ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 2, pp. 137-144. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 2, pp. 172-180.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-148, November 9, 2008.

Molecular Cloning and Expression in *Escherichia coli* of an Active Fused *Zea mays* L. D-Amino Acid Oxidase

A. Gholizadeh[1]* and B. B. Kohnehrouz[2]

[1]*Research Institute for Fundamental Sciences (RIFS), University of Tabriz, Tabriz, Iran; fax: (98-411) 336-3137; E-mail: aghz_bioch@yahoo.co.in*
[2]*Department of Plant Breeding and Biotechnology, University of Tabriz, Tabriz, Iran*

Received April 23, 2008

Abstract—D-Amino acid oxidase (DAAO) is an FAD-dependent enzyme that metabolizes D-amino acids in microbes and animals. However, such ability has not been identified in plants so far. We predicted a complete DAAO coding sequence consisting of 1158 bp and encoding a protein of 386 amino acids. We cloned this sequence from the leaf cDNA population of maize plants that could utilize D-alanine as a nitrogen source and grow normally on media containing D-Ala at the concentrations of 100 and 1000 ppm. For more understanding of DAAO ability in maize plant, we produced a recombinant plasmid by the insertion of isolated cDNA into the pMALc2X *Escherichia coli* expression vector, downstream of the maltose-binding protein coding sequence. The pMALc2X-DAAO vector was used to transform the TB1 strain of *E. coli* cells. Under normal growth conditions, fused DAAO (with molecular weight of about 78 kDa) was expressed up to 5 mg/liter of bacterial cells. The expressed product was purified by affinity chromatography and subjected to *in vitro* DAAO activity assay in the presence of five different D-amino acids. Fused DAAO could oxidize D-alanine and D-aspartate, but not D-leucine, D-isoleucine, and D-serine. The cDNA sequence reported in this paper has been submitted to EMBL databases under accession number AM407717.

DOI: 10.1134/S0006297909020035

Key words: D-amino acid oxidase, D-amino acids, *Zea mays*, flavoenzyme, overexpression

D-Amino acid oxidase (DAAO; EC 1.4.3.3) is a flavoenzyme that catalyzes the oxidative deamination of D-amino acids to the corresponding α-keto acids, ammonia, and hydrogen peroxide [1]. It is one of the most studied enzymes discovered more than 60 years ago in pig kidney [2, 3]. Since that time, proteins and coding sequences for DAAOs have been variously reported from different tissues and organs in human and animals [4-10]. DAAO genes have also been identified from different microorganisms including alga, fungi, and bacteria [11-16].

The physiological and biological roles of the enzyme D-amino acid oxidase are diverse and not fully clarified. So far, it has been known to play a role in utilizing exogenous D-amino acids as carbon and nitrogen source in microbial metabolism, acting as regulatory agents in aging mammals, maintaining the critical level of D-serine in the animal nervous system [17-20]. Due to the strict stereoselectivity of DAAO catalysis, its reaction is of considerable importance in research, biotechnology, and industrial fields [21, 22]. The main interests are mainly due to prospects of its use in the production of several cephem antibiotics from the natural antibiotic cephalosporin C and in biosensors [23, 24].

Although DAAO is almost ubiquitous in a wide variety of organisms including human, animals, and microorganisms, the ability of DAAO has remained to be clarified in plant kingdom thus far.

Although plants have been found to take up different D-amino acids from their root system, how plants are able to metabolize such compounds remains as a puzzle. The majority of studies within the field of D-amino acid metabolism in plant system were carried out during the 1970s, suggesting different metabolic pathways for D-amino acid metabolism in plants [25].

Recently, on the bases of different responses of plants to different D-amino acids, Erickson's group introduced the *daao1* gene into *Arabidopsis thaliana* to develop a novel positive and negative selectable marker system in plants based on D-amino acid metabolism [25]. Their achievement suggested that the substrate specificity of D-amino acid oxidase enzyme varies towards different D-enantiomers of amino acids in *A. thaliana* plants. This specificity indicated the possibility of DAAO activity in a plant system.

Abbreviations: DAAO, D-amino acid oxidase.

* To whom correspondence should be addressed.

We predicted the existence of DAAO activity in plant systems after finding two mRNA structures containing a DAAO domain in GenBank databases, using different bioinformatic tools on expressed sequences. The corresponding sequences belong to *A. thaliana* (GenBank accession No. NM_126129) and *Oryza sativa* (GenBank accession No. BAD31345.1), respectively. This could help us to conduct our research in this field on *Zea mays* after finding a number of maize ESTs (express sequence tags) containing a DAAO domain in GenBank databases too.

The catalytic activity of DAAO and its effectiveness have mostly been achieved using recombinant DNA technology. D-Amino acid oxidase enzymes from different sources were expressed in prokaryotic and eukaryotic cells of an active recombinant or fused proteins. The most popular and efficient approach to produce DAAO recombinant proteins was their expression in an *Escherichia coli* system [26-28]. We looked for similar recombinant experimental evidence for the presence of DAAO activity in a plant system in this work.

Here we describe the molecular cloning and expression procedures of a cDNA encoding DAAO from *Z. mays* for the first time and hope plant DAAOs will be used as a good biotechnological tool in the future. Such ability has already been proved and used in the case of microbial and animal systems.

