Cloning and molecular characterization of a soluble epoxide hydrolase from *Aspergillus niger* that is related to mammalian microsomal epoxide hydrolase

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Aspergillus niger strain LCP521 harbours a highly processive epoxide hydrolase (EH) that is of particular interest for the enantioselective bio-organic synthesis of fine chemicals. In the present work, we report the isolation of the gene and cDNA for this EH by use of inverse PCR. The gene is composed of nine exons, the first of which is apparently non-coding. The deduced protein of the *A. niger* EH shares significant sequence similarity with the mammalian microsomal EHs (mEH). In contrast to these, however, the protein from *A. niger* lacks the common N-terminal membrane anchor, in line with the fact that this enzyme is, indeed, soluble in its native environment. Recombinant expression of the isolated cDNA in *Escherichia coli* yielded a fully active EH with similar characteristics to the fungal enzyme. Sequence comparison with mammalian EHs suggested that Asp^{192}, Asp^{348} and His^{374} constituted the catalytic triad of the fungal EH. This was subsequently substantiated by the analysis of respective mutants constructed by site-directed mutagenesis. The presence of an aspartic acid residue in the charge-relay system of the *A. niger* enzyme, in contrast to a glutamic acid residue in the respective position of all mEHs analysed to date, may be one important contributor to the exceptionally high turnover number of the fungal enzyme when compared with its mammalian relatives. Recombinant expression of the enzyme in *E. coli* offers a versatile tool for the bio-organic chemist for the chiral synthesis of a variety of fine chemicals.

Key words: α/β hydrolase fold, catalytic triad, enzymic mechanism, 4-nitrostyrene oxide, xenobiotic metabolism.

INTRODUCTION

Epoxide hydrolases (EHs; EC 3.3.2.3) comprise a group of functionally related enzymes that catalyse the addition of water to oxirane compounds (epoxides), thereby usually generating vicinal *trans*-diols [1]. EHs have been found in all types of living organisms, including mammals, invertebrates, plants, fungi and bacteria. In animals, the major interest in EH is directed towards their detoxification capacity for epoxides since they are important safeguards against the cytotoxic and genotoxic potential of oxirane derivatives that are often reactive electrophiles because of the high tension of the three-membered ring system and the strong polarization of the C—O bonds. This is of significant relevance because epoxides are frequent intermediary metabolites which arise during the biotransformation of foreign compounds.

Interest in microbial EH has recently arisen because of the potential of this class of enzymes as enantioselective biocatalysts [2]. Due to their chemical reactivity, epoxides represent versatile chemical building blocks. Of particular interest is the potential of EH for chiral resolution, since enzymes are most often intrinsically enantioselective.

Over the last decade EHs have been cloned from animals, plants and bacteria [3–8]. Most of these turned out to be members of a large superfamily of enzymes with a common three-dimensional structure, the α/β hydrolase-fold enzymes [9]. Amino-acid sequence similarity between the different members of this family is usually very low and mostly restricted to the α/β hydrolase-fold domain. For instance, direct comparison between the two mammalian xenobiotic EHs, namely the microsomal EH (mEH) and the soluble EH (sEH), does not indicate any phylogenetic relationship between these two enzymes [4]. Only by the significant sequence similarity of both proteins to the same bacterial enzyme, haloalkane dehalogenase [10], does their common phylogeny become evident [11–13].

The α/β hydrolase-fold enzymes hydrolyse their substrates by the action of a catalytic triad in a two-step mechanism, best known from acetylcholine esterase, a prototypic α/β hydrolase-fold enzyme [14] or haloalkane dehalogenase [15,16]. Catalytic triads have also been proposed to constitute the active centres of EHs [11–13], and this was recently proved by experiments with mammalian sEH [17–19] and mEH [20–22], as well as for an EH from *Agrobacterium radiobacter* [7]. For the latter enzyme, the three-dimensional structure has very recently been solved [23] and supports the previous biochemical findings. In all the above EHs, an aspartic acid residue acts as a catalytic nucleophile that attacks the substrate epoxide ring, which leads to the intermediate formation of a substrate–enzyme ester. This intermediate is subsequently hydrolysed by a water molecule in the catalytic centre of the enzyme, which is activated by a so-called charge-relay system composed of a proton-abstracting histidine residue that is supported by hydrogen bonding to an acidic residue. In almost all EH/haloalkane dehalogenase-related enzymes known to date this acidic residue is an aspartic acid, only the mEHs of mammals and invertebrates invariably possess a glutamic acid

Abbreviations used: EH, epoxide hydrolase; sEH, soluble epoxide hydrolase; mEH, microsomal epoxide hydrolase.

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The nucleotide sequences reported in this paper have been deposited in the EMBL Nucleotide Sequence Data Bank with accession numbers AJ 238459 (*A. niger EH* gene) and AJ238460 (*A. niger* EH mRNA).

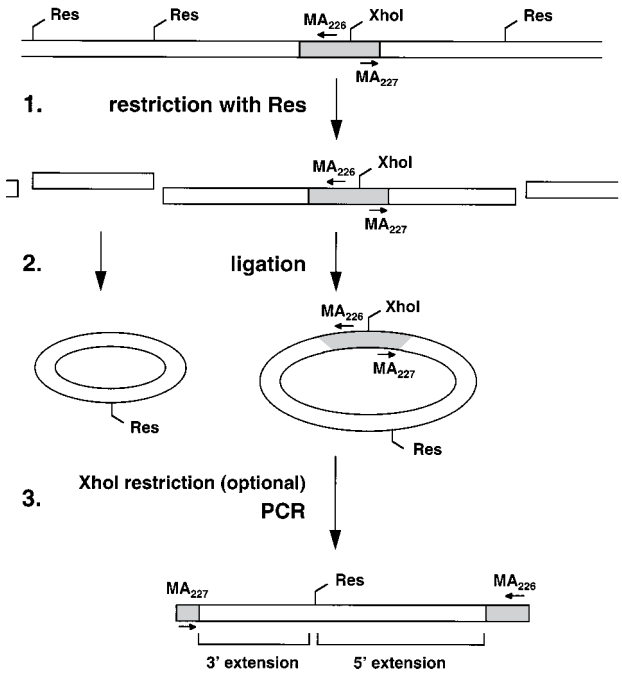
residue in this position [24]. Notably, the substitution of this glutamate with aspartate strongly increases the substrate turnover number of the enzyme [22].

