Vol. 161, No. 2, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 15, 1989 Pages 865-872

# PORCINE D-AMINO ACID OXIDASE : PRODUCTION OF THE BIOLOGICALLY ACTIVE ENZYME IN *Escherichia coli*

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Received May 2, 1989

DNA molecules coding either for mature porcine D-amino acid oxidase or for truncated forms of the enzyme have been obtained by stepwise addition of synthetic oligonucleotides to a partial cDNA. Under the control of the $\lambda$ $P_L$ thermoregulatable promoter, these DNAs were respectively expressed in *Escherichia coli* as 36, 28 and 25 kilodalton polypeptides, specifically recognised by antibodies raised against the natural enzyme. None of the truncated proteins were biologically active whereas the mature recombinant species was able to hydrolyze D-alanine *in vitro* as efficiently as the natural product. 

D-amino acid oxidase (DAO, EC1.4.3.3), a peroxisomal flavoenzyme, catalyses the oxidative deamination of D-amino acids (1). It occurs as a multimer in various mammalian tissues and most abundantly in liver and kidney (2). The basic subunit of the enzyme consists of a single polypeptide containing 347 amino acid residues (3).

Relatively few data are available concerning the physiological significance of DAO. Some evidence suggest that it participates to the metabolism of D-amino acids arising from the breakdown of intestinal bacteria and other informations point to the role of DAO as an effector in nervous tissue metabolism (4,5).

Several attempts have been made to clarify the role of DAO at the molecular level. Bacterial clones carrying the cDNA for DAO have been isolated and characterized (6,7,8); in addition, catalytic properties of the enzyme were studied using site-specific mutagenesis of the cDNA coding for DAO (9).

We report here the construction and expression in *Escherichia coli* of semi-synthetic DNAs encoding partial or complete DAO. It was found that none of the truncated products were biologically active whereas the mature recombinant protein had a catalytic efficiency comparable to that one of the natural enzyme.

0006-291X/89 $1.50

## MATERIALS AND METHODS

General
Restriction endonucleases, T4 DNA polymerase, *Escherichia coli* DNA polymerase I (Klenow fragment), T4 DNA ligase and T4 DNA polynucleotide kinase were purchased from Boehringer-Mannheim, Amersham or New England Biolab's and used as recommended by the manufacturers.
Oligonucleotides were synthesized by the phosphoramidite method with an Applied Biosystems model 380A DNA synthesizer (10). DNA fragments were cloned into M13 mp18 vector (11) and sequenced by the dideoxy chain termination method (12). Analysis of the DNA sequences was aided by the SASIP programs (13). Protein concentrations were measured according to standard protocols (14).

Strains, plasmids and media
*Escherichia coli* strain AR58, a cryptic lysogen [(galE:Tn10, Δ-8(chlD-pgl) Δ-H1(cro-chlA),N$^+$, cI857)], was used for bacterial transformations (15). Plasmids pAS1 (16), pOTS-Nco (17), pULB9301 (6) and pULB1221, a derivative of pULB1319 (18) have been described previously. LB medium (Luria broth) for *Escherichia coli* growth was used with the addition of 50 μg/ml ampicillin for the selection of plasmid transformants.

Preparation of bacterial extracts
Extracts for immunodetection and for the activity assay of DAO were prepared as described before (19). Samples were enriched in DAO by precipitation with ammonium sulfate. Precipitates were centrifuged (10 min. at 4000 rpm; Sorvall SS.34) and resuspended in 1 ml of extraction buffer (Tris-HCl 50 mM pH8). Enriched fractions were immediately assayed or kept frozen at -20°C until used.

Immunoblotting and ELISA
The experiments were performed essentially as described before (19,20). Reagents for immunoblotting consisted of a DAO specific rabbit antiserum (dilution 1/3000), a goat anti rabbit immunoglobulin preparation (Amersham, dilution 1/500) and the chromogenic substrate diaminobenzidine. The quantification of DAO in enriched fractions was performed by ELISA (20). In short, immunoplates I (Nunc) were coated with rabbit anti-DAO immunoglobulins, washed, saturated with bovine serum albumin and then incubated either with a DAO standard (Boehringer-Mannheim) or with the appropriate samples diluted serially. Thereafter, mouse anti DAO serum was added and immune complexes were detected by the addition of peroxidase-labeled rabbit anti mouse immunoglobulins and the chromogenic substrate orthophenylene diamine. Absorbance was read at 490 nm in a Dynatech AM120 microelisa automatic reader.

