We cloned and expressed two genes encoding azoreductase homologs, AzrB and AzrC, from *Bacillus* sp. B29. Purified recombinant AzrB and AzrC were homodimers with 23 kDa identical subunits, and were flavoproteins. NADH was preferred as electron donors for both azoreductases. The azoreductases showed optimal activities at 70 °C (AzrB) and 55 °C (AzrC), and retained activities up to 55 °C (AzrB) and 50 °C (AzrC) after incubation for 1 h. Other enzymatic properties, including the substrate specificities of both azoreductases, were also investigated.

**Key words:**azo dyes; azoreductase; substrate specificity; flavoprotein; *Bacillus* sp. B29

Azobenzene reductase (EC 1.7.1.6) catalyzes the reduction of azo compounds in the presence of NAD(P)H.\(^1\) These enzymes are generally called azoreductases. They catalyze reductive cleavage of the azo group to transform various azo dyes into colorless metabolites corresponding to aromatic amines. They are favorable for the development of biodegradation process for azo dyes.

The bacterial azoreductases are classified into two families based on their cofactor requirement and molecular weight. NADPH-dependent azoreductases have been found in *Bacillus* species such as *Bacillus* sp. OY1–2\(^3\) and *B. subtilis*,\(^3,4\) as well as other bacteria, such as *Geobacillus steaothermophilus*,\(^6\) *Rhodobacter sphaeroides*,\(^5\) and *Staphylococcus aureus*.\(^6\) The azoreductases prefer NADPH as an electron donor and have subunits of about 18 kDa. On the other hand, NADH-dependent azoreductases require FMN as a prosthetic group for activity. They have been found in several *Bacillus* species that have multiple copies of the homologous genes of the NADH-dependent azoreductase in individual locus. Some NADH-dependent azoreductases were thought to play an important role in electron transport during redox reaction into the cells.\(^7\)

However, the enzymatic properties and physiological roles of NADH-dependent azoreductases are barely understood. In this study, we attempted to clarify the enzymatic properties of two novel NADH-dependent azoreductases from *Bacillus* sp. B29. Here we describe the expression, purification, and characterization of two azoreductases, designated AzrB and AzrC, in term of thermal stability and substrate specificity.

Two azoreductase genes, *azrB* and *azrC*, were amplified by PCR from *Bacillus* sp. B29 genomic DNA using two primer pairs: 5′-ataaaaagtgtgatctttatatgcctgc-3′ and 5′-cattatgecatataaactcccc-3′ for *azrB*, and 5′-ggtagagggagggatttcatt-3′ and gacgccatctcggggtg-3′ for *azrC*. Primer design was based on the nucleotide sequences of both neighbor genes of the targeted azoreductase, as described previously.\(^8\) The PCR products were subcloned using a pGEM-T vector (Promega, Madison) yielding pGEM-azrB and pGEM-azrC. For expression of the *azrB* and *azrC* genes, both genes were amplified again by PCR using two primer pairs to introduce restriction enzyme sites: 5′-acatatctgaaggtattgtagattaagacagac-3′ and 5′-ggatccctggtggttttcc-3′ for *azrB*, and 5′-ctatgacagacagacagattaaragac-3′ and 5′-aggtcagacagacagattaaragac-3′ for *azrC*. The *NdeI* and *BamHI* sites are underlined. Both PCR products were inserted via their unique *NdeI* and *BamHI* restriction sites into the corresponding sites of pET3a vector, yielding pET3a-azrB and pET3a-azrC respectively.

The *azrB* (accession no. AB471797) and *azrC* (accession no. AB471798) genes encoded protein consisting of 208 and 211 amino acids corresponding to molecular weights of 23 kDa and 22.9 kDa respectively, very close to that of *AzaR* (22.8 kDa).\(^3\) The proteins displayed 30% (AzrB) and 25% (AzrC) primary structure identities against *AzaR*. The putative FMN-binding site and a substrate binding site characterized in *AzOR* from *E. coli*\(^7\) are conserved among *AzaR*, AzrB, and AzrC.

Recombinant strains *E. coli* BL21 (DE3) pLysS carrying expression plasmids pET3a-azrB and pET3a-azrC were grown at 37 °C in 1 liter of LB medium with 1 mM IPTG induction, and the cells were harvested by centrifugation. Crude cell extracts were prepared by sonication of the cells in 100 ml of Tris–HCl buffer (pH 7.4), and the extracts were analyzed by SDS–PAGE (Fig. 1). The clued cell extracts displayed large amounts of recombinant proteins corresponding to the deduced sizes of the gene product.