A particularly interesting EH, in terms of its biocatalytic properties, has been identified in the fungus *Aspergillus niger*, and high enantioselectivity with chiral epoxides of commercial interest has been demonstrated [25–27]. Very recently, the protein has been purified to homogeneity [28]. Subsequent peptide analysis finally resulted in the generation of a DNA probe for the enzyme by genomic PCR. In the present work, the cloning and analysis of the *A. niger EH* gene and cDNA is reported.

EXPERIMENTAL

Isolation of nucleic acids from *A. niger*

A. niger (strain number LCP 521; Museum of Natural History, Paris, France) was grown in aqueous medium containing 10 g glucose and 20 g corn steep liquor (Sigma) per litre of culture. Culture medium (100 ml) at 28 °C was inoculated with *A. niger* spores and incubated for 3 days in a shaking flask. The mycelium was harvested by filtration over cheesecloth and stored at −70 °C in aliquots, after determination of the wet weight. RNA extraction was performed by the method of Chomczynski and Sacchi [29], using 10 ml of denaturing solution/g of mycelium and the typical yield was 300 μg of total RNA/g of mycelium. For the isolation of DNA, 2 g of mycelium were homogenized in a Potter–Elvehjem glass homogenizer in 15 ml of lysis solution (6 M guanidinium chloride/0.1 M sodium acetate, pH 5.5). After centrifugation at 10000 ***g*** for 10 min, the supernatant was transferred to a fresh tube and 2.5 vol. of ethanol were added. Precipitated nucleic acids were harvested by centrifugation at 10000 ***g*** for 10 min and the resulting pellet was redissolved overnight in 10 ml of lysis buffer after brief drying. Insoluble material was removed by centrifugation and nucleic acids were again precipitated by the addition of 25 ml of ethanol. The pellet resulting after a final centrifugation step was briefly washed with 70% (v/v) ethanol, air dried for 30 min and dissolved in TE buffer (10 mM Tris/HCl/1 mM EDTA, pH 8.0).



Scheme 1 Cloning of the *A. niger* EH by inverse PCR

The horizontal bar represents the genomic DNA of *A. niger*, with the grey block indicating the fragment of the *A. niger EH* gene obtained from our previous work [28]. In step 1, the DNA is restricted with an enzyme of choice (Res), leading to multiple fragments that were subsequently circularized via ligation in step 2. This enabled PCR amplification in step 3 of a specific contiguous fragment, using the two primers MA226 and MA227. The PCR runs over the junction between the 5′ end and the 3′ end of the initial restriction fragment. Therefore the 3′ part of the gene situated downstream of the priming site for oligonucleotide MA227 in the genomic DNA is now located upstream of the 5′ part of the gene in the resulting PCR fragment. In the present case, the restriction enzyme *Xho*I was used to relinearize the circular template before the PCR, which slightly increased the yield of the amplification as long as additional *Xho*I recognition sites were absent between the two priming sites in the resulting linear fragment.

Cloning of the *A. niger EH* gene and cDNA via PCR

Inverse PCR [30] for the amplification of the *A. niger EH* gene was performed according to the following procedure (see also Scheme 1): 500 ng of genomic DNA were digested with a suitable restriction enzyme (best results were obtained with *Bam*HI or *Cfo*I) and recovered by ethanol precipitation after phenol/chloroform (1:1, w/v) extraction [31]. Of these, 100 ng were circularized by ligation with T4 DNA ligase (Life Technologies) in a volume of 20 μl, using the conditions specified by the supplier. The resulting preparation (1 μl) was amplified in a PCR reaction over 30 cycles (1 min at 94 °C, 1 min at 60 °C, 3 min at 72 °C) with *Taq* DNA polymerase (Perkin–Elmer) under standard reaction conditions as recommended by the supplier. The primers used (MA226: 5′-ATGCGATCGGACTGCTGGACA-3′; and MA227: 5′-CGCGGGCAATCCACACCTAC-3′) were deduced from the sequence of a genomic fragment obtained earlier [28]. An *Xho*I restriction site, situated between the two priming sites in the genomic sequence, was optionally used to relinearize the circularized DNA before inverse PCR in order to release the torsional stress and thus improve the amplification efficiency from the genomic template. PCR products were separated by agarose gel electrophoresis and *A. niger* EH-specific amplicons were subsequently identified by Southern blotting [31], using the above mentioned genomic fragment as a probe. *A. niger EH* gene fragments identified this way were purified by agarose gel electrophoresis and subsequent gel extraction using the Quiaex II kit (Qiagen), and cloned into the pGEM-T vector (Promega) for sequence analysis by the chain termination method [32].

From the sequence information obtained, two primers (MA290: 5′-cggaattccATGgTCACTGGAGGAGCAATAAT-TAG-3′, and MA291: 5′-ttgaatTCCCTACTTCTGCCACAC-3′; residues printed in capital letters are complementary to the template sequence) flanking the predicted protein coding region of the *EH* gene were deduced and used to amplify respective fragments from genomic DNA and reverse transcribed mRNA with the high fidelity *Pfu* DNA polymerase (Stratagene) over 40 cycles (1 min at 94 °C, 1 min at 50 °C, 6 min at 72 °C). The resulting DNA fragments were digested with *Eco*RI (note the *Eco*RI recognition sites underlined in the sequences of the two primers) and inserted into pUC19 (New England Biolabs) for final sequence analysis.

