Journal of Biochemistry and Molecular Biology, Vol. 31, No. 2, March 1998, pp. 130–134

JOURNAL OF
Biochemistry
and
Molecular Biology

Overproduction, Purification, and Characterization of Heat Stable Aldolase from *Methanococcus jannaschii*, a Hyperthermophic Archaea

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Received 24 October 1997

An aldolase gene has been cloned from *Methanococcus jannaschii*. The coding region of the gene has been expressed in *E. coli* using a pET system to a level of 30% of total cellular proteins. The protein was purified to more than 95% homogeneity by heat treatment and ion exchange chromatography. The protein performed an aldol condensation reaction with glyceraldehyde as substrate and dihydroxyacetone phosphate as a carboxyl donor. The protein was determined to be a type II aldolase which requires the Zn^{2+} ion as a metal cofactor. This enzyme has a broad range of optimum pH (7–9) and temperature (50–80°C). It shows strong stability against heat, chemical denaturants, as well as a high percentage of organic solvents. The half-life of this enzyme at 85°C is more than 24 h and it maintains more than 90% of aldolase activity in the presence of 6 M urea, 50% acetonitrile, or 15% isopropyl alcohol.

Keywords: Aldolase, Fuculose, Hyperthermophile, *Methanococcus jannaschii*

Introduction

Aldolases catalyze the aldol condensation of an aldehyde and a ketone to produce a stereo-specific carbohydrate compound. These enzymes are grouped into two types. Type I aldolases are found mostly in animals or plants and they do not require any metal cofactors. The lysine in the active site of the type I enzyme forms a Schiff base with the donor substrate. Most aldolases from microorganisms are type II aldolases which require metal ions such as Zn^{2+} ion as a cofactor. The Zn^{2+} ion in type II aldolase serves as the Lewis acid in the active site. Fructose-1,6-diphosphate aldolase (Osten *et al.*, 1989), rhamnulose-1-phosphate aldolase, and fuculose-1-phosphate aldolase (Fessner *et al.*, 1991) have been tested for synthesis of stereo-selective enzymatic aldol addition. Among them, fuculose-1-phosphate aldolase, belonging to type II aldolase, catalyzes aldol condensation between dihydroxy acetone phosphate (DHAP) and L-lactaldehyde to produce fuculose-1-phosphate. This enzyme has been identified and cloned in *E. coli* and assumed to be involved in the dissimilation of fuculose (Ghalambor and Heath, 1962; Chen, *et al.*, 1989). The fuculose-1-phosphate aldolase accepts various aldehydes to produce vicinal diols with D-threo configuration (Fessner *et al.*, 1991).

Chemical synthesis using enzymes as reaction catalysts has several advantages over conventional synthetic methods, especially for environmental safety and stereo-selectivity of products. Aldolase has been attractive as a synthetic catalyst due to its ability to form a C–C bond and to produce stereo-specific carbohydrates (Wong *et al.*, 1995). Until now, only a few aldolases, including fuculose-1-phosphate aldolase from *E. coli*, have been tested for their possible application in synthetic chemistry (Osten *et al.*, 1989; Ozaki *et al.*, 1990; Fessner *et al.*, 1991).

Recently, organisms grown in extreme conditions have been the target of interest as a new sources of enzymes for industrial applications (Adam, 1993). For heat stable enzymes, hyperthermophiles that grow at temperature higher than 80°C are considered as good resources for such enzymes. *Methanococcus jannaschii*, one of the hyperthermophilic archaea whose optimum growth temperature is 85°C, has been studied since it was isolated from deep sea vents. The whole genome sequence has been determined, as well as its open reading frames, and their possible functions were assigned according to their sequence similarity to functionally characterized genes (Bult *et al.*, 1996). This organism was revealed to have only one aldolase that shows a strong sequence similarity

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to fuculose-1-phosphate aldolase from *E. coli*. Aldolase is an essential enzyme in central energy metabolism, such as fructose-1,6-diphosphate aldolase in glycolysis. Although *M. jannaschii* also has several enzymes related to the glycolytic pathway, based on the whole genome analysis, its only aldolase is not fructose-1,6-diphosphate aldolase but fuculose-1-phosphate aldolase.