Assay for DAO activity
The assay measures the capacity of DAO to hydrolyse D-alanine into pyruvic acid with concomittant release of water peroxide. The amount of peroxide is determined using peroxidase and O-dianisidine as a dye.
The assay mixture contained 200 μl of D-alanine (220 mM), 100 μl of horseradish peroxidase (grade I, Boehringer; 10 u/ml), 100 μl of O-dianisidine (Sigma, 10 mg/ml), 200 μl of Tris-HCl pH 8 (200 mM), 10 μl of Flavin adenine dinucleotide (FAD; United States Biochemicals, 0.5 μg/ml) and 200 μl of the sample to be assayed. The reaction took place for 15 minutes at 25°C and was blocked with 50 μl of 50% sulfuric acid. Increases in absorbance were read at 520 nm.
Standard porcine DAO (Boehringer-Mannheim) had a specific activity of 15 u/mg (at 25°C , using D-alanine as substrate).

## RESULTS

(a) Construction of plasmid pNIV1802

Plasmid pULB9301, which has been described recently (6), was cleaved with the restriction enzymes *Ava*II and *Pvu*II. This generated a fragment coding for amino acids 104 to 347 of DAO and including the stop codon. The fragment was treated with T4 DNA polymerase to flush protruding ends, then introduced by ligation into the blunted *Nco*I site of the expression vector pOTS-Nco. The resulting recombinant plasmid, pNIV1802, carried the sequence coding for aa Asp104 to Leu347 of DAO in frame downstream to an ATG initiation codon (Fig.2).

