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Expression and Functional Analysis of Aminotransferase Involved in Indole-3-acetic Acid Biosynthesis in *Azospirillum brasilense* Yu62

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Abstract—In this study, *atrC* (a novel gene from *Azospirillum brasilense* identified in our laboratory) was expressed in *Escherichia coli*, and SDS-PAGE analysis of the expressed AtrC revealed the apparent molecular weight of 45 kD. When analyzed under non-denaturing PAGE conditions and using L-tryptophan as a substrate, the purified AtrC protein exhibited aminotransferase activity, while crude protein extracts from *A. brasilense* Yu62 showed two activity bands with molecular masses estimated as 44 and 66 kD. Thus, we deduced that AtrC protein is identical to the 44 kD band of crude protein extracts. The optimal temperature and pH for the catalytic activity of the purified AtrC are 30°C and pH 7.0, respectively.

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Azospirillum brasilense is a rhizosphere bacterium that has a potential use as an inoculant for promoting plant growth in grass and cereals [1]. The phytohormone indole-3-acetic acid (IAA) is one of the factors released by *Azospirillum*, which might be responsible for the promoting effect on plant growth [2]. There are several biosynthetic pathways for IAA in prokaryotes, and a given bacterial species may use more than one pathway [3]. In *Azospirillum*, attempts to isolate a mutant completely deficient in IAA production have not been successful [4-6], suggesting that more than one pathway might be involved in IAA synthesis. Feeding experiments with radioactively labeled indole-3-acetamide and tryptophan demonstrated that *A. brasilense* possesses at least three IAA biosynthetic pathways. These are two Trp-dependent pathways, i.e. the indole-3-acetamide pathway and the indole-3-pyruvic acid (IPA) pathway, and a Trp-independent pathway [7]. Biochemical and genetic evidence for the IPA pathway has been provided, and this way was found to be the main route for IAA production in the presence of exogenous Trp in *A. brasilense* [8, 9]. In general, the first step in the IPA pathway is the conversion of Trp to IPA catalyzed by multispecific aminotransferases. Indole-3-pyruvate decarboxylase then catalyzes the conversion of IPA to indole-3-acetaldehyde, and the latter product can be oxidized to IAA by a non-specific aldehyde dehydrogenase (also called aldehyde oxidase) [10].

Two aromatic amino acid aminotransferases with molecular masses ranging from 44 to 66 kD have been identified in *A. brasilense* strains Sp7, Sp245, UAP14, and R07 [5, 6, 11]. However, the genes encoding aminotransferases in *A. brasilense* have not been reported. Recent reports from our laboratory [12, 13] identified a novel gene, designated as *atrC*, whose deduced products showed high similarity to aminotransferases of various bacteria, and mutagenesis and complementation studies demonstrated that *atrC* was involved in IAA production. In this study, *atrC* gene was expressed and purified in *Escherichia coli*, and the function of the expressed AtrC protein as an aminotransferases was established.

MATERIALS AND METHODS

Strains and plasmids. *Azospirillum brasilense* Yu62 was a nitrogen-fixer isolated from the rhizosphere of

Abbreviations: IAA, indole-3-acetic acid; IPA, indole-3-pyruvic acid.

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maize in Beijing, China [14]. *Escherichia coli* DH5α was used as host for cloning and sequencing, and *E. coli* BL21(DE3) was used as host for expressing AtrC protein. Plasmid pET28(a)+ was used as an expression vector.

Construction of expression plasmid (pET-AtrC) for AtrC protein. A 1329-bp DNA fragment (from the start codon to 12 bp downstream of the stop codon) containing the *atrC* gene was amplified by polymerase chain reaction (PCR) from chromosomal DNA of *A. brasilense* Yu62. Primers P_{atrC}-1 (5′-CGCTGAATTCATGACCATGATCAACGCCT-3′; an *Eco*RI site underlined) and P_{atrC}-2 (5′-CGTTAAGCTTAAGCGCCGTCACTTACAGC-3′; a *Hind*III site underlined) were used. The PCR product was digested with *Eco*RI and *Hind*III, and cloned in frame into the pET28(a)+ expression vector digested with the same restriction enzymes, yielding plasmid pET-AtrC.