Expression and analysis of recombinant EH

The bacterial expression vector pGEF+ [33] was modified by introducing a polylinker (5′-CCATGGGAATTCTCGAGATC-TAAGCTTATGCATCAGCTGCATGG-3′) into its *Nco*I recognition site, which contained the start codon of the pGEF+ vector in the proper context of a ribosomal binding site, downstream of the T7 RNA polymerase promoter. The resulting plasmid was termed pGEF II.

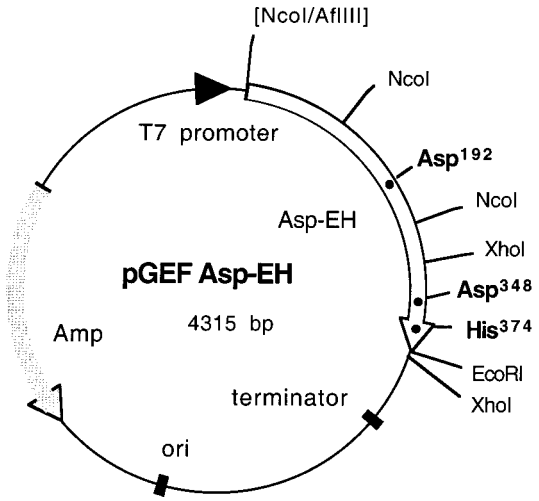


Figure 1 Expression construct for the recombinant production of *A. niger* EH in *E. coli*

The positions of restriction sites used for the construction of the expression vector and its derivatives for the analysis of active-site mutants, as well as the positions of the active-site amino-acid residues of the *A. niger* EH are indicated. Asp-EH, *A. niger* EH coding region; T7 promoter, transcription start site for the DNA-dependent RNA polymerase of bacteriophage T7; Amp, bacterial β-lactamase gene, conferring ampicillin resistance; ori, pMB1 origin of replication from pBR322; terminator, ϕ transcription terminator of bacteriophage T7

For recombinant expression in *E. coli*, the *A. niger* EH cDNA fragment was amplified with *Pfu* DNA polymerase using primer MA291 (see above) and primer MA318 (5′-gctgaattcacATGTCCGCTCCGTTCGCCAAG-3′) in order to introduce an *Nco*I-compatible *Afl*III recognition site (underlined) into the putative start codon of the *A. niger EH* gene, revealed by sequence analysis. The *Afl*III/*Eco*RI-restricted *A. niger* EH PCR fragment was ligated into the *Nco*I/*Eco*RI site of pGEF II to yield the expression construct pGEF–AspEH (see Figure 1). The *E. coli* strain BL21(DE3) (Novagen) was transformed with pGEF–AspEH and grown in Luria–Bertani medium at 30 °C. In the late exponential phase, induction of recombinant protein expression was afforded by addition of isopropyl β-D-thiogalactoside (100 μM). After 2 h, bacteria were harvested by centrifugation, resuspended in 0.02 culture vol. of STE buffer (10 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) and stored at −70 °C for further analysis. The enzymic activity was determined by monitoring the conversion of the *R* enantiomer of 4-nitrostyrene oxide [28] into the corresponding diol. The reaction was carried out at a substrate concentration of 880 μM in 500 μl STE buffer at 37 °C for up to 30 min, in the presence of 10 μl acetonitrile, which was used as a solvent for the 4-nitrostyrene oxide. Subsequently, substrate turnover was terminated by the extraction of the incubation mixture with an equal volume of chloroform. Under these conditions, more than 99.9 % of the substrate was extracted into the organic phase and 60 % of the diol remained in the aqueous phase. Product formation was quantified by adding 400 μl of the aqueous supernatant to 800 μl water and reading the A_{277} (molar absorption coefficient 9.1×10^3 $M^{-1} \cdot cm^{-1}$).

Purification of recombinant *A. niger* EH

A. niger EH was purified to homogeneity by a three-column procedure. Briefly, the bacterial pellet was suspended in STE, supplemented with 100 μg/ml lysozyme, and incubated on ice for 30 min. After addition of 5 mM dithiothreitol (final concentration), cells were broken by sonication. The resulting suspension was cleared by centrifugation (10000 ***g*** for 10 min), and *A. niger* EH was recovered in the 30 %–60 % pellet of a fractionated $(NH_4)_2SO_4$ precipitation. The pellet was resuspended in buffer A (10 mM Tris/HCl, 20 mM NaCl, 1 mM EDTA, pH 7.0), dialysed twice against excess buffer A and subjected to anion-exchange chromatography on a QA52 column. Elution of *A. niger* EH was accomplished by a linear NaCl gradient (20 mM–500 mM) in buffer A. *A. niger* EH activity was eluted between 150 and 180 mM NaCl. The pooled active fractions were dialysed against 30 % $(NH_4)_2SO_4$, loaded on to a phenyl-Sepharose column and eluted with a linear gradient of $(NH_4)_2SO_4$ (30 % to 0 %). The enzyme was eluted between 4 % and 1.5 % $(NH_4)_2SO_4$. The pooled active fractions were brought to 70 % $(NH_4)_2SO_4$ and the precipitated protein was redissolved in 3 ml of buffer A after centrifugation (10000 ***g*** for 10 min). The resulting solution was passed over a HiLoad 26/60 Superdex 200 pg gel-filtration column (Pharmacia Biotech) previously equilibrated with buffer A. The *A. niger* EH was eluted as a single homogeneous peak at an elution volume equivalent to a molecular mass of 100 kDa. Antibodies were raised against the purified protein by immunizing rabbits as described previously [34]. The purified protein was analysed by SDS/PAGE, followed by Coomassie Blue staining or immunoblotting according to previously described procedures [19].