MATERIALS AND METHODS

Materials. *Zea mays* L. seeds were prepared by the Department of Plant Breeding and Biotechnology, University of Tabriz, Iran. *Escherichia coli* strains DH5α and TB1 were used in molecular cloning experiments. Plasmid vectors of pGEM-T easy (Promega, USA) and pMALc2X (New England Biolabs, USA) were used for PCR product cloning and expression studies, respectively. Trizol reagent (CinnaGen, China) was used for total RNA isolation. AcessQuick™ RT-PCR System was purchased from Promega. Restriction enzymes *Eco*RI and *Bam*HI were provided by CinnaGen. Fermentas (Lithuania) DNA Extraction Kit was used for the purification of the PCR product from the agarose gel. All the other chemicals used in this research work were of molecular biology grades.

Cultivating maize plants on D-alanine containing media. Seeds of *Z. mays* L. were surface sterilized using 20% NaOCl, germinated up to seedling stage, and then transferred to experimental tubes each containing 16 ml of Hoagland solution. Four treatments of D-alanine at the concentrations of 0, 100, 1000, and 10,000 ppm were taken, and for each treatment three repetitions were included. Experiments were conducted in nitrate-free as well as nitrate-containing solutions in the same growth conditions for the period of 36 days. The growth of the plants was relatively analyzed by comparing their heights and weights at days 14 and 36. Data are presented as mean values.

The same experiment was repeated under sterile growth conditions inside a laminar flow apparatus for a period of 10 days. All the materials were sterilized. For leaf disc assay, leaf discs of maize plants with the same size and vigor were prepared and placed into Petri plates in three repetitions. Three different treatments of D-alanine (0, 1000, and 10,000 ppm) were taken in 8 ml of nitrate-free Hoagland solution and poured into each Petri plate. Petri plates were then kept under similar growth condition as for the whole plants. Treated leaf discs were then observed for their morphological changes every day.

Molecular cloning of maize *DAAO*. Poly(A^+) RNA was purified from total RNA using Qiagen (USA) mRNA purification kit. *DAAO* was cloned by RT-PCR using sequence-specific primers (Fw, 5′CCTACGAATTCTG-CACGGCCTACTTCCTC3′; Rv, 5′ATTAGGATCCT-CAACGCCTGCTCCTTCTC3′) designed based on 5′ and 3′ ends of the predicted maize *DAAO*. To do the directional cloning of the PCR-amplified cDNA in an *E. coli* expression vector, *Eco*RI and *Bam*HI restriction sites were included at the 5′ ends of the primers. RT-PCR reaction was performed using the one-step AcessQuick™ RT-PCR System (Promega). About 0.5 μg of mRNA was mixed with 25 μl Master Mix (2×) and 1 μl of each primer. The mixture was adjusted to a final volume of 50 μl using nuclease-free water. The reaction mixture was incubated at 45°C for 45 min and processed with PCR cycling. The PCR was carried out after a pre-denaturation stage at 95°C for 3 min in 25 cycles of 1-min denaturation at 95°C, 1-min annealing at 60°C, and 1.5-min extension at 72°C. The reaction was finally extended at 72°C for 10 min. Amplified PCR product was extracted from the agarose gel using a Fermentas DNA Extraction Kit, cloned in pGEM-T easy cloning vector, and processed for sequencing in "Microsynth" DNA sequencing center in Switzerland.

Expression of DAAO in *E. coli*. The cDNA insert in pGEM-T easy vector was restricted by restriction enzymes *Eco*RI and *Bam*HI and the fragment of our interest was purified from the 0.8% agarose gel and was subcloned in pMALc2X *E. coli* expression vector as a fusion protein. *Escherichia coli* cells (strain TB1) were transformed according to the recommendations by the kit suppliers (protein fusion and purification system kit; New England Biolabs). The transformed cells were grown in rich broth containing glucose and ampicillin at 37°C under uninducible/inducible condition with isopropyl-1-thio-β-D-galactoside at a final concentration of 0.3 mM. When A_{600} of the culture reached 0.5, the *E. coli* cells were harvested after 2.5 h of incubation and disrupted by sonication as described by the manufacturer. Total soluble protein was extracted from the bacterial lysate in extraction buffer containing 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM azide, and 10 mM β-mercaptoethanol. Total protein fractions were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% running gel [29]. Since maltose-binding protein forms a part of the

fusion protein, the approximate size of the recombinant product was expected to be about 86 kDa on the gel.

Purification of recombinant protein. The fusion protein was purified from the crude extract of induced bacterial culture by maltose-affinity column chromatography according to the recommendations of the kit suppliers (protocol in protein fusion and purification system kit; New England Biolabs). The purification was carried out in a 2.5 × 10 cm column packed with amylose resin specific for the maltose-binding protein, which forms a part of the fusion protein. The recombinant protein was eluted from the column with protein extraction buffer containing 10 mM maltose. The purified product was subjected to SDS-PAGE on a 10% running gel [29].