Expression and purification of AtrC protein. *Escherichia coli* strain BL21(DE3) was transformed with the expression plasmid pET-AtrC carrying the *A. brasilense atrC* gene and the transformant, named BL21-pET-AtrC, was chosen for protein expression. The bacterial cells were grown in an LB medium up to the end of the log phase and then a final concentration of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the culture, and the cells were harvested after growing for another 5 h at 20°C. Then the cells were disrupted by sonication on ice. Sonicated supernatant was collected and loaded onto nickel-nitrotriacetic acid (Ni^{2+}-NTA) agarose columns (Qiagen, USA). Tagged fusion protein was eluted with 250 mM imidazole and collected in 0.5 ml fractions. Protein eluates were used for aminotransferase activity assay.

Aminotransferase activity assay. Aminotransferase activity was detected by a non-denaturing gel enzyme activity assay [15] with a minor modification. Electrophoretic analyses under native conditions were performed in a continuous system, using Tris-glycine running buffer (25 and 192 mM, pH 8.3) and 10% acrylamide running gel [16]. Protein (20-40 μg) extract was loaded in each well. Gels were run at 4°C at 30 mA for 4-5 h until the blue bromophenol colorant of the loading buffer reached the bottom of the gel. Then gels were stained at room temperature in the dark. The staining solution contained 0.1 mM Tris-HCl buffer (pH 8.6), 5.5 mM amino acid, 12.5 mM α-ketoglutaric acid, 0.2 mM pyridoxal phosphate, 98 μM phenazine methosulfate, 0.6 mM nitroblue tetrazolium, 3 mM NAD, and 3-6 U/ml glutamate dehydrogenase. When darkly stained protein bands appeared, the staining was stopped.

RESULTS

Expression and purification of AtrC protein. The *atrC* is a novel gene identified by our lab for its involvement in IAA production, and its predicted product is a protein of 439 amino acid residues with a calculated molecular mass of about 45 kD [12, 13]. The *atrC*$^-$ mutant whose *atrC* gene was knocked out produced lower IAA level than the wild type did, and IAA production was nearly completely restored by complementation with the *atrC* gene.

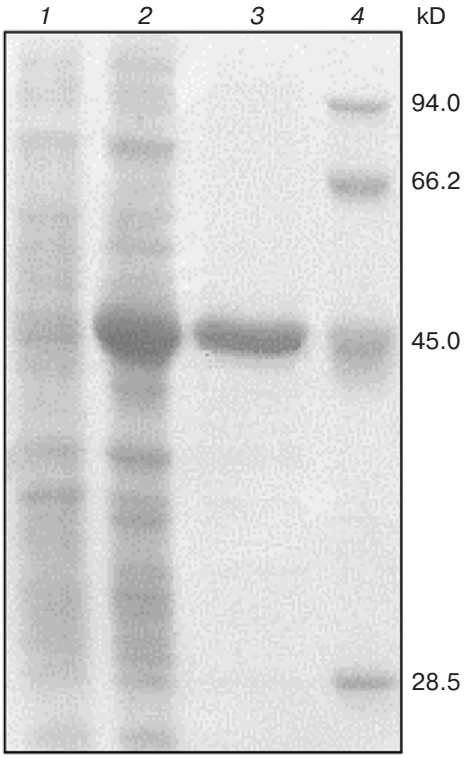

Fig. 1. SDS-PAGE analysis of the expressed AtrC protein in *E. coli* BL21. Lanes: *1*) *E. coli* BL21 containing pET28a vector as a negative control; *2*) induced AtrC protein; *3*) purified AtrC protein; *4*) protein markers.

In this study, the AtrC protein fused with a His-tag peptide at its N-terminus has been successfully overexpressed in *E. coli* BL21 and then was purified under native conditions. SDS-PAGE analysis (Fig. 1) revealed that the apparent molecular weight of AtrC was 45 kD, which was in agreement with its predicted molecular weight.

