

REVIEW OF ACTIVITY OF HALOALKANE DEHALOGENASE DBJA FROM *BRADYRHIZOBIUM JAPONICUM USDA 110*

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Abstract

The present work is a review of activity of haloalkane dehalogenase (DbjA). Bacterial strain *Bradyrhizobium japonicum USDA 110* produces such enzyme, which degrades halogenated aliphatic pollutants. The DbjA dehalogenase is highly tolerant to pH and temperature changes. *Bradyrhizobium japonicum USDA 110* displays wide substrate specificity towards 1,2 – dibromoethane.

Key words: *Bradyrhizobium japonicum USDA 110*, haloalkane dehalogenase (DbjA), biodegradation

1. INTRODUCTION

Halogenated compounds are ubiquitous in the environment. Short-chain halogenated aliphatic compounds are manufactured in bulk quantities by the chemical industry. Some of these pollutants are very toxic and cause enormous problems to human health.

1,2-dibromoethane (1,2-DBE) is a synthetic organic chemical that was used primarily in an antiknock additive to gasoline. It is also one of the most effective and widely used pesticide. For a better understanding of the fate and persistence of 1,2-DBE in the environment and for development of bioremediation techniques for the cleanup of polluted locations, it is important to study the physiology and ecology of bacteria that degrade this toxic compound.

Many different organisms have been found that are capable of using halogenated compounds as a growth substrate. Sfetsas et al. 2009 studied different *Rhizobium* strains for their ability to produce stable and active DBE-degrading dehalogenase. Their results showed that *Bradyrhizobium japonicum USDA 110* strain produces the most potent dehalogenase.

Chaloupkova et al. 2011 have recently shown that newly isolated haloalkane dehalogenase DbjA from *Bradyrhizobium japonicum USDA110* possesses new substrate specificity with high catalytic activity towards b-methylated haloalkanes and sufficient enantioselectivity for industrial scale synthesis of optically pure compounds. The efficient utilization of enzymes in industrial processes requires that a number of criteria are fulfilled, e.g. high activity, stability under process conditions, appropriate substrate specificity and enantioselectivity (Bornscheuer et al. 2001, Woodley 2008). Understanding the effect of physical parameters on the structure and activity of an enzyme is important for optimization of the operational conditions of a biocatalytic process.

2. BACTERIA GROWTH CONDITIONS

The bacteria *Bradyrhizobium japonicum USDA 110* was originally isolated from a soybean nodule in Florida, USA in 1957. It is Gram-negative, rod shaped, nitrogen fixing bacteria that forms a symbiotic relationship with *Glycine max*, a soybean plant. Carbon sources come in the form of dicarboxylic acids, succinate, fumarate, and malate. The bacteria provide the plant with fixed nitrogen, which is nitrogen gas that has been reduced and is readily usable by the plant. *Bradyrhizobium japonicum USDA 110* has a symbiotic relationship with legumes, or more specifically soybean plants. It carries out a process called nitrogen fixation in the plant, so that the plant has a usable form of nitrogen.

Bradyrhizobium japonicum USDA110 strain was grown at 28°C on YMB or TY supplemented or not with the appropriate concentration of 1,2-DBE (0.2, 2.0, 5.0 mM). The YMB medium contained the following constituents: K₂HPO₄, 0.5 g/L; Mg₂SO₄·7H₂O, 0.2 g/L; NaCl, 0.1 g/L; yeast extract, 1 g/L; mannitol, 10 g/L. The TY medium contained: 5 g/L tryptone; 3 g/L yeast extract; 10 mM CaCl₂. For

the construction of the growth curves, 25mL starter cultures were used to inoculate 200 mL of YMB to an initial OD₆₀₀ of 0.05 (Sfetsas et al. 2009).