DAAO activity assay. DAAO activity of the isolated fusion protein was estimated using a peroxidase-coupled system by measuring the increase in A_{435} resulting from the oxidation of D-amino acids. To carry out this assay, 3 ml blank solution was prepared with 2.8 ml of 0.05 M D-amino acids (prepared in 20 mM Tris-HCl extraction buffer), 0.0065% *o*-dianisidine, and 1 μM FAD mixture, 0.1 ml of 1% peroxidase enzyme, and 0.1 ml of fusion protein free buffer. Three test samples were prepared by the addition of purified fusion product at the concentrations 0.05, 0.1, and 0.2 mg/ml. The increase in the A_{435} was recorded spectrophotometrically at 37°C after 10 min for samples as well as blank. Three repetitions were taken for each sample and the data were analyzed using ANOVA at the significance level of $P < 0.01$. The presented data correspond to mean values.

RESULTS AND DISCUSSION

Effect of D-alanine on maize growth. The growth of maize plants in four different concentrations of D-alanine (0, 100, 1000, and 10,000 ppm) in nitrate-free and nitrate-containing media were relatively assayed by comparing their shoot and root heights and weights at two different time intervals (Fig. 1, a and b). As shown in the diagram, there is a significant difference between the growth (heights and weights) of plants grown in D-alanine free (0 ppm), D-alanine excess (10,000 ppm), and in the media containing D-alanine at the concentrations of 100 and 1000 ppm. Plants grown in the media containing 100 and 1000 ppm D-alanine showed normal growth, while the growth of the plants in D-alanine free and D-alanine excess media were stunted after 10 days.

This experiment was similarly repeated under sterile conditions and the same results were observed as for non-sterile conditions. The relative growth of 10-day-old plants grown in three different concentrations of D-alanine (0, 1000, and 10,000 ppm) under sterile conditions is shown in the photograph Fig. 1c.

Comparison of the growth of plants in different media revealed that maize plants can uptake, utilize, and accumulate D-alanine up to toxic levels. Utilization of D-alanine as a nitrogen source by maize plants confirmed the *in vivo* activity of D-amino acid metabolizing enzyme in this plant.

The effect of D-alanine on maize cells was further confirmed by leaf disc assay experiment results in nitrate-free solution media (Fig. 1, d-h). Leaf discs placed in solution containing 1000 ppm D-alanine (Petri plate No. 2) remained green, while the leaf discs in solutions containing 0 and 10,000 ppm of D-alanine (Petri plates Nos. 1 and 3, respectively) started to become brownish, looking like necrotic tissues in cell death phenomenon, after 24 h. Closer views of the leaf discs from solutions 1, 2, and 3 are presented in Figs. 1e, 1f, and 1g, respectively. Figure 1h shows an interesting photograph of leaf discs in solutions containing 1000 and 0 ppm D-alanine after 36 days of treatment time, indicating the functioning of D-alanine metabolizing enzyme in maize cell leaf tissues.

Based on the presence in plant systems of D-amino acids [30-35] and due to the different responses of plants to different D-amino acids [35, 36], it had already been accepted as a fact that plants contain D-amino acid-metabolizing enzyme. But if this enzyme a D-amino acid oxidase or not still remains to be determined.

Prediction of maize DAAO. Since the discovery of DAAO enzyme in pig kidney and its wide characterization in different animal species and microorganisms, the presence of a *DAAO* gene and its activity in plant systems has not been investigated in detail. Recently, the use of D-amino acids as negative and positive selective agents for developing transgenic plants showed the substrate specificity of D-amino acid oxidase enzyme in *A. thaliana*. The experiment had been designed according to the fact that there have been some exogenously tested D-amino acids acting as less toxic or non-toxic compounds in plants [25].

Later on, finding two mRNA sequences containing a DAAO domain in *A. thaliana* and *O. sativa* from GenBank database helped us to conduct our experiments towards identification of DAAO ability in a plant system. We planned to search for DAAO activity in maize plants as a rich source in FAD-dependent redox system. The sequence data survey for this system by bioinformatic tools revealed the presence of candidate ESTs for DAAO. The complementarity of 3′ overlapping plus- and minus-strands of the *DAAO* gene was observed using 3′ and 5′ ESTs. This could bring about a putative coding region for maize DAAO named predicted *Zm*DAAO. The characteristics of the nucleotide and deduced amino acid sequences of this coding region are shown in Fig. 2. This region spans 1158 bp in size, starts from ATG start codon and ends by TGA termination codon.

The bioinformatic studies showed the presence of the N-terminal amino acid residues GXGXXG located between amino acid residues 13 and 20 (Fig. 2). It is the characteristic feature for nucleotide binding domain and is conserved in all D-amino acid oxidases (known in