Aminotransferase activity assay. Aminotransferase activity of the purified AtrC protein and crude protein extracts of *A. brasilense* Yu62 was determined by a non-denaturing gel enzyme activity assay. When analyzed under non-denaturing PAGE conditions and using L-tryptophan as a substrate, the purified AtrC protein with its predicted molecular mass of 45 kD exhibited aminotransferase activity, and crude protein extracts showed two activity bands with molecular masses of 44 and 66 kD (Fig. 2). Figure 2b (lane *1*) also shows that the 66-kD band of crude protein extracts is more intense than the 44-kD band. When α-ketoglutaric acid or L-tryptophan

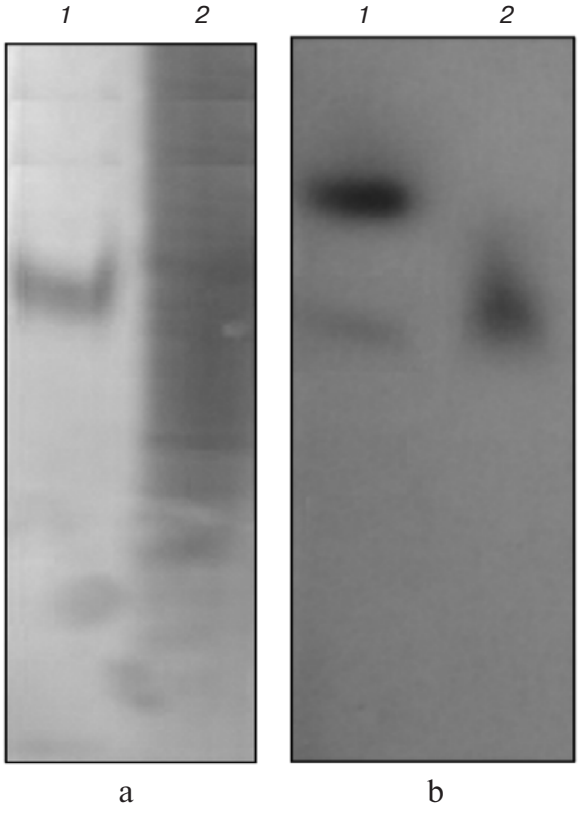

Fig. 2. Non-denaturing PAGE analysis (a) and aminotransferase activity staining (b) of AtrC protein and crude protein extracts of *A. brasilense* Yu62. a: *1*) purified AtrC protein; *2*) crude protein extracts of *A. brasilense* Yu62. b: *1*) crude protein extracts of *A. brasilense* Yu62; *2*) purified AtrC protein.

was omitted from the staining reaction mix, the darkly stained protein bands indicative of aminotransferase activity were not detected in AtrC protein or crude protein extracts, confirming that the assay is specific for aminotransferase enzymes. When using L-phenylalanine or L-tyrosine as the substrates, the AtrC protein showed no activity, even if the His-tag peptide was removed from the fused AtrC protein (data not shown), indicating that the AtrC protein is an L-tryptophan aminotransferase with a high substrate specificity. The data are in agreement with the report that *Azospirillum* strains displayed two aminotransferase isoforms with molecular masses of 44 and 66 kD [5, 6] and the band of 66 kD is stronger than that of 44 kD [11]. We deduced that AtrC protein is identical to the 44-kD band of crude protein extracts. This is the first report on cloning of an aminotransferase gene from *A. brasilense*.

Effect of temperature and pH on the catalytic activity of the purified AtrC. The effects of temperature and pH on the catalytic activity of the purified AtrC were investigated. Figure 3a shows that high level of IAA was produced at 25, 30, and 37°C and the optimum is 30°C, while no IAA was produced at 18 or 45°C. Figure 3b shows that IAA was produced at pH 6.0-10.0 and the optimum pH is 7.0, while no IAA was produced at pH 5.0.