Azoreductase assay was spectrophotometrically carried out according to measurement of the decolorization of Methyl red (MR) at 430 nm, as described previously.\(^8\) The reaction mixture contained 25 µM MR, 100 µM NADH, and 25 mM Tris–HCl buffer (pH 7.4). One unit of enzyme activity was defined as the amount of enzyme required to decolorize 1 µmol of MR per min at 30 °C under the assay conditions. About 5.4 unit/ml (for AzrB)

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and 3.3 unit/ml (for AzrC) of the activity were detected in 1 liter of crude extracts.

For purification of an azrB gene product, crude cell extracts were applied on a DEAE cellulose column equilibrated with 25 mM Tris–HCl buffer (pH 7.4). After extracts were applied on a DEAE cellulose column in 1 liter of crude extracts.

and 3.3 unit/ml (for AzrC) of the activity were detected in SDS–PAGE of Purified AzrB and AzrC of Fig. 1.

using a linear gradient of NaCl (0 to 0.6M) in the buffer.

for AzrA are homologous with those of azoreductases from other bacteria.7) and from Enterococcus faecalis,8) have been published recently.

1-(2-pyridylazo)-2-naphthol 91.4 0.64 23.2
Acid red 88 27.8 0.12 12.2
Orange I 43.1 0.03 1.26
Orange II 41.1 0.01 0.29
New cossin ND trace 0.1
Sunset yellow FCF 0.26 trace 0.16
Orange G ND ND ND

The values agreed with both enzyme subunit molecular weights as deduced from the gene sequences (23 kDa each). The molecular weights of the native enzymes were calculated to be 48 kDa each by gel filtration (TSK G300SW, Tosoh, Tokyo). These results indicate that AzrB and AzrC acted as homodimeric forms as well as AzrA. The specific activities for MR of the purified enzymes were 38.6 units/mg for AzrB and 38.2 units/mg for AzrC. These values were higher than that for AzrA (26.6 units/mg).

Reaction mixtures containing several concentrations of substrate were used to determine the kinetic parameters of the two enzymes. The Km values for NADH were 36.4 μM for AzrB and 105 μM for AzrC, while those for MR were 37 μM for AzrB and 61.1 μM for AzrC. The activities of both enzymes decreased significantly when NADPH were used to measure enzyme activity (data not shown).

The absorbance spectrum of the purified AzrB and AzrC showed two distinct peaks, at 445 nm and 385 nm, clearly indicating the presence of a flavin prosthetic group. To identify the flavin prosthetic group, AzrB and AzrC were denatured with TCA, and the supernatants including cofactor were analyzed by HPLC, as described previously.23 The retention times of the flavin prosthetic group from supernatants of the two enzymes were identical to that of the FMN standard. These results indicate that AzrB and AzrC bind non-covalently to FMN. Quantitation analysis of FMN and protein revealed that 1 mole of FMN bound per subunit of the enzymes.

The effects of temperature and pH on the activity and stability of the enzymes were examined (Fig. 2). The optimum temperatures were 70 °C for AzrB and 55 °C for AzrC. The enzymes were stable up to 55 °C for AzrB and 50 °C for AzrC after incubation for 1 h at pH 7.4. During incubation at 30 °C for 1 h, the enzymes were stable at pH 5–11 for AzrB, and pH 6.5–10 for AzrC.

The substrate specificities toward several different azo dyes were determined (Table 1). AzrB and AzrC were highly active toward Ethyl red and MR. AzrB had a narrow range of the substrate specificity as compared with AzrA and AzrC. It is of interest to investigate the structural factors affecting the different substrate specificities. The primary structures of AzrB and AzrC with AzrA are homologous with those of azoreductases from E. coli7) and from Enterococcus faecalis,30 and thus the tertiary structures of these enzymes appear to have close similarity. Crystallization and X-ray analysis of the azoreductases from E. coli (AzOR)30,31 and from E. faecalis (AzoA)12) have been published recently.

Here, we describe for the first time detailed enzymatic properties of azoreductases, AzrB and AzrC, from Bacillus sp. B29. Although azoreductases have been found and their enzymes characterized in several
Bacillus strains including Bacillus sp. OY1–2,2) Bacillus sp. strain SF,13) and B. subtilis,4) their enzyme properties were quite different in molecular weight, subunit structure, cofactor requirement, prosthetic group interaction, and several other enzymatic properties from those of AzrA, AzrB, and AzrC from Bacillus sp. B29. Further studies based on structural interpretation are necessary to understand the different enzymatic properties, including substrate specificities.

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References