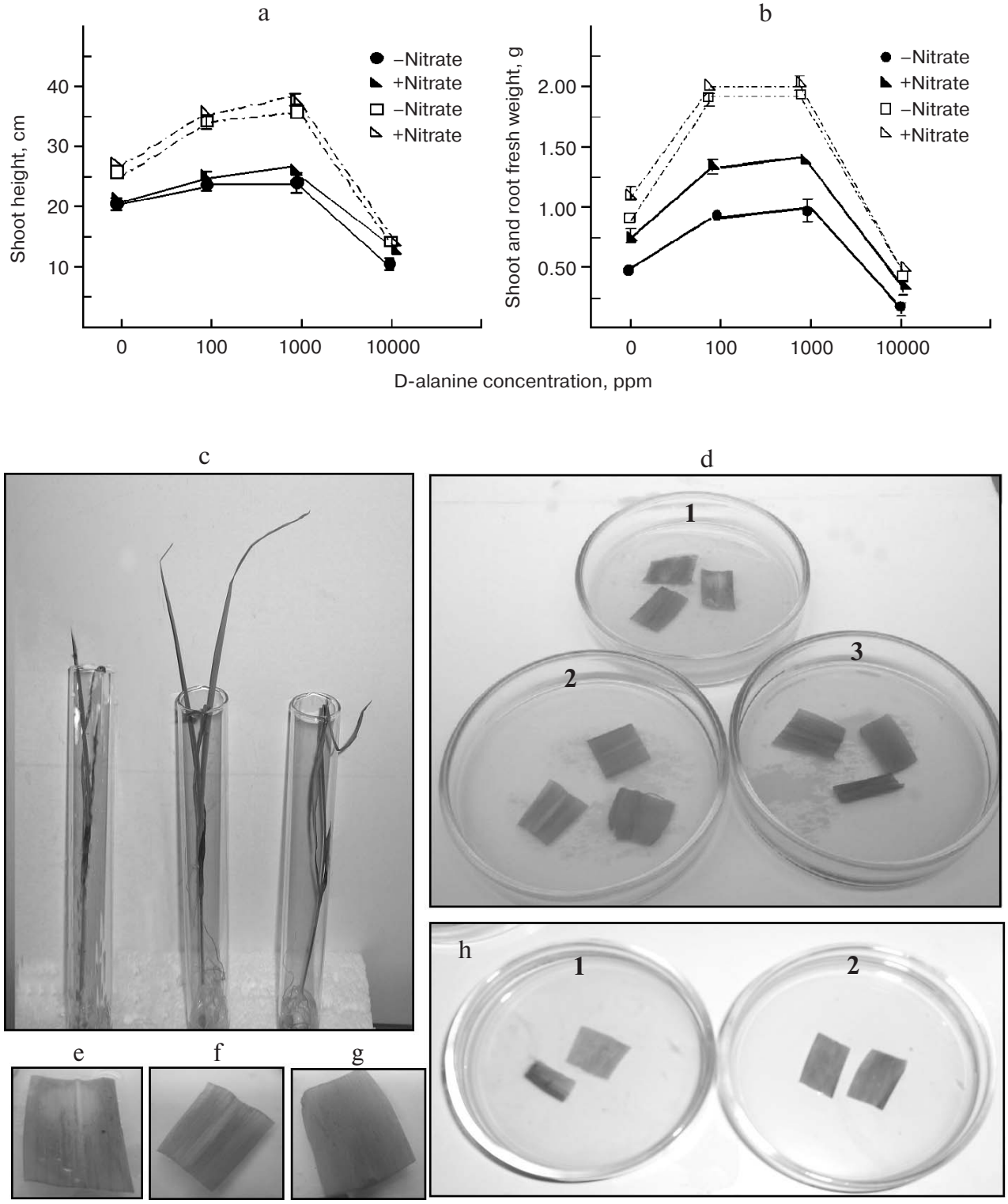

Fig. 1. a) Relationship between shoot height of plants and D-alanine concentration in the growth media after 36 days. b) Relationship between shoot and root fresh weights of plants and D-alanine concentration in the growth media. c) Relative growth of plants in media containing 0, 1000, and 10,000 ppm D-alanine (tubes No. 1, 2, and 3, respectively) under sterile growth conditions. d) Morphological color changes in plants leaf discs placed in D-alanine-free and D-alanine-excess media. Petri plates No. 1, 2, and 3 containing 0, 1000, and 10,000 ppm D-alanine, respectively. e) Closer view of a leaf disc taken from D-alanine-free medium. f) Closer view of a leaf disc taken from D-alanine-containing medium at the concentration of 1000 ppm. g) Closer view of a leaf disc taken from the medium containing 10,000 ppm D-alanine. h) An interesting photograph of leaf discs in the media containing 1000 and 0 ppm D-alanine.

FAD-dependent oxidoreductases) identified so far [37]. Also, different clusters of internal and C-terminal conserved amino acid sequences have been found to act as the catalytic sites of the active center. Presence and location of these catalytic regions, presented as regions (2), (3), and (4), are shown in *Zm*DAAO compared to those of already characterized DAAOs from different origins (Fig. 2). No conserved peptide has been observed for substrate-binding domains in DAAO primary structures, reflecting the wide variation in the substrate specificity for the enzymes of different origins [37].

The comparative studies among predicted *Zm*DAAO and those of known D-amino acid oxidases from different sources revealed about 20% homology with microbial sources and about 14% with those of animal species. However, this score for predicted DAAO mRNAs from *O. sativa* and *A. thaliana* was found to be 88 and 62%, respectively (Fig. 3).

