Volume 323, number 1,2, 135–140 FEBS 12483 May 1993
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# A bacterial enzyme degrading the model lignin compound β-etherase is a member of the glutathione-*S*-transferase superfamily

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Received 25 March 1993

Cleavage of β-aryl ether linkages is essential in lignin degradation. We identified another β-etherase gene (*ligF*), which contains an open reading frame of 771 bp and lies between genes coding Cα-dehydrogenase (*ligD*) and β-etherase (*ligE*). The β-etherase activity of LigF expressed in *Escherichia coli* was more than 80 times as high as that of LigE. *ligF* and *ligE* are homologous to glutathione-*S*-transferase, and upon addition of glutathione a remarkable acceleration of β-etherase activity was found in *E. coli* carrying *ligF*. It is concluded that LigF plays a central role in β-aryl ether cleavage and that glutathione is the hydrogen donor in this reaction.

Lignin degradation; Arylglycerol-β-aryl ether linkage; β-Etherase; Glutathione-*S*-transferase; *Pseudomonas paucimobilis*

## 1. INTRODUCTION

Lignin is the most abundant aromatic material in the biosphere, and the substance therefore plays a major role in the earth's carbon cycle. *Pseudomonas paucimobilis* SYK-6 is able to assimilate various types of dimeric lignin compounds [1,2]. Protocatechuate 4,5-dioxygenase is a key enzyme in this process [3], and the nucleotide sequence of this gene is known [4]. Cleavage of arylglycerol-β-aryl ether is the most important step in lignin degradation. Detection of β-etherase activity in *P. paucimobilis* SYK-6 and characterization of the β-etherase gene (*ligE*) was reported [5,6]. The substrate specificity of the β-etherase is for a β-aryl ether which contains a carbonyl group at the Cα-position [5,6]. β-Aryl ether (Cα-alcohol type) was therefore oxidized by Cα-dehydrogenase and cleaved at the β-aryl ether linkage by β-etherase. We also characterized the Cα-dehydrogenase gene (*ligD*) [7] and this study indicated that the 3-kbp *Sal*I fragment carrying *ligD* was adjacent to a 1.9-kbp *Sal*I fragment carrying *ligE* [5,7]. Here we report that another β-etherase gene is located in the 5-kbp *Sal*I fragment containing *ligD* and *ligE*. The structure of this gene was determined and functional information on the β-etherase obtained.

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*Abbreviations.* IPTG, isopropyl-β-D-thiogalactopyranoside; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

## 2. MATERIALS AND METHODS

### 2.1. *Model compounds*

Model compounds used in this study were prepared as in a previous study [5].

### 2.2. *Bacterial strains and plasmids*

*Escherichia coli* MV1190 was used as the host strain. pBE10 containing the 24-kbp *Sal*I fragment carrying the β-etherase gene (*ligE*) and Cα-dehydrogenase gene (*ligD*) in pVK100 was isolated as in a previous study [6] pBE10 was partially digested with *Sal*I (Takara Shuzo) and a 5-kbp *Sal*I fragment carrying *ligD* and *ligE* was obtained. The 5-kbp *Sal*I fragment was cloned into pUC19 (pUBX7 and pUBX5). Various deletion derivatives, shown in Fig. 1, were constructed with restriction enzymes, the Kilosequence kit (Takara Shuzo), and pUC18.

### 2.3. *Enzyme assay*

Cell extracts were prepared as described earlier [6]. β-Etherase was assayed using a fluorogenic substrate synthesized in an earlier study (Fig. 1) [5]. Assay for glutathione-*S*-transferase was performed essentially according to the method of Habig et al. [8].

### 2.4. *DNA sequence analysis*

To determine the nucleotide sequence of *ligF*, various deletion derivatives were constructed using the Kilosequence kit. The nucleotide sequence of these deletion derivatives were determined by the dideoxy method [9] with Sequenase 2.0 (US Biochemical Corp.). [α-$^{32}$P]dCTP (specific activity, 110 TBq/mmol) was from Amersham. The nucleotide sequence presented here has been submitted to the DDBJ, EMBL and GenBank databases under the accession number D11473.

