Volume 311, number 3, 206–208 FEBS 11657 October 1992

Isolation, sequencing and expression in *E. coli* of the urocanase gene from white clover (*Trifolium repens*)

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Received 31 August 1992

The urocanase gene was detected in a clone obtained from a genomic library of white clover. The entire gene has been sequenced and expressed in the pT7-7/*E. coli* BL 21 (DE 3) system. The deduced sequence of the plant urocanase is 72% homologous with that of the well-characterized urocanase from *Pseudomonas putida*. The purification procedure, as well as kinetic and electrophoretic behaviour, of the new enzyme are described.

Plant urocanase gene; Sequencing; Expression; Enzyme purification

1. INTRODUCTION

Urocanase (4′-imidazolone-5′-propionate hydrolyase, EC 4.2.1.49) catalyzes an unusual hydration of urocanate, representing the second step in histidine degradation. The enzyme has been isolated and characterized from different prokaryotes, as well as from mammalian and chicken liver [1–6]. The best examined urocanase is the *Pseudomonas putida* urocanase which contains a tightly bound NAD^+ in each subunit of the homodimer (M_r 122,000). The urocanase gene from *P. putida* has been sequenced [7]. A computer-aided search for homologous sequences revealed about 80% homology between this gene and a 370-bp region upstream from the gene encoding the small subunit of white clover ribulose bisphosphate carboxylase [8]. In a corresponding clone we discovered an open reading frame of 1,695 bp that showed 69% homology with the urocanase gene from *P. putida*. Expression of this putative urocanase gene from a plant in *E. coli* yielded a homodimeric product with high urocanase activity.

2. MATERIALS AND METHODS

2.1. *Sequencing*

A 3.5 kbp fragment of *Trifolium repens* genomic DNA in the plasmid pGEM-3Z (containing the upstream region of the rubisco small subunit gene), that had been subcloned from a 15 kbp clone isolated from a λ-EMBL 3 genomic library, was generously provided by Dr. N.W. Ellison [8]. Digestion with *Bam*HI yielded a 1.7 kbp fragment which was cloned into M13 mp18. Both possible orientations were obtained so that the fragment could be sequenced from both ends. Sequenase 2.0 standard protocols for dGTP and dITP reactions were followed. Another 97 bp fragment lying between adjacent *Bam*HI sites was sequenced directly after alkaline denaturation of double-stranded DNA. Specific primers were supplied by Dr. R. Frank, Center for Molecular Biology, University of Heidelberg, Germany.

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2.2. *Expression in* E. coli

The 3.5 kbp fragment was cut out from the recombinant plasmid by digestion with *Eco*RI/*Sal*I and inserted into M13 mp19. Single-stranded DNA was used to introduce an *Nde*I site at the start codon by oligonucleotide-directed in vitro mutagenesis [9] (Amersham kit version 2.1). The complete coding region of the urocanase gene, as well as 249 bp of the 3′ non-coding region, were subsequently cloned into the expression vector pT7-7 [10]. *E. coli* BL 21 (DE 3) [11] was transformed with the recombinant vector. The host cells were cultured in an LB medium containing 50 mg/l ampicillin.

2.3. *Purification and assay of urocanase*

The purification procedure followed a protocol established for *P. putida* urocanase over-expressed in the same system (M. Lenz, unpublished). Host cells were collected by centrifugation and resuspended in TE (10 mM Tris-Cl, 1 mM EDTA, pH 7.5) containing 30–40 IU of Benzonase. Sonification (Branson Sonifier, on ice for 5 min at maximum power) was followed by ultra-centrifugation at 344,000 × *g* for 105 min at 15°C.

The filtered supernatant (Minisart N 0.2 μm) was applied to a Hi Load 26/60 superdex 200 prep. grade column and fractionated with an FPLC (Pharmacia-LKB) system (150 mM potassium phosphate buffer, pH 7.2, was used for elution). The final purification was performed with the same system using a TSK DEAE-3SW (21.5 × 150 mm) Ultrapac column as previously described [12].

The urocanase assay was carried out according to [13]; K_m was determined using a double-reciprocal plot. The spectrophotometric protein determination followed a method adapted to the urocanase from *P. putida* [12].

3. RESULTS AND DISCUSSION

A search for sequences homologous to the urocanase gene from *P. putida* [7] revealed that Ellison et al. [8] had, without realising it, published the first 411 bp, including the start codon, of a potential urocanase gene from a plant. It was detected in its reverse-complementary form upstream from the white clover rubisco gene.