Sato et al. 2005 confirmed that the rhizobial strains and dehalogenases converted 11 halogenated substrates (1-chlorobutane, 2-chloropropane, chlorocyclohexane, 1-chloropropane, 1-chlorodecane, 2-chlorobutane, 1-chloropentane, 1,2-dichloroethane, 1,2-dichloropropane, 1,2,3-trichloropropane, and 1,3-dibromopropane) to the corresponding alcohols. These results suggested that the rhizobial dehalogenases are not used for the assimilation of haloalkanes. Recently, they demonstrated that the products of haloalkane dehalogenase-like genes in other rhizobial strains, also exhibited the dehalogenating activity. Further study of haloalkane dehalogenases in these rhizobial strains will provide some clues to the origin and physiological function of these enzymes. Oligomerization of DbjA is strongly pH dependent. Monomer, dimer, tetramer and a high molecular weight cluster of the enzyme were distinguished in solution at different pH conditions. The ability of biodegradation of *Bradyrhizobium japonicum* USDA110 is shown on Table 1.

Substrate	Product	Commentary Substrate	Literature Substrate	Literature Product
1,2-dibromoethane + H ₂ O	2-bromoethanol + bromide	1100% of the activity with 1-chlorobutane	Sato et al. 2005	Sato et al. 2005
1,3-dibromo-2-methylpropane + H ₂ O	3-bromo-2-methylpropan-1-ol + bromide	desymmetrisation of a dihaloalkane is followed by kinetic resolution of the chiral haloalcohol that is produced in the first step, increase of the enantiomeric excess of the respective haloalcohol	Westerbeek et al. 2012	Westerbeek et al. 2012
1-bromobutane + H ₂ O	n-butanol + bromide	180% of the activity with 1-chlorobutane	Sato et al. 2005	Sato et al. 2005
1-chlorobutane + H ₂ O	n-butanol + chloride	-	Sato et al. 2005	Sato et al. 2005
1-chlorobutane + H ₂ O	1-butanol + chloride	-	Koudelakova et al. 2011	Koudelakova et al. 2011
1-chlorohexane + H ₂ O	n-hexanol + chloride	210% of the activity with 1-chlorobutane	Sato et al. 2005	Sato et al. 2005
1-iodobutane + H ₂ O	1-butanol + iodide	-	Sato et al. 2005	Sato et al. 2005
1-iodohexane + H ₂ O	1-hexanol + iodide	-	Chaloupkova et al. 2011	Chaloupkova et al. 2011
2-bromobutane + H ₂ O	2-butanol + bromide	-	Chaloupkova et al. 2011	Chaloupkova et al. 2011
2-bromopentane + H ₂ O	2-pentanol + bromide	-	Chaloupkova et al. 2011	Chaloupkova et al. 2011
bromocyclohexane + H ₂ O	cyclohexanol + bromide	84% of the activity with 1-chlorobutane	Sato et al. 2005	Sato et al. 2005

Table 1. Biodegradation by *Bradyrhizobium japonicum* USDA110

3. HALOALKANE DEHALOGENASE DBJA

Sfetsas et al. investigated the haloalkane dehalogenase (DbjA) from *Bradyrhizobium japonicum* USDA110. The dehalogenase activity of *B. japonicum* USDA110 and RT-PCR analysis revealed that the DbjA gene expression is induced by 1,2-dibromoethane (1,2-DBE) during the early exponential phase. The DbjA gene was cloned, expressed in *Escherichia coli* BL21 (DE3) and characterized. The results showed that the DbjA displays wide substrate specificity towards haloalkanes and particular high activity towards 1,2-DBE. In addition, consistent with its broad specificity, the DbjA has a substantially larger and more polar active site cavity compared to the *Xanthobacter* and *Rhodococcus*

enzymes and as a consequence. DbjA is able to dehalogenate longer and polar compounds. These properties make this enzyme very promising bioremediation tool for environmental applications. Enzyme biotechnology can provide an attractive solution for the removal of pollutants of such high toxicity.