## 3. RESULTS

### 3.1. *Detection of the high* β-*etherase activity in* E. coli *carrying the 5-kbp* Sal*I fragment*

We earlier isolated the β-etherase gene (*ligE*) and Cα-dehydrogenase gene (*ligD*) as the 1.9-kbp and 3-kbp

*Sal*I fragments, respectively [6,7]; these fragments were adjacent to each other [7]. pBE10 containing a 24-kbp *Sal*I fragment was partially digested with *Sal*I, and we isolated the 5-kbp *Sal*I fragment containing *ligD* and *ligE*. The 5-kbp *Sal*I fragment was cloned into pUC19 and was designated pUBX7 (Fig. 1). β-Etherase activity of the cell extract of *E. coli* MV1190 containing pUBX7 cultured with IPTG was 330 pmol of 4-methylumbelliferone/mg of protein/min, which is more than 100 times higher than the activity of *E. coli* MV1190 containing pUBE13 which was carrying the 1.9-kbp *Sal*I fragment containing *ligE*.

### 3.2. *Deletion analysis of the 5-kbp* SalI *fragment*

Deletion analysis of the 5-kbp *Sal*I fragment was carried out to determine the reason for the increase in β-etherase activity when this fragment was present in *E. coli* (Fig. 1). The increase in β-etherase activity was lost when the 5′ terminus of the 5-kbp fragment was deleted to the *Sma*I site located downstream of *ligD* (pUBX74). When the deletion entered the 3′ terminus of *ligE*, the increase in β-etherase activity was retained (pUBX75). β-Etherase activity was lost in *E. coli* containing pUBX79. These results indicate that the gene encoding another β-etherase with strong activity existed between *ligD* and *ligE*; the gene was designated *ligF*.

### 3.3. *Nucleotide sequence of the other* β-*etherase gene (*ligF*)*

The nucleotide sequence of a 1,250-bp *Xho*I–*Pst*I fragment containing *ligF* was determined (Fig. 2). There is an open reading frame of 771 bp, with the initiation codon (ATG) at position 317 and the termination codon (TGA) at position 1,088, encoding 257 amino acids. This gene had a high G+C content (61%) and the occurrence of G or C in the third base of each codon was extremely high (87%). There is a sequence similar to a ribosome-binding site upstream of the initiation codon (GGAG) [10]. The molecular weight of the deduced protein is 29,685 Da. Homology search (SWISS-PROT, release 21.0) revealed that LigF showed significant homology with glutathione-*S*-transferase III of *Zea mays* (Fig. 3A) [11].

### 3.4. *Acceleration of the* β-*etherase activity by the addition of glutathione*

A homology search showed the similarity between LigF and glutathione-*S*-transferase (GST). We further found that LigE was homologous to GST. We examined the possibility that glutathione (GSH) was actually involved in the cleavage of β-aryl ether by β-etherase. When 2 mM of GSH was added to a reaction mixture including cell extracts of *E. coli* MV1190 containing pUBX78, there was about a 15-fold increase of β-etherase activity (Table I). On the other hand, only a 2-fold stimulation was observed upon addition of GSH to the cell extract of *E. coli* MV1190 containing pUBE14 (Table I). LigF and LigE were tested with 1-chloro-2,4-dinitrobenzene (CDNB), which is the universal substrate for GST. There was only very weak activity (< 0.020 ($\Delta A_{340}$/mg·min)) in cell extracts of *E. coli* MV1190 containing pUBX78 or pUBE14.