Construction and analysis of EH mutants

PCR-mediated site-directed mutagenesis of the *A. niger* EH cDNA was carried out by the method of Tomic et al. [35], as described earlier for the mammalian sEH and mEH [19,22]. Primers used for the introduction of the different mutations are given in Table 1. Mutations affecting the catalytic nucleophile Asp^{192} were introduced by exchanging the internal *Nco*I cassette of the *A. niger* EH cDNA (see Figure 1) against a PCR-modified fragment. Similarly, mutations targeting the residues of the charge-relay system, namely Asp^{348} and His^{374}, were introduced by substituting the *Xho*I fragment of pGEF–AspEH with a respective PCR fragment. PCR modifications were generated using *Pfu* DNA polymerase to minimize the introduction of unwanted sequence modifications. Furthermore, all PCR-generated fragments were sequenced to assess their correctness. After recombinant expression, the mutant proteins were tested for their solubility as an indicator for their structural integrity. After sonication of the bacterial pellets, the resulting suspensions were centrifuged at 10000 ***g*** for 20 min, and pellets and supernatants were checked for the presence of *A. niger* EH by immunoblotting. Enzymic activity was monitored in the supernatant as described above.

RESULTS

Cloning of the Aspergillus *EH* gene and cDNA

Starting from earlier work that lead to the isolation of a genomic fragment of the *A. niger EH* gene [28], we have isolated the complete gene and cDNA for the enzyme. Initial efforts to obtain an *A. niger* EH cDNA by conventional hybridization screening of an *A. niger* cDNA library failed (results not shown). In an alternative approach, we first isolated the *A. niger EH* gene by means of inverse PCR, as described in the Experimental section. Using amplification of genomic DNA recircularized after *Bam*HI restriction, we obtained a 1.8-kb fragment that gave a positive hybridization signal with the above genomic fragment (Figure 2). Sequence analysis proved that this fragment harboured the complete 5′-region of the *A. niger EH* gene. However, the

Table 1 Oligonucleotides used for site-directed mutagenesis

The nucleotide sequences of the mutation primers and the matching adapter primers are shown. For a detailed description of the procedure see [19] or [22]. The restriction-enzyme-recognition sites used for the recombination of the mutated fragment and the adapter fragment are shown in **bold** letters. Mutated residues are in ***bold italic*** letters. The parts of the sequence removed by enzymic digestion is presented in lower case. The resulting 5′-cohesive ends are underlined.

Mutation	Mutation primer	Adapter primer
$Asp^{192} \rightarrow Ala$	cca**gaagac**ggAGGTG***CA***ATTGGTAGCTTTGTTGGACGA	cca**gaagac**tcACCTCCCTGAATAATATAACCAC
$Asp^{192} \rightarrow Ser$	cca**gaagac**ggAGGT***TCA***ATTGGTAGCTTTGTTGGACGA	cca**gaagac**tcACCTCCCTGAATAATATAACCAC
$Asp^{192} \rightarrow Asn$	cca**gaagac**ggAGGT***A***ATATTGGTAGCTTTGTTGGACGA	cca**gaagac**tcACCTCCCTGAATAATATAACCAC
$Asp^{348} \rightarrow Ala$	cca**gaagac**ccAAGG***C***CCTTTGTCCTGTGCCTC	cca**gaagac**gtCCTTGGGGAAGAAGGAGAAC
$Asp^{348} \rightarrow Glu$	cca**gaagac**ccAAGGA***G***CTTTGTCCTGTGCCTC	cca**gaagac**gtCCTTGGGGAAGAAGGAGAAC
$His^{374} \rightarrow Ser$	aaa**g*GATC*C**TCCCTCTGCATGATCCC	gga**g*GATC*C**TTTGCCGCATTGGAGCGTCC
$His^{374} \rightarrow Lys$	ggc**aAATTT**TCCTCCCTCTGCATGATCCC	agga**aAATTT**GCCGCATTGGAGCGTCC
$His^{374} \rightarrow Asn$	ggc**aAATTT**CCTCCCTCTGCATGATCCC	agg**aAATTT**TGCCGCATTGGAGCGTCC

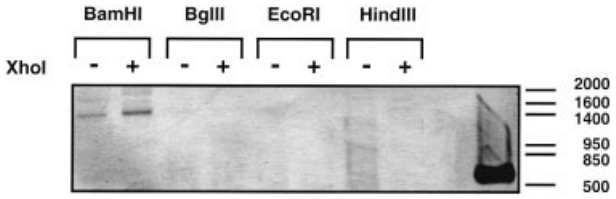


Figure 2 Southern blot of the inverse PCR products

The inverse PCR on the *A. niger* genomic DNA and subsequent Southern-blot analysis were carried out as described in the Experimental section. Each lane contained 5 μl of PCR sample. The restriction enzyme used for the initial fragmentation of the genomic DNA (step 1 in Scheme 1) is given above the respective lanes. Below this label, the use of *Xho*I restriction (+) in step 3, before amplification, is indicated. In the extreme right lane, 1 ng of genomic fragment obtained previously [28] served as a positive control for the hybridization. The positions of molecular-mass markers are indicated on the right. As is evident from the blot, a signal for the amplification of a specific fragment was obtained when *Bam*HI was used as the initial excision enzyme. Restriction of the template with *Xho*I before the PCR step moderately increased the amplification efficiency. No significant amplification products were obtained on using *Bgl*II, *Eco*RI or *Hin*dIII as the initial excision enzyme.

amplified sequence obviously lacked the 3′-end of the *A. niger EH* gene. Therefore a second inverse PCR reaction was performed, using *Cfo*I for the initial restriction of the genomic DNA, which resulted in the generation of a 1-kb amplicon that contained the missing part of the gene. From the sequences of the two PCR fragments, primers were designed to amplify the complete *A. niger EH* gene as one contiguous fragment, using the high-fidelity *Pfu* DNA polymerase. The same set of primers was used to amplify the full length cDNA for the *A. niger* EH after reverse transcription of *A. niger* mRNA. From each amplification, two independent clones were subjected to sequence analysis. No conflict was observed on comparison of the four resulting sequences, indicating that no mutations were introduced into either fragment during preparative PCR. An open reading frame was identified in the cDNA, coding for a polypeptide of 398 amino acids (Figure 3) with a calculated molecular mass of 44.53 kDa and a calculated pI of 5.2, which is in good agreement with the data reported for the biochemical characterization of the native protein [28]. Comparison of the deduced *A. niger* EH amino-acid sequence with those of other known EHs showed

