc ttc acc tac tac ags aat ttc acc tt gtaattttct gattctgcgt
Pro Leu Aan Tyr Tyr Arg Aan Phe Aan Leu 255 260

atcggccggt gtatattgtt ttcaactgtc ctatagtttaat gtttttcttt ttggggaaaata
1297

tgttgttaac atgtcgggat ccacatadasa aagaaccttt attaattttaa ttattgtgat
1357
			
tattttgacg a aat tgg gag tgt acg gca cca tgg aca gga ggg cga atc
Aen Trp Glu Leu Thr Ala Pro Trp Thr Gly Gly Glu Ile 265 270

aag gtc ccc gta aac tat ata aca aat ggt ggg gag tgt gac atg gta tac aac
Lys Val Pro Val Lys Tyr Ile Thr Gly Glu Leu Asp Met Val Tyr Aan 275 280 285

tcg ctc acc aac tgt aag tgt tct ccc ggc cga ggg ttc aag cca gat
Ser Leu Aen Leu Lys Tyr Ile His Gly Gly Phe Lys Glu Asp 290 295 300 305

gtt cca aat tta gaa cca gtg gtt att tgt cag cca aca gga ggt gtt gct ccc
tc Val Pro Aen Leu Glu Gln Val Ile Glu Gly Val Ala His Phe 310 315 320

aat aat cca gaa gaa gca gag cag cag aat cec ata tca gat ttt
Aen Aen Gln Ala Ala Gln Glu Ile Asp Aen Tyr Ile Tyr Asp Phe 325 330 335

atc acc aag ttc tgtcttgtgca casnasaogaa ttcasacgoa tataaattc
Ile Aen Lys Phe 340

cagctgctag gnaaggggtg tataattgcat ctttgttttt tgtatattag gtagcggagat
1710

catattcatg ccagacgtct acacatgtcga gaaacctcgc atcactcgc cttctctgat
1770

cattgttgtgac ttaattacatg gataaattc actgtatgtgc ttgtactctgc taaattacatc
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tagctctgtg atcgsaatgg atatccttaa aaatgttttt gaaatgttgc tatttggaac
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<213> ORGANISM: Glycine max

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Thr Gln Arg Ile Val Glu Phe Asn Glu Met Glu Gln Ile Lys His Arg
20 25 30
Thr Val Glu Val Asn Gly Ile Lys Met His Gly Ala Glu Lys Gly Glu
35 40 45
Gly Pro Val Leu Val Phe Pro Gly Glu Leu His Tyr Arg Ser
50 55 60
Trp Arg His Glu Ile Leu Ser Leu Ser Leu Gly Tyr Arg Ala Val
65 70 75 80
Ala Pro Asp Leu Arg Glu Tyr Gly Asp Thr Glu Ala Pro Pro Ser Ile
85 90 95
Ser Ser Tyr Asn Cys Phe His Ile Val Gly Asp Leu Val Ala Leu Ile
100 105 110
Asp Ser Leu Gly Val Gln Glu Val Phe Leu Val Ala His Asp Trp Gly
115 120 125
Ala Ile Ile Glu Trp Tyr Leu Cys Met Phe Arg Pro Asp Lys Val Lys
<210> SEQ ID NO: 5
<211> LENGTH: 23
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<221> NAME/KEY: misc_feature
<222> LOCATION: (.....)
<223> OTHER INFORMATION: Description of Artificial Sequence: primer
<211> NAME/KEY: misc_feature
<222> LOCATION: (.....)
<223> OTHER INFORMATION: N is A, G, C, or T

<400> SEQUENCE: 5

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Description of Artificial Sequence: primer

<400> SEQUENCE: 6

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<210> SEQ ID NO: 7
<211> LENGTH: 34
<212> TYPE: DNA
What is claimed is:

1. A cDNA encoding the plant-derived epoxide hydrolase having the amino acid sequence of SQ ID No. 1 in the Sequence Listing.

2. A gene encoding the plant-derived epoxide hydrolase having the amino acid sequence of SQ ID No. 2 in the Sequence Listing.

3. A plasmid vector carrying a cDNA encoding the plant-derived epoxide hydrolase according to claim 1.

4. A transformant (FERM BP-6624) retaining a plasmid vector according to claim 3.