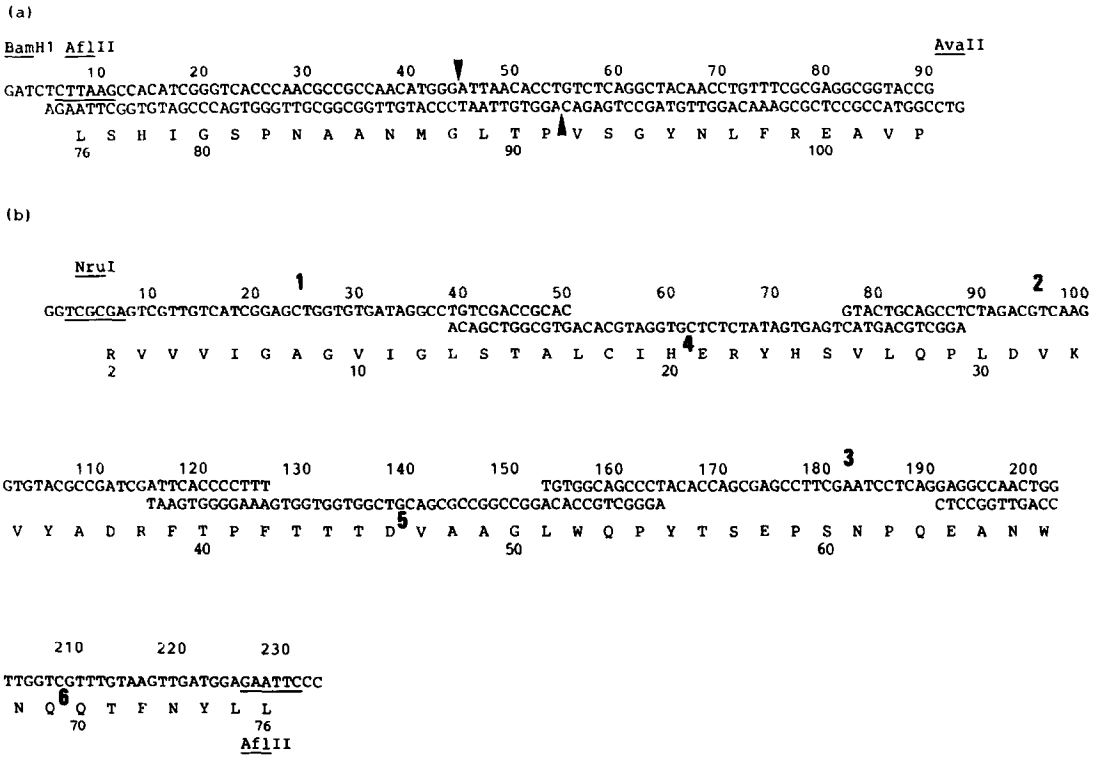


Figure 1 : DNA sequence of synthetic oligonucleotides used to construct plasmids pNIV1804 and pNIV1805.
a) sequence of the four oligonucleotides coding for amino acids Leu76 to Pro103 of DAO. When assembled, these synthetic DNAs extend for 90 bp and are flanked 5' by a *Bam*H1 protruding end and 3' by an *Ava*II site. The 5' proximal *Afl*II site used later on is underlined. The deduced amino acid sequence is shown below in the single letter code and residues are numbered. Arrows point to the junction of the two sets of oligonucleotides.
b) sequence of the six oligonucleotides coding for amino acids Arg2 to Leu76 of DAO. When assembled, these synthetic DNAs extend for 233 bp. *Nru*I and *Afl*II sites used to reconstruct plasmid pNIV1805 are indicated. The deduced amino acid sequence is shown below in the single letter code and residues are numbered. Bold numbers, 1 to 6, identify each oligonucleotide.

(b) Construction of plasmid pNIV1804

A 90 bp synthetic DNA fragment, flanked by the restriction sites *Bam*H1 and *Ava*II, was assembled by pairwise annealing of oligomers then ligated to the promoter of vector pAS1 and to the 5' end of the *Ava*II-*Pvu*II fragment derived from plasmid pULB9301. Oligonucleotides were designed to present a unique 5' internal *Afl*II restriction site which can be used for further extension. (Fig.1a). The resulting recombinant plasmid, pNIV1804, carried the sequence encoding aa Leu76 to Leu 347 of DAO, in frame downstream to an ATG initiation codon (Fig.2).

(c) Construction of plasmid pNIV1805

Six synthetic oligonucleotides, flanked by the restriction sites *Nru*I and *Afl*II, were prepared in order to reconstruct the sequence coding for mature DAO (Fig.1b). The fragments were annealed pairwise and cloned