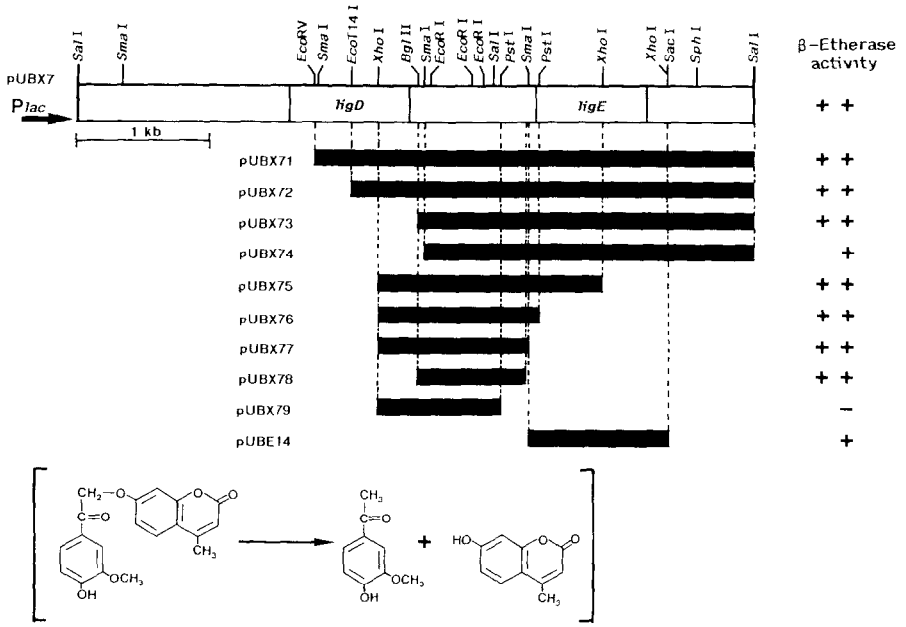


Fig. 1. Deletion analysis of the 5-kbp *Sal*I fragment. The β-etherase activity of *E. coli* MV190 containing pUBX7, pUBX71-79, and pUBE14 was assayed. Abbreviations (++, +, and −) indicate the level of β-etherase activity: (+) shows the level of *E coli* having *ligE* (pUBE13), (++) shows the level of *E coli* having pUBX7, and (−) indicates no β-etherase activity. The assay for β-etherase is indicated by the chemical formula surrounded by brackets.

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CTCGAGCCGGAGAAGCTGGCGGAAGCGATCAAGAAGGGTGTCGAGGACAATGCTCTCTACATCATTCCCTAT   72
LeuGluProGluLysLeuAlaGluAlaIleLysLysGlyValGluAspAsnAlaLeuTyrIleIleProTyr

CCCGAAGTGCGCGAAGGACTGGAGAAGCATTTTCAGGCCATCATCGATTCGGTCGCGCCGATGGAGAGCGAT  144
ProGluValArgGluGlyLeuGluLysHisPheGlnAlaIleIleAspSerValAlaProMetGluSerAsp

CCGGAAGGCGCCCGCCAGCGGGTCGAGGCACTGATGGCCTGGGGACGGGACCGCACGCGGGTCTTCGCCGAG  216
ProGluGlyAlaArgGlnArgValGluAlaLeuMetAlaTrpGlyArgAspArgThrArgValPheAlaGlu

GGCGACAAGAAAGGCGCCTGACGAAATGCACGGGCGTGGCGGCTTAACCGCCGCGCCCTTTTCGCTCATGAC  288
GlyAspLysLysGlyAla***
                SD          ligF
GAGATGACAAGAGATCTGGGAGACAGGA ATG ACG TTG AAA CTC TAC AGC TTC GGT CCC GGG   349
                             Met Thr Leu Lys Leu Tyr Ser Phe Gly Pro Gly

GCG AAC TCG CTC AAG CCG CTC GCG ACG CTC TAC GAG AAG GGC CTC GAA TTC GAG   403
Ala Asn Ser Leu Lys Pro Leu Ala Thr Leu Tyr Glu Lys Gly Leu Glu Phe Glu

CAG GTC TTC GTC GAT CCG AGC AAG TTC GAG CAG CAT TCG GAC TGG TTC AAG AAG   457
Gln Val Phe Val Asp Pro Ser Lys Phe Glu Gln His Ser Asp Trp Phe Lys Lys

ATC AAT CCG CGC GGT CAG GTG CCG GCG CTC TGG CAT GAC GGC AAG GTC GTC ACC   511
Ile Asn Pro Arg Gly Gln Val Pro Ala Leu Trp His Asp Gly Lys Val Val Thr

GAA TCG ACG GTG ATC TGC GAA TAT CTG GAG GAC GTG TTC CCC GAG TCC GGC AAT   565
Glu Ser Thr Val Ile Cys Glu Tyr Leu Glu Asp Val Phe Pro Glu Ser Gly Asn

TCG CTG CGC CCG GCC GAC CCC TTC AAG CGC GCC GAA ATG CGG GTG TGG ACC AAG   619
Ser Leu Arg Pro Ala Asp Pro Phe Lys Arg Ala Glu Met Arg Val Trp Thr Lys

TGG GTC GAT GAA TAT TTC TGC TGG TGC GTC TCC ACC ATC GGC TGG GCC TTC GGC   673
Trp Val Asp Glu Tyr Phe Cys Trp Cys Val Ser Thr Ile Gly Trp Ala Phe Gly