Here, we report a rapid cloning of the aldolase homologue from *M. jannaschii*, by an over-expression and simple purification procedure. The purified protein has aldolase activity and shows unusually strong stability against heat, denaturants, and organic solvents.

Materials and Methods

Cell culture and plasmids *M. jannaschii* was obtained from Deutsch Sammlung von Mikroorganismen (DSM #2661). The cells were inoculated in 20 ml of medium in 120-ml bottles under 3 bar of CO_2 and H_2 gas in 1:4 (v/v) ratio, as previously described (Ferrante *et al.*, 1990). Cells were harvested after growth at 85°C for 12 h with moderate shaking and stored at −80°C. *E. coli* strains DH5α and BL21(DE3) were used for plasmid amplification and protein expression, respectively. The *E.coli* cells were grown in LB medium containing 100 μg/ml ampicillin at 37°C with vigorous shaking. For the construction of the expression vector, pET21a (Novagene, Madison, USA) was used. The general method for handling plasmid DNA was used as previously described (Sambrook *et al.*, 1989).

Genomic DNA isolation and PCR amplification The genomic DNA of *M. jannaschii* was obtained from whole cell extract, as described below. About 0.1 g (wet weight) of *M. jannaschii* cell paste was resuspended with 11 ml of lysis buffer (0.2 mg/ml RNase A, 50 mM EDTA, 50 mM Tris-HCl, 0.5% Tween-20, 0.5% Triton X-100, pH 8.0) and incubated at 50°C for 12 h in the presence of 1 mg/ml of proteinase K and 2.5 mg/ml of lysozyme. After removing the cell debris by centrifugation at 12,000 × *g* for 10 min, nucleic acids were precipitated by adding 2 volumes of ethanol. The DNAs were centrifuged and resuspended with TE buffer. The coding region of *Mj* aldolase (MJ#1418, Bult *et al.*, 1996) was amplified by PCR reaction directly from the purified genomic DNA of *M. jannaschii* using a 5′-primer (5′-GGGCCCGGGCATATGGACAAAAAGCAATTTATTAAAAT-3′) and a 3′-primer (5′-CCGGGCCGGATCCCTATTTC-TTTACAAGAAGGTTTAAAAGTG-3′) with *Pfu* DNA polymerase (New England Biolab., Beverly, USA) with 25 cycles of denaturation (1 min, 95°C), annealing (1 min, 55°C), and polymerization (1.5 min, 72°C) reaction. The amplified DNA was further purified by ethanol precipitation.

Construction of expression vector The amplified DNA fragment and pET21a plasmid DNA were restricted with *Nde*I and *BamH*I enzymes. The restricted DNAs were mixed and ligated with T4 DNA ligase and incubated overnight at 16°C. The DNAs were transformed into *E. coli* (DH5α) and the colony with recombinant plasmid isolated, and the sequence of the inserted DNA was determined by the dideoxy chain termination method using a DNA sequencer (Applied Biosystems, Foster City, USA).

Expression and purification of *M. jannaschii* fuculose-1-phosphate aldolase The constructed expression vector was transformed into BL21(DE3) cells and grown in LB medium at 37°C with vigorous shaking to an OD_{600} of 0.7. Induction of protein expression was initiated by adding IPTG (isopropylthio-β-galactoside) to a final concentration of 0.5 mM. After 3 h growth, the cells were harvested by centrifugation at 4000 × *g* for 10 min. About 10 g of cell pellet was resuspended in 30 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) and passed through a French pressure cell three times at 12,000 psi. The cell lysate was then centrifuged for 10 min at 12,000 × *g* and the cell debris in the pellet fraction was discarded. The crude extract was incubated at 90°C for 20 min and the denatured proteins then removed by centrifugation at 15,000 × *g* for 20 min. The supernatant was loaded onto a Q-sepharose column (1.5 × 15 cm; Pharmacia, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl, pH 8.0. 400 ml of a 0–0.5 M NaCl linear gradient in the equilibrating buffer was applied and the eluted proteins were collected in 5 ml fractions.