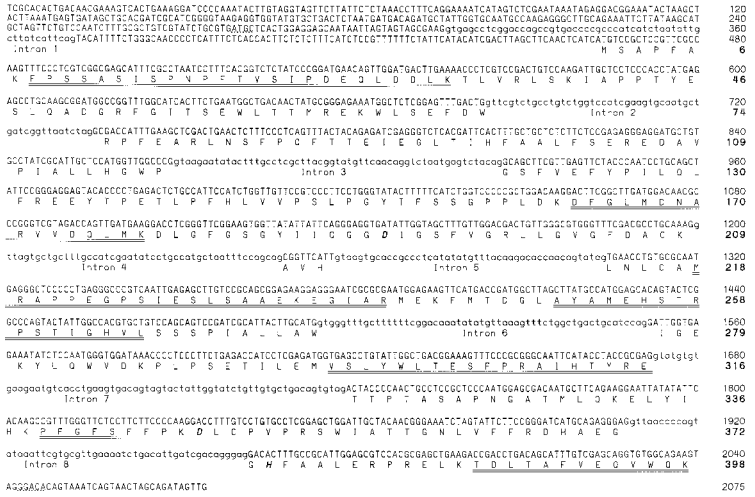


Figure 3 Sequence of the *A. niger EH* gene

The nucleotide sequence of the exons is underlined. Intronic sequences are printed in lower case letters. Below the coding region, the deduced amino-acid sequence is given using the IUPAC single-letter code. The parts of the sequence that were previously determined by Edman degradation [28] are doubly underlined. Residues of the catalytic triad are printed in bold italic letters. The previously isolated genomic fragment [28] extends from positions 1062 to 1664 in the present sequence. Exon/intron structure was obtained by comparison of the genomic sequence with that of the cDNA.