ATC AAG GCG ATC GCG CAG AAG ATG AGC GAC GAG GAA TTC GAG GAG CAC ATC AAC   727
Ile Lys Ala Ile Ala Gln Lys Met Ser Asp Glu Glu Phe Glu Glu His Ile Asn

AAG AAT GTG CCG ATC CCC GAG CAG CAG CTC AAA TGG CGC CGC GCG CGC AAC GGA   781
Lys Asn Val Pro Ile Pro Glu Gln Gln Leu Lys Trp Arg Arg Ala Arg Asn Gly

TTC CCG CAG GAG ATG CTG GAC GAG GAA TTC CGC AAG GTC GGC GTC TCG GTG GCG   835
Phe Pro Gln Glu Met Leu Asp Glu Glu Phe Arg Lys Val Gly Val Ser Val Ala

CGG CTG GAA GAG ACG CTC TCG AAG CAG GAC TAT CTG GTC GAC ACG GGT TAC AGC   889
Arg Leu Glu Glu Thr Leu Ser Lys Gln Asp Tyr Leu Val Asp Thr Gly Tyr Ser

CTC GCG GAC ATC TGC AAT TTC GCC ATC GCC AAT GGC CTG CAG CGC CCC GGC GGC   943
Leu Ala Asp Ile Cys Asn Phe Ala Ile Ala Asn Gly Leu Gln Arg Pro Gly Gly

TTC TTC GGC GAC TAT GTG AAC CAG GAA AAG ACG CCC GGC CTG TGC GCC TGG CTC   997
Phe Phe Gly Asp Tyr Val Asn Gln Glu Lys Thr Pro Gly Leu Cys Ala Trp Leu

GAC CGG ATC AAT GCG CGT CCG GCG ATC AAG GAA ATG TTC GAG AAA TCG AAG CGC  1051
Asp Arg Ile Asn Ala Arg Pro Ala Ile Lys Glu Met Phe Glu Lys Ser Lys Arg


GAG GAC CTG CTC AAG CGG CAG AAC GAG AAA GTC GCC TGA ACAGGAAAGCGCGTCCCGG  1109
Glu Asp Leu Leu Lys Arg Gln Asn Glu Lys Val Ala ***

CGCCCGCCGGACAAGGCCAGCCGGTTGACGTTCGCCTTGTCCGGCAGCGCCGTTCGCGCGGCCCGGGCGCG  1180


CCGCTGCGTTGAATGATGGAGAGGATTGCCGATGGCCAGGAACAACACCATCACTCTGTATGACCTGCAG  1250
                                MetAlaArgAsnAsnThrIleThrLeuTyrAspLeuGln
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Fig. 2. Nucleotide sequence of the *Xho*I–*Pst*I fragment carrying *ligF*. The open reading frame of *ligF* had the initiation codon (ATG) at position 317 and the termination codon (TGA) at 1,088. Partial sequences of *ligD* and *ligE* are also indicated upstream and downstream of *ligF*, respectively. The Shine–Dalgarno (SD) sequence is indicated by a thick underline.

**A**

```
LigF:1    M-TLKLYSFG PGANSLKPLA TLYEKGLEFE QVFVDPSKFE QHSDWFKKIN PRGQVPALWH
          * :****: :  ::* ::  :  * ****:**  * ** :     :   *  :* * **:***
GT31:1'   MAPLKLYGMP LSPNVVRVAT VLNEKGLDFE IVPVDLTTGA HKQPDFLALN PFGQIPALVD

60        DGKVVTESTV ICEYLEDVFP ESGNSLRPAD PFKRAEMRVW TKWVDEYFCW CVSTIGWAFG
            *: **   *  *:   ::   *  * **  :   * : **        *      : * : :
61'       GDEVLFESRA INRYIASKYA SEGTDLLPA- TASAAKLEVW LEVESHHFYP NRVAAGVPAA

120       IKAIAQKMSD EEFEEHINKN VPIPEQQLKW RRARNGFPQE MLDEEFRKVG VSVARLEETL
            * : : :*            *    :**              :**   *    * *    :
120'      REAAPGRRPD -------AAV VDKHAEQL-- --------AK VLDVYER--- TSPATSTSPG

180       SKQDYLVDTG YSLADICNFA IANGLQRPGG FFGDYVNQEK TPGLCAWLDR INARPAIKEM
          :      *:  *       :    :  **               * : ** :  * ****
160'      TSSRSPTPTT RSYLLYLSKT PRPARRRP-- ---------- -PHVKAWWEA IVARPA----

240       FEKSKREDLL KRQNEKVA
          *:*:     *        *
203'      FQKTVAAIPL PPPPSSSA
```