DISCUSSION

Like in higher plants and most microorganisms, the IPA pathway is the main pathway in the IAA biosynthesis of *A. brasilense* [3, 8] and IAA was produced from L-tryptophan in the following three steps: L-tryptophan → indole-3-pyruvic acid → indole-3-acetaldehyde → IAA. It is generally believed that the first step of conversion of Trp to IPA is catalyzed by multispecific aminotransferases.

The recent report from our lab [12, 13] identified a novel gene, designated as *atrC*, whose deduced products showed high similarity to aminotransferases of various bacteria, and mutagenesis and complementation studies

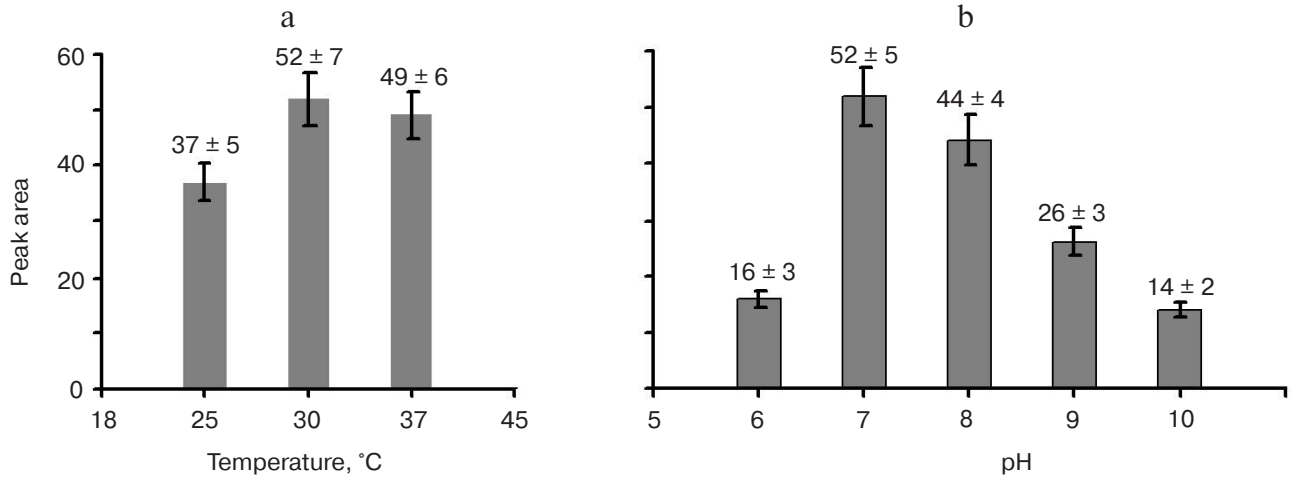

Fig. 3. Temperature (a) and pH (b) optima for AtrC protein as an aminotransferase in IAA synthesis.

demonstrated that *atrC* was involved in IAA production. In this study, the *atrC* gene was expressed in *E. coli* and purified. When analyzed under non-denaturing PAGE conditions and using L-tryptophan as a substrate, the purified AtrC protein with its predicted molecular weight of 45 kD exhibited aminotransferase activity, and crude protein extracts of *A. brasilense* Yu62 showed two activity bands with molecular masses of 44 and 66 kD. We deduced that AtrC protein is identical to the 44 kD band of crude protein extracts, and *atrC* is responsible for encoding the protein. The data from strain Yu62 are in agreement with the report that *A. brasilense* strains Sp7, Sp245, UAP14, and R07 displayed two aminotransferase isoforms with molecular masses of 44 and 66 kD when using tryptophan, phenylalanine, and tyrosine as substrates [6]. When using histidine as a substrate, strains Sp245 and UAP14 exhibited two isoforms, while strains Sp7 and R07 displayed the isoform of 66 kD. In this study, when using L-phenylalanine or L-tyrosine as the substrates, the AtrC protein showed no activity, indicating that the AtrC protein is a highly specific L-tryptophan aminotransferase. An aromatic aminotransferase with a novel substrate specificity was also reported in *Pyrococcus horikoshii* [17].

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