Assay The aldolase activity was measured by determining the remaining DHAP, as described by Ghalambor *et al.* (1962), with the following modification. The reaction mixture contained 1.25 mM dihydroxy phosphate, 2.5 mM DL-glyceradehyde in 100 mM Tris-HCl, 0.1 mM $ZnCl_2$, pH 8.0. The reaction was initiated by adding 1–5 μg of protein to the reaction mixture and incubated at 37°C. After 10 min incubation, one tenth of the sample was transferred to 1 ml of the secondary reaction solution containing 0.1 units of α-glycerophosphate dehydrogenase (Sigma, St. Louis, USA), 0.2 mM NADH in 100 mM Tris-HCl, 50 mM EDTA, pH 7.5. After 5 min incubation at 37°C, the absorption of the reaction mixture at 340 nm was measured using a UV–Vis spectrophotometer (Shimadzu, Tokyo, Japan). The amount of DHAP was calculated from the reduced amount of NADH by dehydrogenase. By comparison of the amount of DHAP before and after the aldolase reaction, the specific activity of aldolase was calculated. The protein assay was performed using the Bradford method as described in the instruction manual (Bio-Rad, Hercules, USA).

Results and Discussion

Cloning and sequence analysis of fuculose-1-phosphate aldolase The aldolase gene from *M. jannaschii* was cloned directly from genomic DNA by PCR amplification into an expression vector. The PCR primers were designed from the reported sequence of whole genomic DNA of *M. jannaschii*. The sequence of the cloned aldolase was identical to the reported sequence. The amino acid sequence of the cloned gene was compared with those of other reported genes in the PIR database. Among them, the aldolases were found to be closely related genes and a fuculose-1-phosphate aldolase from *H. influenza* had the highest value of sequence identity (34.3%). The fuculose-1-phosphate aldolase is a type II aldolase and requires Zn^{2+} ion as a metal cofactor. The crystal structure of *E. coli* enzyme complexes with Zn^{2+} ion showed three His residues as metal binding sites (Dreyer and Schulz, 1996).

The amino acid sequence around these three histidines was well conserved among the type II aldolase genes as well as the *M. jannaschii* aldolase gene (Fig. 1). It is noticeable that *Mj* aldolase is about 30 amino acids shorter than the *E. coli* enzyme. Since the C-terminus region is important for determining substrate specificity (Sygusch *et al.*, 1987), *Mj* aldolase lacking that region may have broader substrate specificity than the *E. coli* enzyme. If the cloned aldolase is the only aldolase gene in *M. jannaschii*, this enzyme may perform aldol condensation or a reverse reaction with diverse forms of aldehydes in the cell to generate a variety of carbohydrates.