Koudelakova et al. 2011, described a systematic analysis of the substrate specificities of nine wild-type and four engineered haloalkane dehalogenases. Haloalkane dehalogenases (HLDs) catalyze the hydrolysis of chlorinated, brominated and iodinated alkanes, alkenes, cycloalkanes, alcohols, carboxylic acids, esters, ethers, epoxides, amides and nitrites (Prokop et al. 2010, Damborsky et al. 2001, Schanstra et al. 1996). Analysis of the activity data showed that the most universally-useful substrates in the assessment of haloalkane dehalogenase activity are 1-bromobutane, 1-iodopropane, 1-iodobutane, 1,2-dibromoethane and 4-bromobutanenitrile. Functional relationships among the enzymes were explored using Principal Component Analysis. Analysis of the untransformed specific activity data revealed that the overall activity of wild-type haloalkane dehalogenases decreases in the following order: LinB~DbjA > DhlA~DhaA~DbeA~DmbA > DatA~DmbC~DrbA. After transforming the data, we were able to classify haloalkane dehalogenases into four substrate specificity groups. Structurally, HLDs belong to the α/β -hydrolase superfamily. The HLD family currently includes 14 distinct enzymes with experimentally confirmed dehalogenation activity. A recent analysis of the sequences and structures of these HLDs and their homologs divided the family into three phylogenetic subfamilies, HLD-I, HLD-II and HLD-III, Fig. 1-A, shown differ mainly in the composition of the catalytic pentad and cap domain (Chovancova et al. 2007).

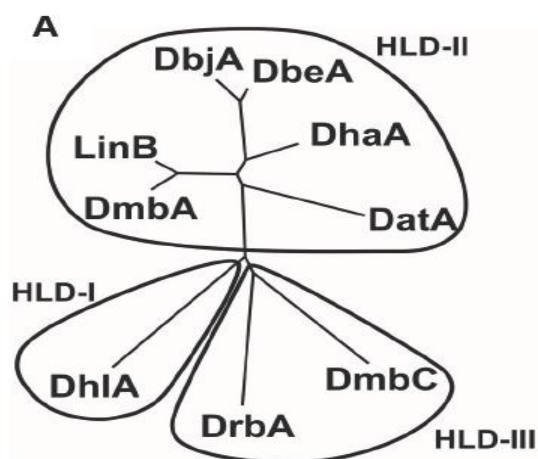


Fig. 1. A-differ mainly domain

4. ANALYTICAL METHODS

The haloalkane dehalogenase activity was assayed by the method of Iwasaki et al. 1952. The halide ions released were measured spectrophotometrically at 460 nm with mercuric thiocyanate and ferric ammonium sulfate. One unit of enzyme activity was defined as that required for the release of 1 μ mol of halide ion per minute. Data are expressed by subtraction of background activities.

Michaelis-Menten kinetic constants were determined by initial velocity measurements (Chaloupkova et al. 2003). The substrate concentration was assessed by a gas chromatography system equipped with a flame ionization detector (Trace GC 2000; Thermo Finnigan) and a DB-FFAP capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m; J&W Scientific). The method described previously by Iwasaki et al. 1952 was used for the determination of the product concentration. The steady-state kinetic constants K_m and k_{cat} were calculated using the computer program Origin 6.1 (OriginLab) (Sato et al. 2005).

The dehalogenase activity of the purified His-tagged DbjA protein in the presence of 18 halogenated compounds was measured spectrophotometrically. The CD spectra of all tested enzymes showed two

negative maxima at 210 and 222 nm that are characteristic for α -helical content. Compared to other haloalkane dehalogenases, DbjA exhibited more intensely negative maxima than the other enzymes.

CD spectra were recorded at room temperature (22 °C) using a Jasco J-810 spectrometer (Jasco, Tokyo, Japan). All the spectra were obtained at an interval of 0.1 nm with a scanning speed of 100 nm·min⁻¹, 1 s response time and 2 nm bandwidth. Cuvettes of 0.1 and 1 cm path length were used in the far and near UV regions, respectively. The protein concentrations for the far UV and the near UV spectra acquisition were 0.23 mg·mL⁻¹ and 1.15 mg·mL⁻¹, respectively (Sato et al. 2005).