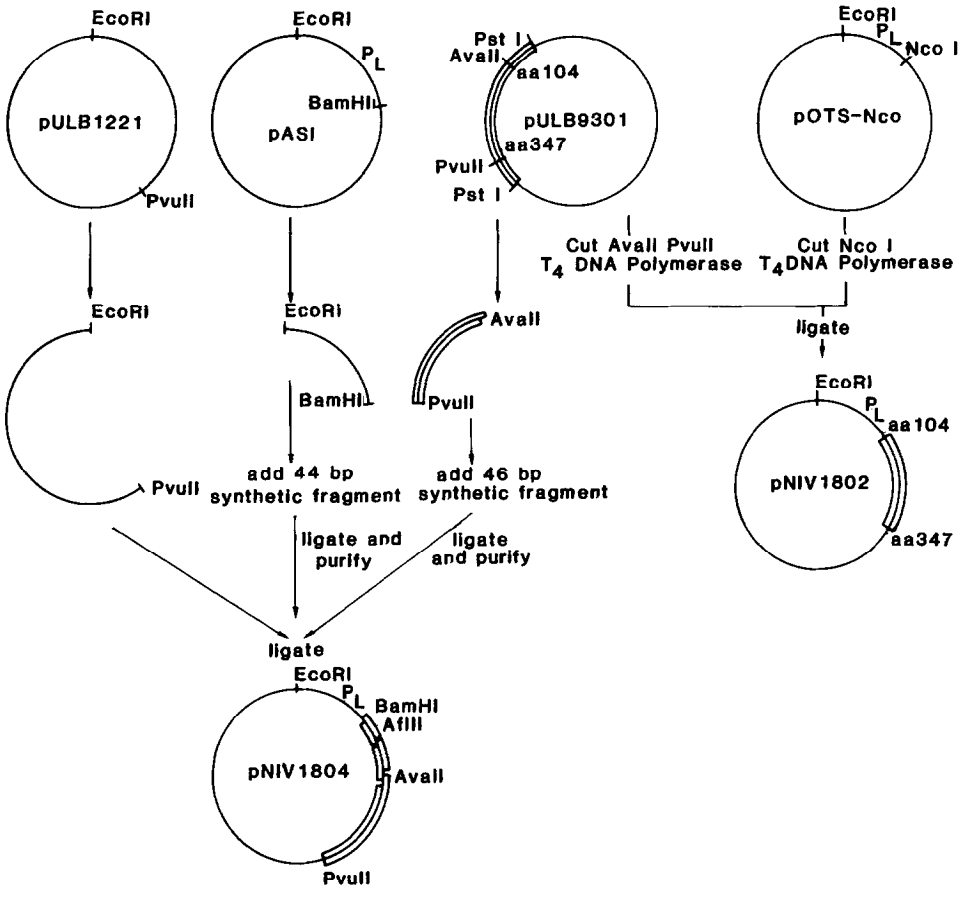


Figure 2 : Construction of plasmids pNIV1802 and pNIV1804.
pNIV1802 results from the introduction of the cDNA encoding amino acids 104 to 347 of DAO into the expression plasmid pOTS-Nco.
pNIV1804 derives from the addition of a 90 bp synthetic DNA encoding amino acids 76 to 103 of DAO (see Fig.1a) to the cDNA fragment coding for amino acids 104 to 347 present in pULB9301.
Details on the construction procedures are given in the text.

blunt-ended into the DNA of phage M13 mp18. Clones which proved correct by sequencing were recombined to generate a *NruI-AflII* DNA fragment specifying amino acid residues 2 to 76 of DAO. This piece of DNA was isolated and cloned together with the *AflII-PvuII* fragment derived from plasmid pNIV1804 into the expression vector pOTS-Nco cleaved with *NcoI* and blunted. The resulting recombinant plasmid, pNIV1805, carried the sequence coding for amino acids Met1 to Leu347 of DAO, downstream to the $P_L$ promoter (Fig. 3).

(d) Expression of DAO in bacteria

*Escherichia coli* AR58 cells transformed either with the control plasmids or with the recombinant ones were grown at 30°C then quickly heated at 42°C in order to inactivate the λ repressor and allow induction. Total proteins were separated on 15% NaDodSO4 polyacrylamide gels, transferred