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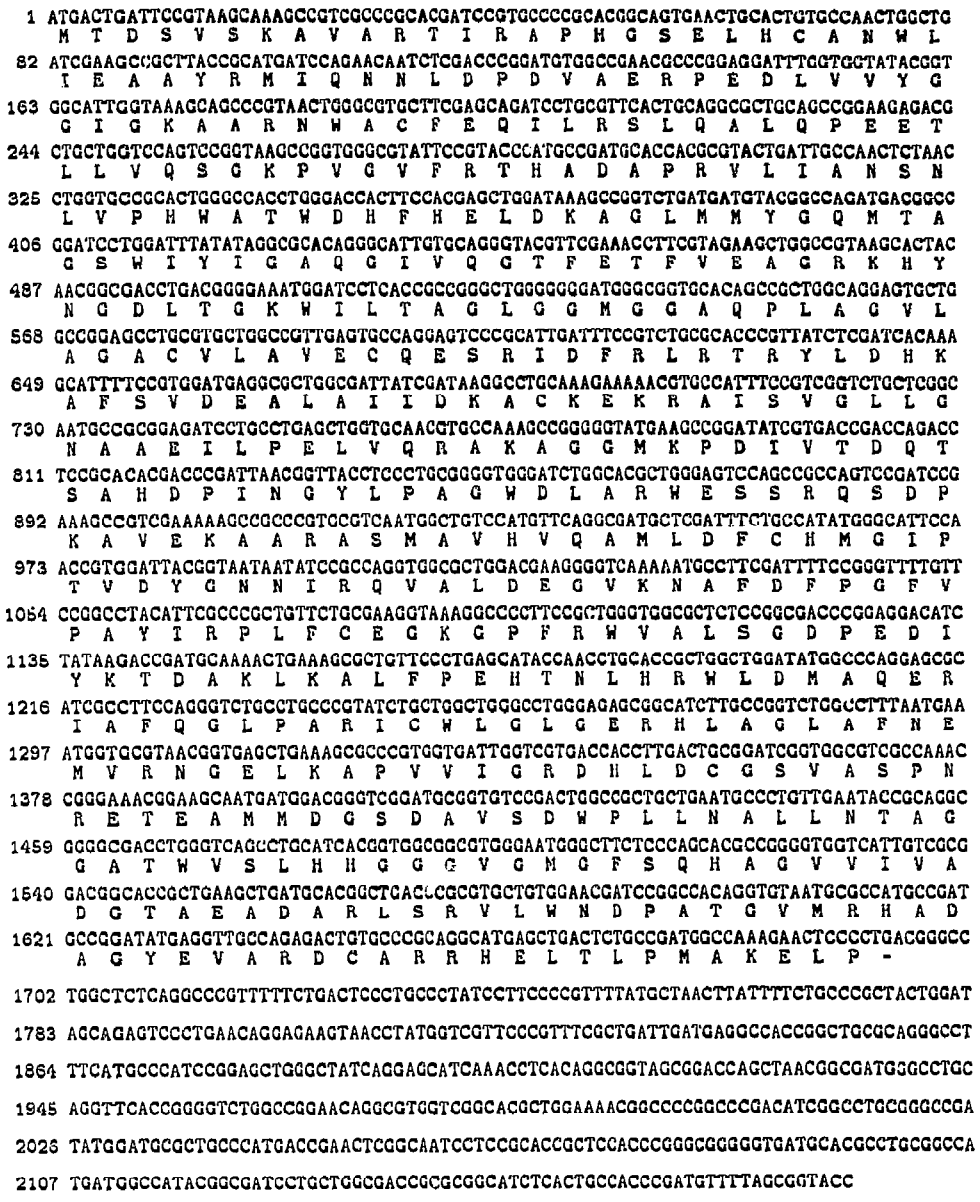

Fig. 1. Base sequence of the urocanase gene from *Trifolium repens* (including the deduced amino acid sequence) and the 3′ non-coding region. Bases 1–411 have already been published by N.W. Ellison et al. [8].

This was the starting point for our sequence analysis that resulted in finding an open reading frame consisting of 1,695 bp. The complete sequence of the gene together with a non-coding region of 481 bp at the 3′ flank is shown in Fig. 1. It is noteworthy that no introns were found. The discovered gene shows 69% homology with the urocanase gene of *P. putida* [7]. The deduced amino acid sequences have over 72% identity. Possible TATA- and CAAT boxes were found 104-bp and 322-bp upstream, respectively (see [8]).

Expression of the new gene in the pT7-7/*E. coli* BL 21 (DE 3) system yielded about 125 IU of urocanase per 500 ml overnight culture (approximately 4 g wet cell paste) without induction by IPTG. Urocanase activity in the crude extract was, however, unstable; after three purification steps (non-optimized) only 6% of the total activity was recovered. On the TSK DEAE-3SW Ultrapac column at least three different forms of urocanase were partially separated resembling the pattern described for the urocanase from *P. putida* [12]. The K_m was determined to be 20 μM, the highest specific activity was 1.6 U/mg (fresh A form [12]). The specific activity may have been reduced due to contamination by some apoenzyme since SDS electrophoresis showed a tight

doublet of bands in the range of M_r 61,000. Addition of NAD^+ to the culture media or to the crude extract may reduce the amount of apoenzyme, as in the case of over-expressed *P. putida* urocanase (M. Lenz and J. Rétey, unpublished).

Gel filtration of the over-expressed plant enzyme showed nearly the same elution volume as urocanase from *P. putida* (M_r of the homodimer ≈122,000 [7]). Therefore the urocanase from *T. repens* also consists of two identical subunits. Its molecular mass was calculated from the deduced amino acid sequence to be 61,401 Da for one polypeptide chain of the homo dimeric protein.

Although it is almost certain that we have detected the first genuine urocanase gene of a plant, we are planning to screen corresponding cDNA libraries with appropriate oligomers in order to prove its transcription in vivo.

Acknowledgements: Special thanks are due to Dr. N.W. Ellison for providing us with subclonal material and additional information. We thank Dr. S. Tabor for generously sending us his pT7-7 expression vector system together with relevant information material. The work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. A.K. thanks the Land Baden-Württemberg for a postgraduate scholarship.

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