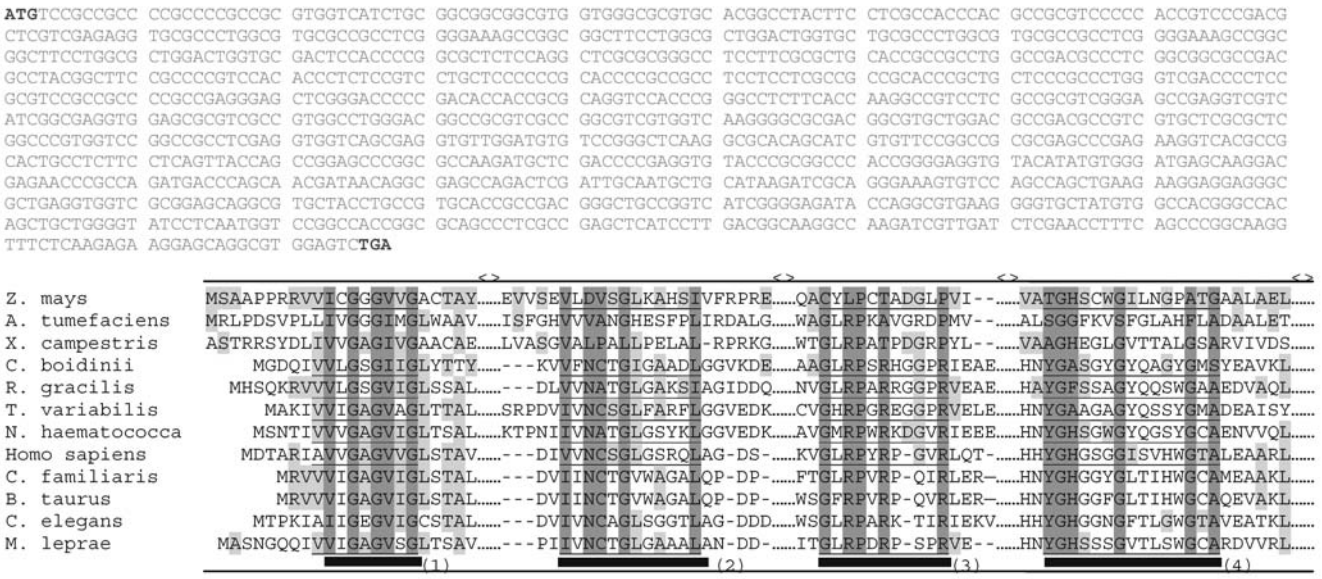

Fig. 2. Prediction of D-amino acid oxidase coding sequence from *Zea mays* L. by a search on various 5′ ends overlapping DAAO ESTs that have been already presented in GenBank databases; the complete coding region has been predicted in maize plants too. Comparison of predicted amino acid sequences of *Zm*DAAO with those of already characterized DAAOs from bacterial and mammalian kingdoms. *Zea mays* L. (Ac. No. AM407717); *Agrobacterium tumefaciens* (Ac. No. AGRC4235); *Xanthomonas campestris* (Ac. No. XCC2414); *Candida boidinii* (Ac. No. AB042032); *Rhdotorula gracilis* (Ac. No. RGU60066); *Trigonopsis variabilis* (Ac. No. AY514426); *Nectria haematococca* (Ac. No. P24552); *Homo sapiens* (Ac. No. BC029057); *Canis familiaris* (Ac. No. 486317); *Bos taurus* (Ac. No. 615334); *Caenorhabditis elegans* (Ac. No. C47A10.5); *Mycobacterium leprae* (Ac. No. MLCOSL672). The highly conserved sequences including nucleotide-binding domain and three catalytic regions (2), (3), and (4) are shaded in gray and underlined.

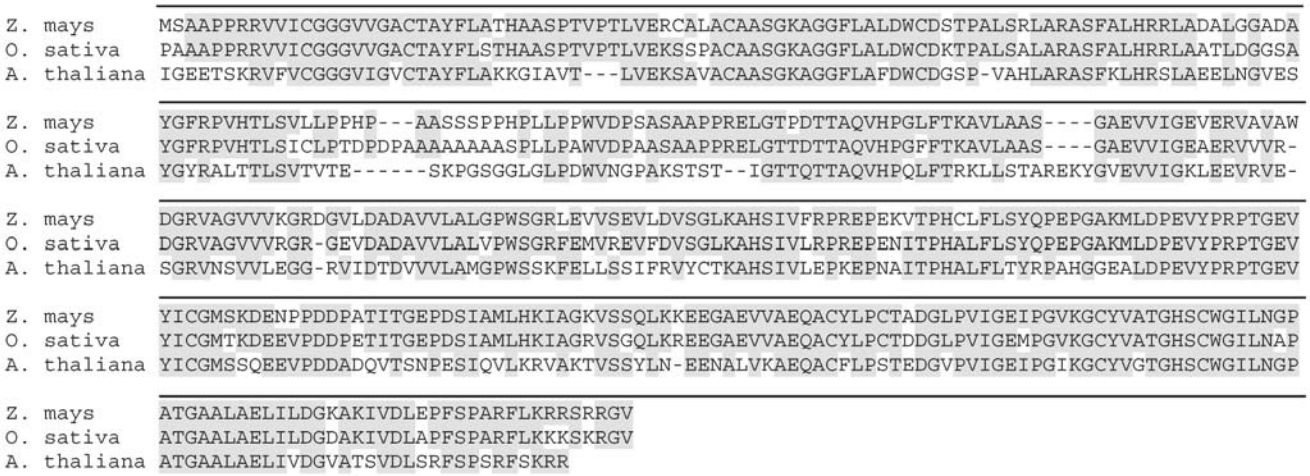

Fig. 3. Amino acid sequence alignment between *Zm*DAAO and those of plant counterparts including *Oryza sativa* (Ac. No. NM_126129) and *Arabidopsis thaliana* (Ac. No. BAD31345.1) DAAO sequences. The identity levels are 88 and 62% for *O. sativa* and *A. thaliana*, respectively.