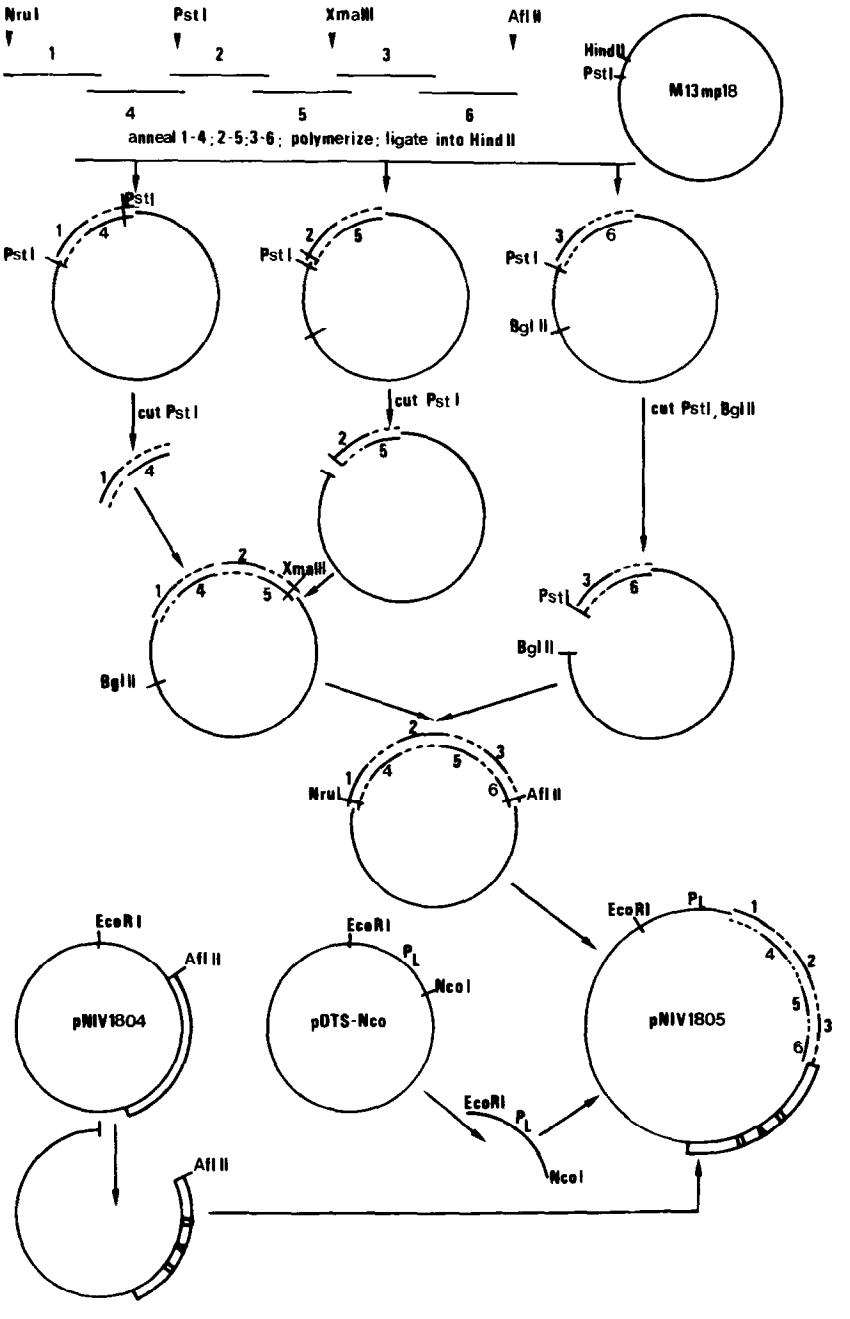


Figure 3 : Construction of plasmid pNIV1805.
Pairs of oligonucleotides (see Fig. 1b) were cloned separately into the *Hind*II site of M13mp18 DNA. Fragment 1/4 was excised by *Pst*I digestion from the relevant M13mp18 recombinant DNA and inserted between the *Pst*I sites of the recombinant M13mp18 carrying the fragment 2/5. The resulting molecule was then digested with *Bgl*II and *Xma*III (blunt) and the appropriate fragment containing the assembled 1/4 and 2/5 pieces was cloned into the *Bgl*II and *Pst*I (blunt) sites of the M13mp18 DNA carrying the fragment 3/6. The final M13 mp18 molecule thus carries the sequence coding for amino acids 2 to 76 of DAO flanked by restriction sites *Nru*I and *Afl*II. This piece of DNA was then excised and assembled together with the *Eco*RI-*Nco*I(blunt) fragment originating from pOTS-Nco and the *Eco*RI-*Afl*II piece derived from pNIV1804 to produce the plasmid pNIV1805. In this last construct, the sequence coding for amino acids Met1 to Leu347 of DAO is placed downstream to the $P_L$ promoter. Relevant restriction sites used in the reconstruction process are indicated. Dotted lines show the enzymatically polymerized sequences.