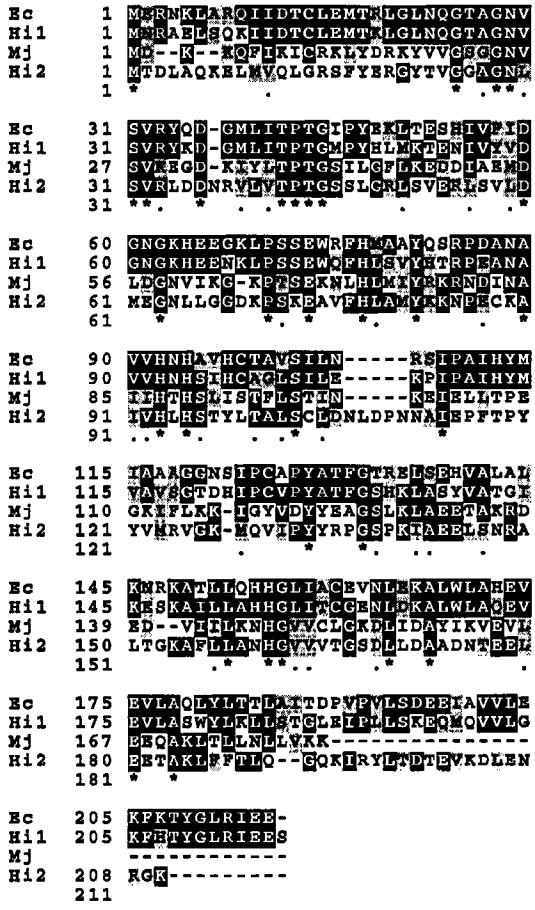

Fig. 1. Sequence comparison of *Mj* aldolase with other aldolases. Each title represents the amino acid sequence as followed. Ec; fuculose-1-phosphate aldolase (fucA) of *Escherichia coli* (PIR #ADECFP), Hi1; fuculose-1-phosphate aldolase (fucA) homologue of *Haemophilus influenza* (PIR #C64081), Mj; fuculose-1-phosphate aldolase homologue of *Methnoccocus jannaschii* (PIR #A64477), Hi2; fuculose-1-phosphate aldolase (fucA) homologue of *Haemophilus influenza* (PIR #B64108). The sequences were aligned using the "clustalw" program (Thompson *et al*, 1994). Conserved regions with identical amino acid sequences are indicated as black boxes and the region with similar amino acids as gray boxes. Amino acids which are identical with *Mj* aldolase are marked as an asterisk.

Expression and purification The coding region of *Mj* aldolase was expressed using a pET expression system. The expressed protein was recovered in a soluble form and the level of expression reached about 30% of total cellular protein after 3 h of induction. Heat treatment of the crude extract for 20 min at 90°C was sufficient to denature most of the *E. coli* proteins except for the expressed protein. After removal of the denatured protein by centrifugation, the purity of expressed protein reached about 80%. Additional ion exchange chromatography was enough to purify the protein with 95% homogeneity (Fig. 2). The final yield of the purified protein was 20 mg/l of culture.

Aldolase activity The purified protein was tested for substrate affinity to DHAP in the presence of glyceraldehyde. The optimum temperature of the enzyme was measured by determining the conversion rate of DHAP at different temperatures. The removal rate of DHAP increased according to the temperature. However, DHAP was also decomposed at high temperatures. When the amount of thermally decomposed DHAP was subtracted, the enzyme showed the highest conversion rate at around 50°C (data not shown). Since the rate of thermal decomposition of DHAP under 37°C was less than 10%, this temperature was used for further characterization. When the activity of the enzyme was measured at different pH, the highest activity was in the range of pH 7.5 to 9 (Fig. 3). The aldolase showed at least 30% of activity at one unit below pH 7 or above pH 9. *Mj* aldolase also required Zn^{2+} ions. Removal of the metal ion by EDTA treatment abolished the aldolase activity whereas addition of 0.1 mM of Zn^{2+} ion restored the activity (data not shown). These indicated that the enzyme is type II aldolase and requires Zn^{2+} ion.

Stability of *Mj* aldolase The thermostability of the *Mj* aldolase was characterized by measuring the residual

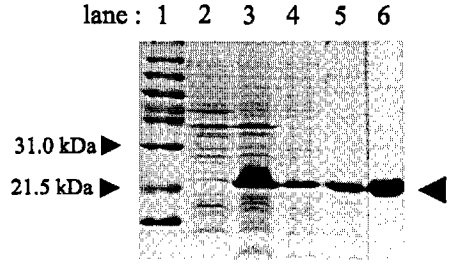

Fig. 2. Expression and purification of *Mj* aldolase. Proteins at different stages of expression and purification were analyzed using 15% SDS-PAGE. Lane 1; size marker, Lane 2; total cell extract of uninduced culture, Lane 3; total cell extract of induced culture, Lane 4; crude extract of induced culture, Lane 5; heat treated crude extract of induced culture at 90°C for 10 min. Lane 6; Purified aldolase after Q-sepharose column separation. The bold arrow indicates the expressed *Mj* aldolase.

