Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 23 (2003) 65–70
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Invited review

Paradoxical thermostable enzymes from psychrophile: molecular characterization and potentiality for biotechnological application

Tadao Oikawa [a,b,*], Takayuki Kazuoka [a], Kenji Soda [a]

[a] *Department of Biotechnology, Faculty of Engineering, Kansai University, Suita, Osaka-fu 564-8680, Japan*
[b] *Kansai University High Technology Research Center, Suita, Osaka-fu 564-8680, Japan*

Received 27 January 2003; received in revised form 26 March 2003; accepted 28 March 2003

On the occasion of the 70th birthday of Kenji Soda

Abstract

NAD(P)$^+$-dependent aldehyde dehydrogenase (EC 1.2.1.5) and aspartase (EC 4.3.1.1) in the cells of an atypical psychrophile from Antarctic seawater, *Cytophaga* sp. KUC-1, were paradoxically thermostable, although they derived from a psychrophile. Both enzymes showed the highest activity at about 55 °C, and also active even under cold conditions. The enzymes contained more Ile residues than the enzymes from mesophiles. The Ile/Ile + Val + Leu ratio of the *Cytophaga* thermostable enzymes was much higher than that of the enzymes from mesophiles. As compared with the enzymes from other microorganisms, the *Cytophaga* thermostable enzymes have the structural differences in the C-terminal region of the enzymes. Therefore, the C-terminal region might be important for the paradoxical thermostability of the enzymes. The psychrophilic microorganism produces not only psychrophilic enzyme, but thermostable enzyme with psychrophilicity. Therefore, the psychrophilic microorganism is one of the candidates for isolation of novel biocatalysts, which have potential for various industrial applications.

Keywords: Thermostable enzyme; Psychrophile; Aldehyde dehydrogenase; Aspartase

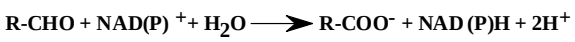
1. Introduction

Psychrophiles and psychrotolerants are found widely in natural and artificial cold environments, and produce a variety of psychrophilic enzymes to carry out metabolism efficiently under cold conditions. Most of the enzymes from psychrophiles so far studied are thermolabile [1–8]. Various enzymes from psychrophiles and psychrotorelants have been studied, but aldehyde dehydrogenase and aspartase from psychrophile have not been known at all. Both enzymes have strong potential for various biotechnological applications, such as biotransformation, bioremediation, and so forth. Schemes 1 and 2 showed the catalytic mechanisms of aldehyde dehydrogenase (aldehyde dehydrogenases, EC 1.2.1.3-7) and aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1). Aldehyde dehydrogenase catalyses the irreversible dehydrogenation of aliphatic and aromatic aldehydes to the corresponding organic acids in the presence of NAD(P)$^+$, and aspartase plays an important function as a key enzyme for conversion between organic and inorganic nitrogen.

A psychrophile, *Cytophaga* sp. KUC-1 isolated from Antarctic seawater, grows optimally at 15 °C and cannot grow above 30 °C. This organism strangely

* Corresponding author. Tel.: +81-6-6368-0812; fax: +81-6-6388-8609.
E-mail address: oikawa@ipcku.kansai-u.ac.jp (T. Oikawa).

1381-1177/03/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1381-1177(03)00073-0



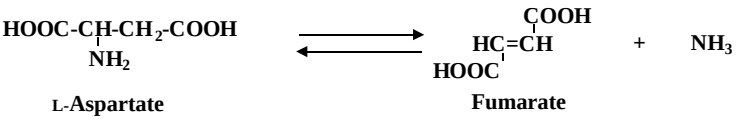
Scheme 1. Enzymatic reaction of aldehyde dehydrogenase.

produces paradoxical thermostable $NAD(P)^+$-dependent aldehyde dehydrogenase and aspartase. Except for our recent studies, there is no report of thermostable enzyme derived from psychrophilic microorganisms. This review describe the enzymological characteristics and functional properties of the thermostable enzymes from the psychrophile and their potential for biotechnological application.