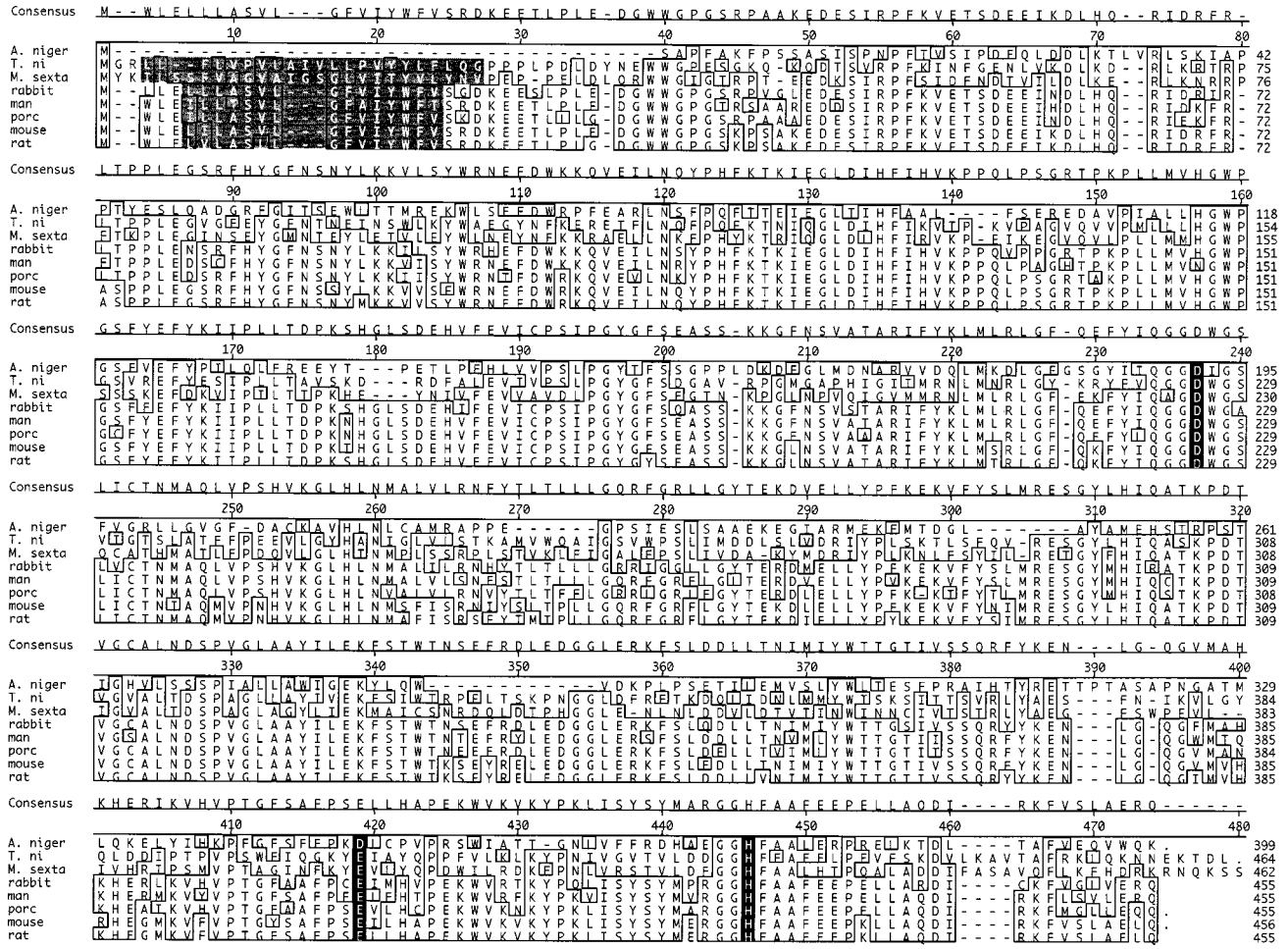


Figure 4 Sequence alignment of the *A. niger* EH with mammalian and insect mEHs

The present alignment was performed using the CLUSTAL V routine [46] of the LaserGene software package (DNASTAR) on a Power Macintosh 8200. All known amino-acid sequences from mammalian mEHs and the two recently analysed mEHs from lepidopterans were simultaneously compared with the EH sequence from *A. niger*. Mammalian mEH were identical in 71% of the residues of the whole sequence. When insect mEHs were included in the alignment, the overall identity was reduced to 26.4%. The *A. niger* EH was identical with all other sequences in 15.5% of the residues and identical to the majority of sequences in 28.1%. Note the absence of the generic N-terminal membrane-insertion signal of mEHs [47], shaded in grey, in the *A. niger* EH sequence. The catalytic residues are highlighted in white-on-black characters. Only the *A. niger* EH has an aspartate residue at the site of the acidic residue of the charge-relay system (position 419 of the consensus); all other sequences have a glutamate residue at this position. The discontinuous α/β hydrolase-fold domain of mEH has been suggested to extend over residues 112–245 and 374–452 of the mammalian mEHs [39], corresponding to the positions 121–256 and 385–471 of the consensus of the present alignment. The amino-acid sequences used for the alignment can be accessed through the respective databases as specified in Table 3.

substantial sequence similarity between the *A. niger* EH and mEH from mammals and insects (Figure 4), with an average sequence identity of approx. 25% with either of these sequences, whereas the similarity to other EHs was much lower (Figure 5). The most obvious difference between the *A. niger* EH and the mEHs is the absence of the N-terminal membrane anchor in the *A. niger* EH, in line with the fact that the native enzyme is found in the soluble fraction of *A. niger* lysates [28]. Like the gene of the mammalian mEH, the *A. niger EH* gene has 9 exons and 8 introns, an unusually high number for an *A. niger* gene (Figure 3). Surprisingly, none of the exon–intron borders is conserved between the *A. niger EH* gene and those from mammals [36,37].

Recombinant expression of *A. niger* EH and analysis of active-site mutants

Recombinant expression of the wild-type and mutant enzyme was achieved using the bacterial expression vector pGEF+,

Table 2 Enzymic properties of the recombinant *A. niger* EH proteins modified by site-directed mutagenesis

b.d., below detection (< 2% of wild-type activity); +, recombinant protein was completely recovered in the 10000 ***g*** supernatant of the bacterial lysate; —, recombinant protein remained in the 10000 ***g*** pellet of the bacterial lysate.

Amino acid position	Mutated residue	Enzymic activity (% of wild-type)	Soluble EH in bacterial lysate
Asp^{192}	Ala	b.d.	+
	Ser	b.d.	+
	Asn	b.d.	+
Asp^{348}	Ala	b.d.	+
	Glu	48	+
His^{374}	Ser	b.d.	—
	Lys	b.d.	+
	Asn	b.d.	+

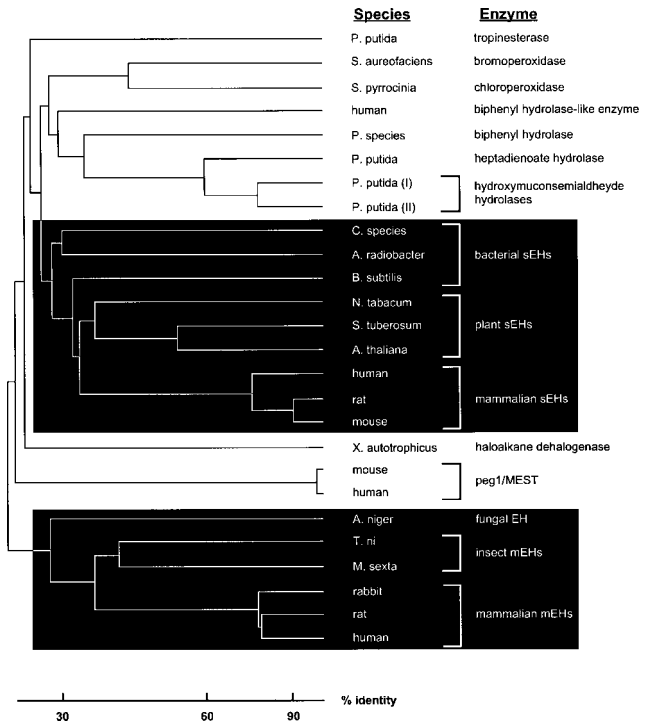


Figure 5 Phylogenetic tree of EH/haloalkane dehalogenase-related α/β hydrolase-fold enzymes

Sequence alignment on a selection of EH-related enzymes was performed on a Power Macintosh 8200 using the Clustal W software package, V 1.6 [48]. The alignment shown displays two clusters of EHs, indicated by the dark boxes. One cluster comprises sEHs from mammals, plants and bacterial EHs. The second cluster contains mEHs from mammals and insects. The soluble EH from *A. niger* is clearly sorted into the second cluster. All sequences above the sEH cluster differ from the rest in that they have a serine residue as the catalytic nucleophile, and are thus esterases or C–C bond hydrolases. Between the two EH clusters are haloalkane dehalogenase and the mammalian peg1/MEST [49], a protein of, as yet, unknown function. The amino-acid sequences used for the alignment can be accessed through the respective databases as specified in Table 3.