Cloning of *DAAO* gene under induced conditions. The gene of *Zm*DAAO was not obtained by RT-PCR method as described in "Materials and Methods". Therefore, we supposed that *Zmdaao* like other DAAO genes might be induced in response to inducing compounds.

As a general rule, the expression of D-amino acid oxidase genes is mostly regulated in eukaryotic and prokaryotic cells. They are usually induced by D-enantiomers of amino acids [38, 39]. D-Alanine is a commonly used DAAO inducer.

We grew maize plants in D-alanine containing media with four different concentrations, i.e. 0, 100, 1000, and 10,000 ppm. The youngest leaves of plants of all treatments at age 36 days were harvested for RNA extraction. Total RNA and mRNA isolations were carried out using a single protocol for all samples, and their equality was tested spectrophotometrically. At the end of the experiment, 5 µl of each RT-PCR end product was run on the 1% agarose gel and photographed. As shown in Fig. 4, in D-Ala-free and D-Ala-excess media grown plants no expressed end products are detectable on the gel. However, sharply visible bands are observed for treatments 100 and 1000 ppm of D-alanine. The amplified fragments were cloned in pGEM-T easy vector and sequenced partially for further confirmation.

We assume that the absence of DAAO pattern in the plant grown under D-Ala excess media may be related to the degradation of its mRNA due to cell death phenomenon induced by the accumulation of excess D-Ala in plant cells. However, this idea needs to be investigated in detail.

As an interesting result, our molecular pattern is consistent with those of morphological results shown in Fig. 1. Significant differences between the growth of plants in D-Ala-free (0 ppm), D-Ala-excess (10,000 ppm), and in the media containing D-Ala at the concentrations of 100 and 1000 ppm, strongly correlate with significant differences shown in the expression pattern of the *DAAO* gene in the experimental plants. This indicates that the D-Ala metabolizing enzyme might be a DAAO in maize plants.

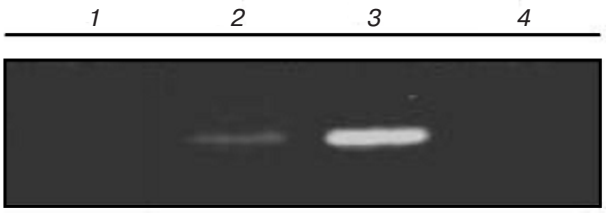

Fig. 4. Expression analysis of *Zm*DAAO under induced conditions. Comparison of RT-PCR end product in plants grown at different concentrations of D-alanine showed that the maize *daao* gene could be induced when plants use D-Ala as the nitrogen source. RT-PCR products in lanes *1-4* are related to the control plant (plant grown in D-Ala-free media) and plants grown in the media containing 100, 1000, and 10,000 ppm D-Ala, respectively.

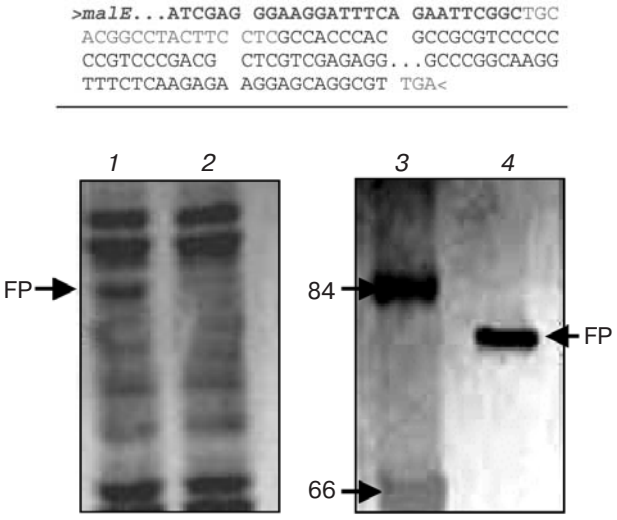

Fig. 5. Presentation of fused *malE–daao* gene in pMALc2X *E. coli* expression vector. Cloned maize DAAO cDNA was inserted downstream of the *malE* gene of *E. coli*, which encodes maltose-binding protein (MBP), resulting in the expression of MBP–DAAO fused protein. SDS-PAGE analysis of the expressed and purified fused protein. The fused product was expressed with a size of about 78 kDa, which is 8 kDa smaller than the calculated size. The gel was stained with Coomassie blue. Lanes: *1*) crude protein extract of TB1 *E. coli* cells containing recombinant plasmid; FP stands for fused protein; *2*) crude extract of TB1 bacterial cells containing non-recombinant plasmid; *3*) protein molecular weight marker; *4*) purified maltose-binding protein fused product.

Expression of *Zm*DAAO in *E. coli* cells. For stronger verification of DAAO ability in maize plants, a recombinant plasmid was produced by the insertion of the putative D-amino acid oxidase cDNA of *Z. mays* into the multiple cloning site of pMALc2X *E. coli* expression vector, downstream of the maltose-binding protein coding sequence. The fused *malE–daao* gene contains eight additional codons located between the *malE* and maize *daao* genes (Fig. 5).