**B**

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GTA8 : 54 | Q | V | P | L | V | E | I | D | G | M | L | L | T | Q | T | R | A |
| GT31 : 54 | Q | I | P | A | L | V | D | G | D | E | V | L | F | E | S | R | A |
| DCMA : 65 | Q | V | P | I | L | V | D | G | E | F | T | V | W | E | S | V | A |
| LigE : 58 | R | V | P | V | I | V | D | D | G | E | W | V | L | D | S | W | V |
| LigF : 53 | Q | V | P | A | L | W | H | D | G | K | V | V | T | E | S | T | V |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GTA8 : 71 | I | L | S | Y | L | A | A | K | Y | N | L | Y | G | K | D | L | K |
| GT31 : 71 | I | N | R | Y | I | A | S | K | Y | A | S | E | G | T | D | L | L |
| DCMA : 82 | I | A | R | Y | V | N | E | K | F | D | G | A | G | N | W | F | G |
| LigE : 75 | I | A | E | Y | L | D | E | K | Y | P | D | R | P | M | L | F | E |
| LigF : 70 | I | C | E | Y | L | E | D | V | F | P | E | S | G | N | S | L | R |

Fig. 3. (A) Homology alignment between LigF (upper line) and GST III of *Z. mays* (GT31). Identical and similar amino acids are indicated by asterisks and colons, respectively. Similar amino acids are Ile, Leu, Val, Met/Lys, His, Arg/Asp, Glu, Asn, Gln/Phe, Trp, Tyr/Ser, Thr, Ala, Gly, Pro. (B) Alignment of amino acid sequence of LigF and LigE with sequences of several GST. GTA8, GST of *R norvegicus*; GT31, GST III or *Z. mays*; DCMA, dichloromethane dehalogenase of *Methylobacterium* spp. Shaded areas show regions of identity. Similar amino acids are underlined.

## 4. DISCUSSION

We identified another β-etherase gene (*ligF*) located between *ligD* and *ligE*. The β-etherase activity of *E. coli* containing *ligF* was more than 80 times as high as that of *E. coli* containing *ligE*. LigF and LigE were homologous to GST, and especially LigF had high similarity with GST III of *Z. mays* (25%) (Fig. 3A) [11]. LigF has 21% homology with LigE. Interestingly, dichloromethane dehalogenase (DCMA) of *Methylobacterium* spp. [12], which is reported to be a member of the GST superfamily, has some similarity with both LigF (22% identical residues) and ligE (19%). To our knowledge, DCMA is the sole bacterial enzyme reported to be a member of the GST superfamily. Comparison of the amino acid sequences of LigF, LigE, DCMA, GST III of *Z. mays*, and GST of *Rattus norvegicus* [13] shows a region containing highly conserved amino acid residues (Fig. 3B). This region (positions 66–82 indicated in LigF) has been hypothesized to represent part of a