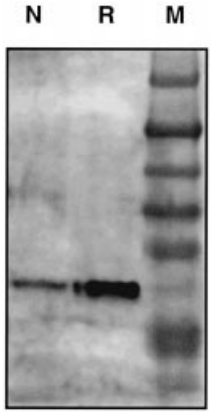


Figure 6 Comparative immunoblot analysis of native and recombinant *A. niger* EH

The supernatant of the crude lysate of *A. niger* grown in liquid culture, corresponding to 10 μg of protein (N) or 50 ng of purified recombinant *A. niger* EH (R) were resolved by SDS/PAGE (10% gel), transferred to nitrocellulose and detected with the rabbit antiserum raised against recombinant *A. niger* EH. No difference in the electrophoretic mobility of the native and recombinant enzyme was observed, indicating that the recombinant *A. niger* EH resembles the native enzyme in size. M, prestained molecular-mass markers.

Table 3 Accession numbers and databases of the amino-acid sequences used for the alignment of *A. niger* EH

Enzyme	Database	Accession number
A. niger EH	EMBL	AJ238460
Trichoplusia ni mEH	SPTREMBL	Q94806
Manduca sexta mEH	SPTREMBL	Q25489
Rabbit mEH	Swiss-Prot	P04068
Human mEH	Swiss-Prot	P07099
Pig mEH	SPTREMBL	P79381
Mouse mEH	SPTREMBL	P97869
Rat mEH	Swiss-Prot	P07687
Tropinesterase	Swiss-Prot	P07383
Bromoperoxidase	Swiss-Prot	P29715
Chloroperoxidase	Swiss-Prot	P25026
Biphenyl hydrolase-like	SPTREMBL	Q13855
Biphenyl hydrolase	Swiss-Prot	P17548
Heptadienoate hydrolase	Swiss-Prot	P23133
Hydroxymuconsemialdehyde hydrolase (I)	Swiss-Prot	P19076
Hydroxymuconsemialdehyde hydrolase (II)	Swiss-Prot	P23106
Corynebacterium species sEH	SPTREMBL	O52866
A. radiobacter sEH	SPTREMBL	O31243
Bacillus subtilis sEH	SPTREMBL	O31581
Nicotiana tabacum sEH	GenBank	U57350
Solanum tuberosum sEH	SPTREMBL	Q41412
Arabidopsis thaliana sEH	SPTREMBL	Q42566
Human sEH	Swiss-Prot	P34913
Rat sEH	Swiss-Prot	P80299
Mouse sEH	Swiss-Prot	P34914
Haloalkane dehalogenase	Swiss-Prot	P22643
Mouse peg1/MEST	SPTREMBL	Q07646
Human peg1/MEST	SPTREMBL	O15007

developed by Janssen and colleagues [33] for the overexpression of bacterial dehalogenase in *E. coli*. The expression yield for the recombinant *A. niger* EH was about 1.5 mg/l of bacterial culture in the strain BL21(DE3) after induction with isopropyl β-D-thiogalactoside, as judged by monitoring of the enzymic activity for the *A. niger* EH in crude bacterial lysates. Antibodies were raised in rabbits against the enzyme after purification to apparent homogeneity. In a subsequent immunoblot analysis using the above antisera the recombinant protein displayed the same electrophoretic mobility as the wild-type EH in lysates from *A. niger* cultures (Figure 6).

By sequence comparison of the *A. niger* EH to mEHs (Figure 4), the potential amino-acid residues of its catalytic triad were identified. Asp^{192} was predicted to be the catalytic nucleophile of the *A. niger* EH, and His^{374} and Asp^{348} evolved as the putative members of the charge-relay system. Respective mutant *A. niger* EH proteins were constructed and produced as described in the Experimental section. Wild-type and mutant recombinant *A. niger* EHs were analysed for their biochemical properties (Table 2). Exchange of either of the predicted members of the catalytic triad of *A. niger* EH with a non-functional amino-acid residue led to a complete loss of enzymic activity, in line with the essential function of the exchanged residues. The only mutant with retained catalytic activity resulted from a substitution of Asp^{348} with a glutamate residue. This exchange reduced the V_{max} of the enzyme by half and at the same time led to an even greater decrease in K_m (Figure 7). The initially inactive $Asp^{192} \rightarrow Asn$ mutant regained enzymic activity after extended storage at 37 °C ($t_{1/2} = 60$ h).

The substitution of His^{374} with serine resulted in the formation of an insoluble expression product, indicated by the inability of

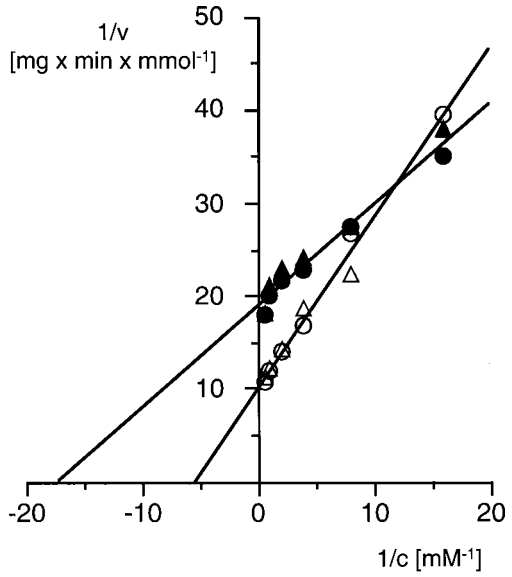


Figure 7 Comparative kinetic analysis of the Asp^{348} → Glu mutant and wild-type *A. niger* EH

Lineweaver–Burk plots of substrate dependence of the catalytic activity of the wild-type *A. niger* EH (△, clone 1; ○, clone 2) and the Asp^{348} → Glu mutant (▲, clone 1; ●, clone 2), using 4-nitrostyrene epoxide as the substrate. Two independent clones were tested for each protein and the results represent the average of three separate measurements. The V_{max} was 98 μmol/min per mg of wild-type enzyme and 53 μmol/min per mg of mutant *A. niger* EH; the K_m was 179 μM for the wild-type enzyme and 57 μM for the mutant EH.

the recombinant protein to fold correctly under our expression conditions. We therefore constructed two additional mutants by replacing His^{374} in the recombinant *A. niger* EH with either lysine or asparagine. Both mutant enzymes were soluble after expression in *E. coli*, but were enzymically inactive.