The pMALc2X-DAAO vector was transformed into TB1 strain of *E. coli* cells. The expression of the fused product was analyzed by SDS-PAGE on a 10% gel and a detectable expressed band was observed with a molecular weight of about 78 kDa, which is 8 kDa smaller than the calculated size (Fig. 5).

The fused protein was purified from the total expressed proteins using maltose-affinity chromatography at the yield of about 5 mg/liter of cell culture (Fig. 5). Although the reasons for such a result could already be argued by the cytotoxicity of the end product of DAAO-catalyzed reactions, the drastic reduction of intracellular pool of D-alanine (as an essential component of the bacterial cell wall) could be due to DAAO action [37].

The purified fusion protein was subjected to *in vitro* DAAO activity assay towards D-alanine, D-aspartate, D-leucine, D-isoleucine, and D-serine using a sensitive

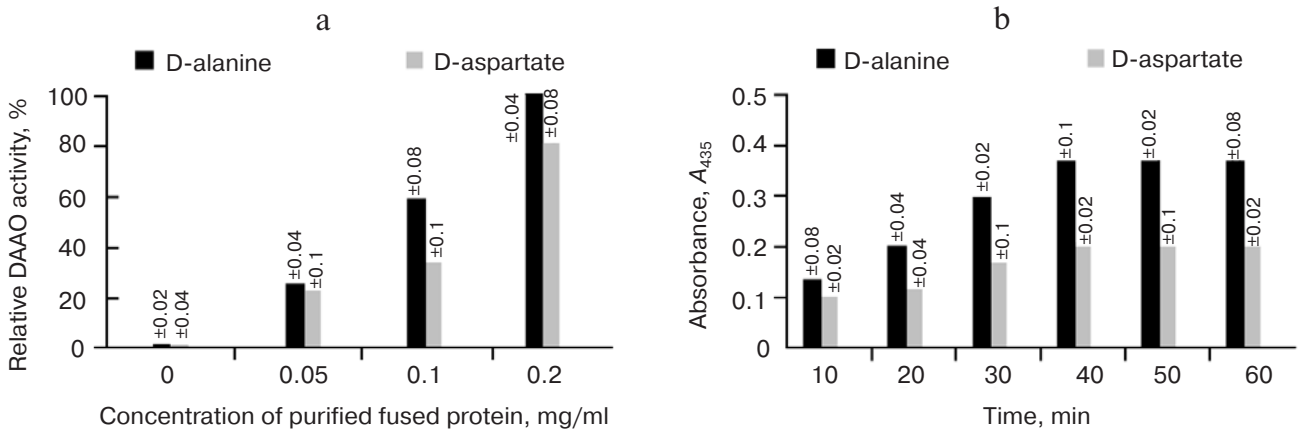

Fig. 6. *In vitro* DAAO activity estimation in purified fused *Zm*DAAO. a) Changes in A_{435} values in solutions of samples containing different concentrations of purified fused protein have been taken as an indicator for DAAO activity. A linear relationship was observed between the concentrations of the *Zm*DAAO and relative DAAO activity expressed in percent of maximum. b) Time course experiment results show that at the concentration of 0.1 mg/ml of purified protein, A_{435} value increases up to 40 min and then reaches constant value 0.38. Data presented as the means of three repetitions. The mean values have been compared at the significant level of $\alpha = 0.01$.

spectrophotometric method based on peroxidase activity assay. The concentrations of purified fused product in test samples were of 0.05, 0.1, and 0.2 mg/ml as described in materials and methods. A linear increase in optical density was observed as the concentration of the fused protein was increased from 0 to 0.2 mg/ml in the test samples containing D-alanine and D-aspartate. Our results showed no increase in optical density for D-leucine-, D-isoleucine-, and D-serine-containing samples. The relative DAAO activity for each sample was calculated based on the absorbance data obtained and graphed as in Fig. 6.

A parallel time course experiment with three replications was also carried out in 10-min time intervals for an hour, at concentration of 0.1 mg/ml of purified recombinant protein. A constant increase in A_{435} was detected as the time of reaction was increased up to 40 min, and then it remained constant at 0.38 in the case of D-alanine and 0.21 for D-aspartate (Fig. 6). These results strongly revealed the *in vitro* activity of expressed D-amino acid oxidase as a fused protein in *E. coli* cells and furthermore the strongest possibility for DAAO activity in the plant system as well.

Generally, the catalytic activity of DAAO and its effectiveness have been demonstrated using recombinant DNA technology. We also believe our recombinant experimental results suggest the existence of DAAO activity in the plant system.

DAAO activity has already been proved and used in the case of microbial and animal systems. Our suggestion is that plant DAAOs will also be used as a good biotechnological tool in the future.

This work was supported by RIFS (Research Institute for Fundamental Sciences), University of Tabriz, Tabriz, Iran.