5. EFFECT OF PH AND TEMPERATURE

Chaloupkova et al. 2011 investigated that the DbjA enzyme is highly tolerant to pH changes. Its secondary and tertiary structure was not affected by pH in the ranges 5.3–10.3 and 6.2–10.1, respectively. Oligomerization of DbjA was strongly pH-dependent: monomer, dimer, tetramer and a high molecular weight cluster of the enzyme were distinguished in solution at different pH conditions. Moreover, different oligomeric states of DbjA possessed different thermal stabilities. The highest melting temperature ($T_m = 49.1 \pm 0.2$ °C) was observed at pH 6.5, at which the enzyme occurs in dimeric form. Maximal activity was detected at 50 °C and in the pH interval 7.7–10.4.

Measurement of DbjA activity was performed to explore whether catalytic function directly relates to conformational stability at various pH values. Experiments were done under different pH conditions and saturated concentrations of substrate 1-iodohexane for which DbjA exhibited the highest catalytic efficiency. The activity profile of this enzyme shows a maximum at pH 9.7. The secondary structure of DbjA remains intact within five pH units. At lower pH levels (pH < 5.0), the enzyme visually aggregates with simultaneous loss of UV signal. On the other hand, at pH 11.0–11.4, the enzyme stays in solution. A strong negative band at 204 nm and a weak band at 220 nm suggest that DbjA enzyme conformation starts to be disordered at these extremely alkaline conditions. When pH is decreased below 5.3, the enzyme suddenly passes from a nearly native state which is soluble to a completely aggregated state (Chaloupkova et al. 2011). On the other hand, alkaline denaturation of DbjA is accompanied by significant modification of both secondary and tertiary structure. At pH conditions 10.3–11.5, the enzyme occurs in disordered conformation and remains soluble. Britton–Robinson buffer solutions were used to cover the pH range 1.7–11.5. The solutions were prepared by mixing 0.04 M phosphoric, boric and acetic acid with the appropriate volume of sodium hydroxide (0.2 M) and sodium perchlorate monohydrate to get a constant ionic strength of 0.15 M. The assays were performed with 1-iodohexane as the substrate for activity measurement at 37 °C or 2-bromopentane as the substrate for enantioselectivity measurement at 25 °C.

Temperature dependence of conformational stability was evaluated by performing a thermal unfolding experiment at different pH conditions. Dependence of the melting temperature on pH was monitored by CD spectroscopy at 222 nm. A decrease in DbjA thermostability at pH below 6.5 possibly corresponds to the loss of tertiary interactions, as indicated by CD spectra determined in the near UV spectral region. On the other hand, the decrease in the enzyme thermostability at a pH above 6.5 could be attributed to the changes in the protonation state of the enzyme, since no changes in enzyme structure were observed in this pH region.

Measurement of enzymatic activity at different temperatures was carried out to study the effect of temperature on the rate of the dehalogenation reaction. The enzyme exhibited the highest activity at 50 °C, although above this temperature it became rapidly inactivated. This observation is in good agreement with similar experiments previously described for other haloalkane dehalogenases possessing the highest activity at temperatures ranging from 35 to 50 °C (Keuning et al. 1985, Yokota et al. 1987, Jesenska et al. 2002, Jesenska et al. 2005, Jesenska et al. 2009). The effect of temperature on DbjA activity and enantioselectivity was determined by performing activity and enantioselectivity assays at different temperatures. The activity measurements were evaluated at temperatures ranging from 20 to 60 °C and the enantioselectivity of the DbjA enzyme was monitored in the temperature range 20–50 °C. Activity measurements were performed with 1-iodohexane, and enantioselectivity

measurements with 2-bromobutane, 2-bromopentane and methyl 2-bromopropionate and ethyl 2-bromobutyrate.

6. CONCLUSION

On the ground of this review we have come to the following conclusions:

1. *Bradyrhizobium japonicum* USDA 110 degrade successfully halogenated aliphatic compounds.
2. The haloalkane dehalogenase (DbjA) is more efficient than other enzymes from α/β -hydrolase superfamily.
3. The highest thermostability occurred when the enzyme is in its dimeric form.
4. The best results were obtained at 50⁰ C and in the pH interval (7,7-10,4).

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