In an attempt to turn the EH into an esterase, we introduced a serine residue in place of the catalytic nucleophile Asp^{192}. However, no significant hydrolysis of either 4-nitrophenyl acetate or 4-nitrophenyl stearate was observed with the mutant enzyme.

DISCUSSION

In the present paper, we describe the isolation of the *A. niger EH* gene and cDNA by inverse PCR. This technique proved to be very useful and efficient for our purpose. Because of its speed and technical simplicity, inverse PCR is a powerful alternative to conventional library screening, especially when those libraries are not readily available, and is particularly useful in the case of genomes of low complexity.

The structure of the *A. niger EH* gene is unexpectedly complex, in view of the simplicity of the organism of origin. The average size of the identified introns is about 60 bp, and thus in good agreement with those found in many other *A. niger* genes, but the number of introns in the *A. niger* gene, 8 in total, is unusually high. None of the corresponding exon–intron borders is conserved between the fungal EH and its mammalian relatives, despite the identical number of introns in these genes. The fungal and the mammalian gene both have a non-coding first exon. In the rat, the existence of at least three first exons has been reported [38]. Here, the non-coding first exon allows the use of different promoters for the synthesis of identical proteins. It remains to be established if a similar function for the non-coding first exon is true for the *A. niger EH* gene.

Within the large superfamily of EH/haloalkane dehalogenase-like α/β hydrolase-fold enzymes, amino acid sequence comparison clearly orders the *A. niger* EH into the family of mEHs (Figure 5). Despite the fact that it shares only about 25% sequence identity with the other members of this family, we would propose to assign the systematic name, HYL1, which was established earlier for the mammalian mEHs [39], since the comparatively low degree of sequence conservation can be explained by the phylogenetic distance between animals and fungi. There are, however, two unique features of the *A. niger* EH when compared with other members of this family. First, it is a soluble enzyme lacking the otherwise common membrane anchor of mEHs. Secondly, it has an aspartic acid as the acidic residue of the charge-relay system within its catalytic triad, whereas all other HYL1 enzymes hitherto have a glutamic acid in this position (Figure 4). We have shown previously [22], that replacing the respective glutamic acid in the rat mEH with aspartic acid increases the hydrolytic step of the enzymic reaction by more than an order of magnitude. It is therefore an intriguing idea that the particularly high turnover number of the *A. niger* EH when compared with those of its mammalian relatives is based on this difference in the charge-relay system.

On first sight, this is not strongly supported by our present results, since substitution of the catalytic Asp^{348} in the *A. niger* EH with glutamic acid led only to a moderate drop in V_{max}, by a factor of 2 (Figure 7). However, at the same time, the K_m was reduced more significantly, by a factor of 3. A possible, although, in view of the present data, still somewhat speculative explanation for this observation could be a switch in the rate-limiting step of the enzymic reaction. The course of the reaction can be represented by the simplified scheme where free enzyme (E) and substrate (S) initially are in equilibrium with a respective Michaelis–Menten complex (ES), which is determined by the equilibrium constant (K).

$$E + S \overset{K}{\leftrightarrow} ES \overset{k_1}{\rightarrow} E \cdot S \overset{k_2}{\rightarrow} E + P$$

Important kinetic parameters of the reaction are the rate constant k_1 for the formation of the covalent intermediate (E·S) and k_2 for the hydrolytic step, finally leading to free enzyme and formation of the product (P). In mammalian mEH and sEH, the second, i.e. the hydrolytic, step of the enzymic reaction appears to be rate limiting. Under such conditions, when the rate constant k_1 for the intermediate ester formation is much larger than the rate constant k_2 for the hydrolytic step, reduction in V_{max} due to a reduced k_2 is paralleled by a similar reduction in K_m, because $K_m = Kk_2/(k_1+k_2)$ [40]. If, however, initially k_1 is rate limiting and k_2 is much larger, the expression $k_2/(k_1+k_2)$ approximates 1 and K_m is thus equal to K. Under such conditions, a reduction of V_{max} by half, because of modulation of the charge-relay system, i.e. the part of the catalytic triad important for the second step of the enzymic reaction, is likely to be due to a decrease in k_2 to half of the value of k_1. In consequence, the term $k_2/(k_1+k_2)$ would now approximate 1/3, i.e. exactly the value observed with the Asp^{348} → Glu mutant of the *A. niger* EH, in form of the reduced K_m. Thus our data are compatible with concept that, in the case of *A. niger* EH with 4-nitrostyrene oxide as the substrate, k_2 is significantly, and possibly by orders of magnitude, higher than k_1, a situation that is abolished by substitution of Asp^{348} with glutamic acid. This is in contrast to the results obtained with other EHs until now, where k_2 has been shown to be rate limiting [41,42].

The observation that mammalian EHs are apparently optimized for a rapid first step in the enzymic hydrolysis of epoxide well suits their function as detoxifying enzymes, since the

genotoxic agent is rapidly inactivated by trapping it in the form of the ester intermediate. A high rate in the second step of the enzymic reaction is important only in cases of significant excess of substrate, and sometimes can even be hazardous, since the generated diols may represent precursors of carcinogens, as is the case in the metabolic formation of diol epoxides from polycyclic aromatic compounds [43], or may themselves mediate toxic effects [44]. In contrast, the *A. niger* EH seems to be optimized for rapid product formation, which suggests a catabolic role in the fungus. This property, together with its high enantioselectivity, makes it an especially interesting tool for enantioselective bio-organic synthesis of fine chemicals and pharmaceutic drugs [45].

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