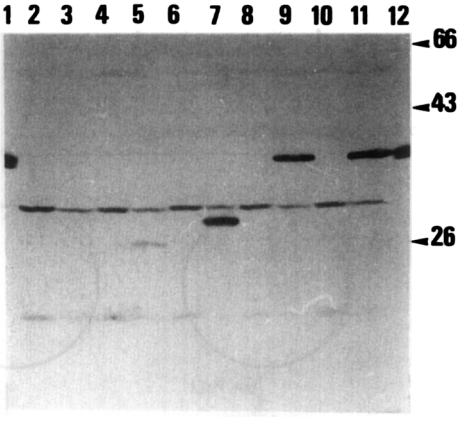


Figure 4 : Immunological characterization of DAO produced in *Escherichia coli*.
Total bacterial proteins were resolved by NaDodSO4-polyacrylamide gel electrophoresis, then transferred onto nitrocellulose sheets and reacted with a DAO-specific rabbit antiserum. Immune complexes were detected with peroxidase-labelled goat anti rabbit serum and the appropriate chromogenic substrate (see Methods).
Lanes 1 and 12, standard porcine DAO
Lanes 2 and 3, control strain NIV158 (pAS1/AR58) grown at 30° and 42°C respectively.
Lanes 4 and 5, recombinant strain NIV60 (pNIV1802/AR58) grown at 30°C and 42°C respectively.
Lanes 6 and 7, recombinant strain NIV82 (pNIV1804/AR58) grown at 30°C and 42°C respectively.
Lanes 8,10 and 9,11, recombinant strain NIV162 (pNIV1805/AR58) grown at 30°C and 42°C respectively.
Molecular weight markers (in kilodalton) are indicated. Denominations of the plasmid and recipient strain, in each case, are given in parentheses.

onto nitrocellulose sheets and detected by antibodies raised against natural DAO. Figure 4 shows that pNIV1802, pNIV1804 and pNIV1805 direct the synthesis of a single product reactive with the antibody against DAO. Apparent molecular weight were respectively 25, 28 and 36 kDa. The product synthesized by cells harboring pNIV1805, as expected, migrated at the same position as the standard DAO species.

(e) Quantification and biological activity of the DAO products synthesized in *Escherichia coli*.

Fractions enriched in DAO by ammonium sulfate precipitation (20 to 40% saturation) were assayed for specific immunoreactivity in the ELISA system, and for specific biological activity in terms of hydrolysis of the D-alanine substrate. Protein concentrations were measured on the crude extracts before ammonium sulfate precipitation. As seen in Table I, plasmid pNIV1805 directs the synthesis of respectively 4 and 25 fold more immunoreactive material than plasmids pNIV1804 and pNIV1802. The products coded for by these two plasmids were totally inactive whereas the mature recombinant DAO, synthesized in bacteria harboring plasmid pNIV1805, was capable to hydrolyse D-alanine with a specific activity equivalent to that one of standard DAO.

**Table I**: Quantification and activity of DAO synthesized in *Escherichia coli*

| Strain | Plasmid | Total proteins (a) (mg/ml) | DAO (b) (µg/ml) | Expression level (%) | Activity (u/ml) | Apparent specific activity (u/mg) |
|---|---|---|---|---|---|---|
| NIV162 | pNIV1805 | 8.1 | 130 | 1.60 | 2.2 | 15.5 |
| NIV82 | pNIV1804 | 7.1 | 30 | 0.41 | < 0.05 | nd |
| NIV60 | pNIV1802 | 7.0 | 5 | 0.07 | < 0.05 | nd |
| NIV158 | pAS1 | nd | < 0.1 | nd | < 0.05 | nd |
| standard DAO (c) | | | 200 | | 3 | 15 |

All plasmids were introduced into the recipient strain *E.coli* AR58
nd : not done
(a) as determined by the Folin reaction (14).
(b) as determined by ELISA.
(c) the specific activity of standard DAO is based on the manufacturer's specifications (Boehringer-Mannheim).

## CONCLUSION

Three forms of D-amino acid oxidase have been produced in *Escherichia coli*. Truncated species were biologically inactive whereas the mature form of the enzyme had the capacity to hydrolyse the D-alanine substrate as efficiently as the material derived from pig kidney. In this respect, engineered bacteria producing DAO may constitute an alternative source of active enzyme for industrial applications, such as the conversion of D-amino acids into α-keto acids or for the elimination of D-amino acids from synthetic racemates (21,22).

ACKNOWLEGDMENTS

Part of this work was carried out under a contract between UCB-Bioproducts, the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (IRSIA) and the University of Brussels. We thank Dr. Degelaen (UCB-Bioproducts) for continuous support of the project.

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