REFERENCES

1. Curti, B., Ronchi, S., and Pilone, S. M. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., ed.) CRC Press, pp. 69-94.
2. Jacobs, P., Brockly, F., Massaer, M., Loriau, R., Guillaume, J., Ciccarelli, E., Heinderyckx, M., Cravador, A., Biemans, R., van Elsen, A., Herzog, A., and Bollen, A. (1987) *Gene*, **59**, 55-61.
3. Krebs, H. A. (1935) *Biochem. J.*, **29**, 1620-1644.
4. Konno, R., and Yasumura, Y. (1981) *Zool. Mag.*, **90**, 368-373.
5. Fukui, K., Watanabe, F., Shibata, T., and Miyake, Y. (1987) *Biochemistry*, **26**, 3612-3618.
6. Momoi, K., Fukui, K., Watanabe, F., and Miyake, Y. (1988) *FEBS Lett.*, **238**, 180-184.
7. Tada, M., Fukui, K., Momoi, K., and Miyake, Y. (1990) *Gene*, **90**, 293-297.
8. Mizutani, H., Miyahara, I., Hirotsu, K., Nishina, Y., Shiga, K., Setoyama, C., and Miura, R. (1996) *J. Biochem.*, **120**, 14-17.
9. Sarower, M. G., Matsui, T., and Abe, H. (2003) *J. Exp. Zool.*, **295**, 151-159.
10. Sarower, M. G., Okada, S., and Abe, H. (2005) *Comp. Biochem. Physiol.*, **140**, 417-425.
11. Pistorius, E. K., and Voss, H. (1977) *Biochim. Biophys. Acta*, **481**, 395-406.
12. Kubicek-Pranz, E. M., and Rohr, M. (1985) *J. Appl. Biochem.*, **7**, 104-113.
13. Pilone, S. M., Verga, R., Fretta, A., and Hanozet, G. M. (1989) *J. Gen. Microbiol.*, **135**, 593-600.
14. Isogai, T., Ono, H., Ishitani, Y., Kojo, H., Ueda, Y., and Kohsaka, M. (1990) *J. Biochem.*, **108**, 1063-1069.
15. Pollegioni, L., Buto, S., Tischer, W., Ghisla, S., and Pilone, M. S. (1993) *Biochem. Mol. Biol. Int.*, **31**, 709-717.
16. Fischer, L., Horner, R., Gabler, M., and Wagner, F. (1996) *Ann. N. Y. Acad. Sci.*, **799**, 683-688.
17. Man, E. H., Fisher, G. H., Payan, I. L., Cadilla-Perezrios, R., Garcia, N. M., Chemburkar, R., Arends, G., and Frey, W. H. (1987) *J. Neurochem.*, **48**, 510-515.

18. D'Aniello, A., D'Onofrio, G., Pischetola, M., D'Aniello, G., Vetere, A., Petrucelli, L., and Fisher, G. H. (1993) *J. Biol. Chem.*, **268**, 26941-26949.
19. Fisher, G. H. (1998) *EXS*, **85**, 109-118.
20. Mothel, J. P., Rouaud, E., Sinet, P.-M., Potier, B., Jouvenceau, A., Dutar, P., Videau, C., Epelbaum, J., and Billard, J.-M. (2006) *Aging Cell*, **5**, 267-271.
21. Fischer, L. (1998) *Recent Res. Devel. Microbiol.*, **2**, 295-317.
22. Riethorst, W., and Reichert, A. (1999) *Chemia*, **53**, 600-607.
23. Conlon, H. D., Baqal, J., Baker, K., Shen, Y. Q., Wong, B. L., Nolles, R., and Rausch, C. W. (1995) *Biotechnol. Bioeng.*, **46**, 510-513.
24. Kulys, J., and Schmid, R. D. (1991) *Biosens. Bioelectron.*, **6**, 43-48.
25. Erickson, O., Hertzberg, M., and Nasholm, T. (2004) *Nature Biotechnol.*, **22**, 455-458.
26. Pollegioni, L., Molla, G., Campaner, S., Martegani, E., and Pilone, M. S. (1997) *J. Biotechnol.*, **58**, 115-123.
27. Molla, G., Vegezzi, C., Pilone, M. S., and Pollegioni, L. (1998) *Prot. Exp. Purif.*, **14**, 289-294.
28. Lin, L., Chien, H. R., Wang, W., Hwang, T., Fu, H., and Hsu, W. (2000) *Enz. Microb. Technol.*, **27**, 482-491.
29. Laemmli, K. (1970) *Nature*, **227**, 680-685.
30. Robinson, T. (1976) *Life Sci.*, **19**, 1097-1102.
31. Manabe, H., Yamauchi, M., and Ohira, K. (1981) *Plant Cell Physiol.*, **22**, 333-336.
32. Gamburg, K. Z., and Rekoslavskaya, N. L. (1991) *Fiziol. Rast.*, **38**, 1236-1246.
33. Bruckner, H., and Westhauser, T. (2003) *Amino Acids*, **24**, 43-55.
34. Patzold, R., and Bruckner, H. (2005) *J. Agric. Food Chem.*, **53**, 9722-9729.
35. Atoh, S., and Esashi, Y. (1980) *Planta*, **149**, 64-68.
36. Monabe, H., and Ohira, K. (1984) *Plant Cell Physiol.*, **25**, 349-354.
37. Tishkov, V. L., and Khoronenkova, S. V. (2005) *Biochemistry* (Moscow), **70**, 40-54.
38. Horner, R., Wagner, F., and Fischer, L. (1996) *Appl. Environ. Microbiol.*, **62**, 2106-2110.
39. Molla, G., Motteran, L., Piubelli, L., Pilone, M. S., and Pollegioni, L. (2003) *Yeast*, **20**, 1061-1069.