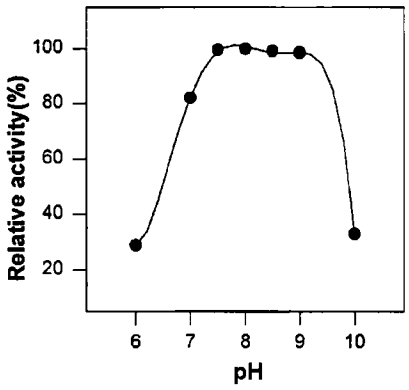

Fig. 3. Optimum pH of *Mj* aldolase activity. The aldolase assay was performed as described in Materials and Methods except that the reaction buffer was 0.1 M Tris-HCl with pH values of between 7 to 9. For buffers of pH 6 and 10, 100 mM sodium phosphate and 100 mM sodium borate were used, respectively.

activity of the enzyme after incubation at different temperatures. At 10 mM of Tris-HCl buffer, the protein denatured rapidly ($t_{1/2}$ = 40 min). However, the stability of the protein dramatically increased in the presence of potassium phosphate ($t_{1/2}$ = 24 h) (Fig. 4). The stabilization of proteins from *Methanopyrus kandleri,* a hyperthermophilic methanogen, by high concentration of lyotropic salts (KH_2PO_4 or ammonium sulfate), has been observed (Ermler *et al.*, 1997). Formylmethanofuran tetrahydromethanopterin formyltransferase (Ftr) from *M. kandleri* has an unusually large number of charged amino acids and an excessively negative charge or highly local concentration of charged amino acids on the protein surface. A high concentration of lyotropic salt may bind to the charged surface and facilitate oligomerization, which is required for activity and stability. Since aldolases are present as tetramers (Dreyer and Schulz, 1996), high concentrations of lyotropic salt may increase the thermostability of *Mj* aldolase by a similar mechanism as for the Ftr of *M. kandleri*. In the presence of 0.5 M KH_2PO_4, the *Mj* aldolase also showed strong resistance against denaturants or organic solvents. It maintained more than 90% activity in the presence of 6 M urea, 50% acetonitrile. However, guanidine ions or isopropyl alcohol affected the stability of *Mj* aldolase at lower concentrations than urea or acetonitrile (Table 1). The stability of *Mj* aldolase against heat, denaturants, or organic solvents may be valuable for utilization of the enzyme in enzyme-catalyzed chemical synthesis. The substrate specificity and stereo-specificity of the *Mj* aldolase will be investigated.

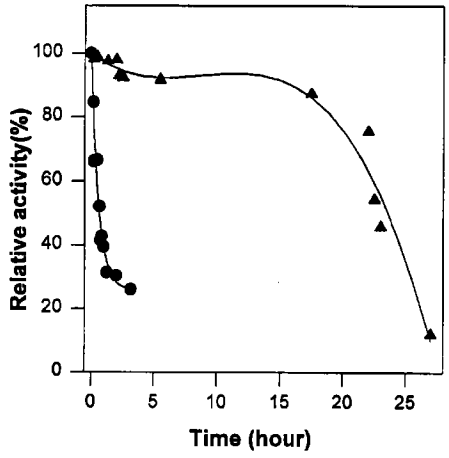

Fig. 4. Thermostability of *Mj* aldolase. The purified *Mj* aldolase was incubated at 85°C in 10 mM Tris-HCl (●) or 0.5 M KH_2PO_4 buffer containing 0.1 mM $ZnCl_2$ (▲) for the indicated time intervals. The remaining activity of the *Mj* aldolase was measured and compared with the *Mj* aldolase activity before heat treatment.

Table 1. Stability of *Mj* aldolase against denaturants and organic solvents.

Denaturants			
Urea (M)	Relative activity (%)	Guanidine HCl (M)	Relative activity (%)
0	100	0	100
0.5	101	0.5	89.6
2	97.4	1	59.9
4	88.5	2	28.6
6	91.1	6	9.9

Organic solvents			
Isopropyl alcohol (%)	Relative activity (%)	Acetonitrile (%)	Relative activity (%)
0	100	0	100
5	94.3	5	109
15	96.9	15	123
30	76.0	30	120
50	53.1	50	96

Acknowledgments This work was supported by a grant from the Ministry of Science and Technology, Korea.

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