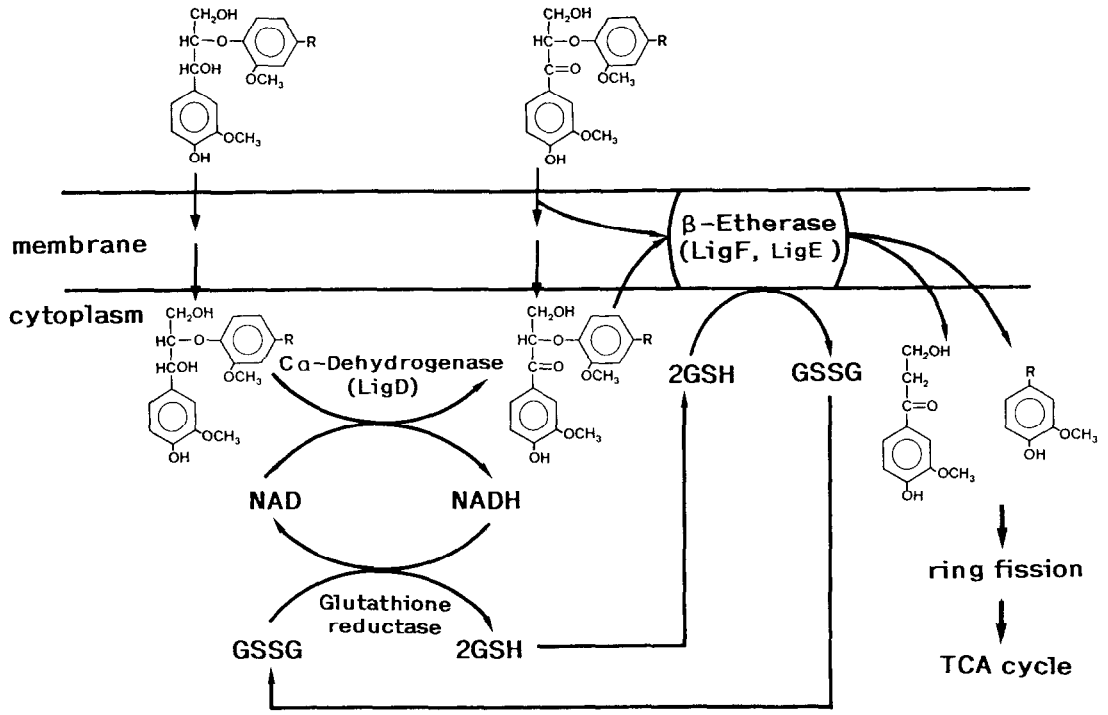


Fig. 4. New proposed model for the cellular assimilation of arylglycerol-β-aryl ether by *P. paucimobilis* SYK-6. GSSG, oxidized glutathione; TCA, tricarboxylic acid.

GSH-binding site common to all GST [12,14–16]. From the result of the homology search, GSH was thought to be involved in β-aryl ether cleavage by β-etherase. Remarkable stimulation of β-etherase activity was observed upon addition of GSH to a reaction mixture containing cell extract of *E. coli* carrying *ligF*. On the other hand, only a 2-fold stimulation was observed in the case of *ligE*. This great difference of β-etherase activity between LigF and LigE suggests that LigF plays a major role in β-aryl ether cleavage. LigE may be an isozyme of LigF with different substrate specificity. Neither LigF nor LigE reacted with CDNB, a compound used as the general substrate for GST, and it has been reported that DCMA also does not react with CDNB [12]. This may represent a difference between these bacterial enzymes and eukaryotic GST. There are few reports about bacterial GST, but it is possible that enzymes homologous to GST in bacteria may be involved in the degradation of a wide variety of aromatic and aliphatic compounds, because lignin is the most abundant aromatic material, and enzymes involved in its degradation seem to be ancestors of those involved in the degradation of various other aromatic materials.

Table I

β-Etherase activity of cell extracts and the effect of glutathione

| Source of extract | GSH[a] | β-Etherase activity (pmol of 4MU/mg of protein/min)[b] |
|---|---|---|
| *E. coli* MV1190 | – | 0 |
| *E coli* MV1190 (pUBE14) | – | 6.4 |
| *E coli* MV1190 (pUBE14) | + | 13.0 |
| *E. coli* MV1190 (pUBX7) | – | 330.0 |
| *E. coli* MV1190 (pUBX78) | – | 540.0 |
| *E coli* MV1190 (pUBX78) | + | 8,100.0 |
| *P. paucimobilis* SYK-6 | – | 2.0 |
| *P. paucimobilis* SYK-6 | + | 40.0 |

[a]GSH, reduced glutathione. 2 mM of GSH was added to the reaction mixture.
[b]4MU, 4-methylumbelliferone

The results that the β-etherase activity was stimulated by GSH leads to the conclusion that GSH is the hydrogen donor in reductive ether cleavage catalyzed by β-etherase. We propose a new functional model for β-aryl ether cleavage by β-etherase in Fig. 4. The arylglycerol-β-aryl ether is taken up into the cell and oxidized by Cα-dehydrogenase to produce NADH and the specific substrate of β-etherase. β-aryl ether (Cα-carbonyl type) is cleaved by β-etherase with consumption of GSH. Oxidized glutathione is reduced to GSH by glutathione reductase, which we detected in *P. paucimobilis* SYK-6, with NADPH or NADH.

*Acknowledgements* This work was supported in part by a Grant-in-Aid for Scientific Research (No. 04454088) from the Ministry of Education, Science and Culture of Japan.

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