2. Molecular strategies for thermal stability

Cytophaga aldehyde dehydrogenase and aspartase were paradoxically thermostable, and showed the highest activity at about 55 °C and were also active even under cold conditions (Fig. 1). However, other enzymes from psychrophiles including *Cytophaga* KUC-1 so far studied are active under only cold conditions, and most of them are naturally thermolabile. For instance, *Cytophaga* valine dehydrogenase is most active at 35 °C, and rapidly inactivated above 40 °C [7]. The activation energy of *Cytophaga* aldehyde dehydrogenase and aspartase were lower than that of mesophiles. The *Cytophaga* aldehyde dehydrogenase showed a discontinuity in Arrhenius plots and a transition temperature is 32 °C (Fig. 1(A)). In the higher temperature range, the value of activation energy was calculated as 27 kJ/mol, whereas in the lower range the value was about 57 kJ/mol. Even in the high temperature range, the activation energy of the *Cytophaga* aldehyde dehydrogenase is lower than that of the enzyme from a mesophile, *Saccharomyces cerevisiae* (76.6 kJ/mol), which shows no transition temperature [9]. The lower activation energy of the *Cytophaga* enzymes is advantageous for catalyzing the reaction under low temperature and even high temperature conditions. The T_m value of *Cytophaga* aspartase was calculated to be 56 °C, which is similar to that of *Cytophaga* aldehyde dehydrogenase (58 °C).

The *Cytophaga* aspartase shows similarity to the *Escherichia coli* enzyme (55%) and the thermostable



Scheme 2. Enzymatic reaction of aspartase.

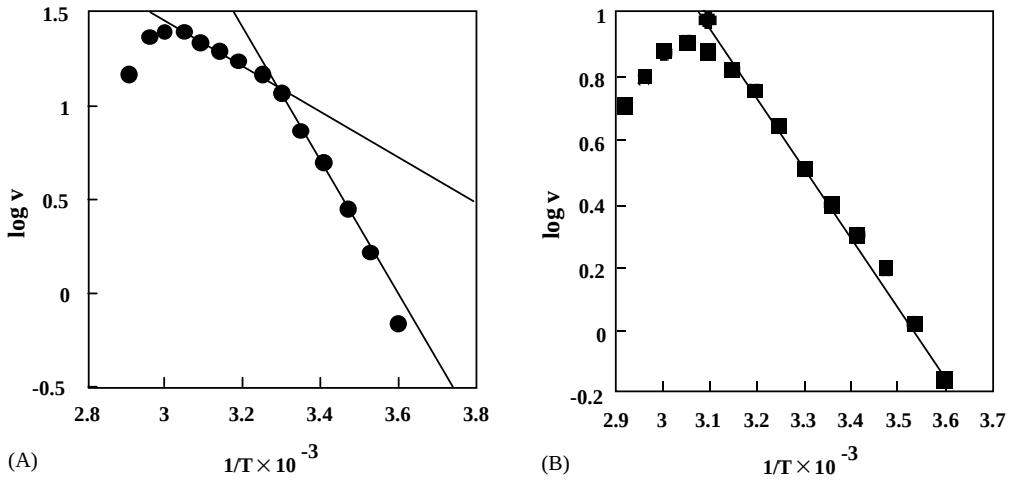


Fig. 1. Arrhenius plots of aldehyde dehydrogenase and aspartase: (A) aldehyde dehydrogenase; (B) aspartase.

Bacillus YM55-1 enzyme (49%), and if similar residues are included, the sequence similarity scores are increased to 70% (the *E. coli* enzyme) and 67% (the *Bacillus* YM55-1 enzyme). It is interesting that the *Cytophaga* enzyme is similar to both thermophilic and mesophilic enzymes. This indicates that three enzymes have similar three dimensional structures with different thermostabilities, and the difference of thermal stability between these enzymes was probably derived from a few changes in primary structure.

The amino acid composition of the *Cytophaga* thermostable enzymes was compared with those of the enzymes from mesophile and thermophile. The amino acid composition of the *Cytophaga* enzyme is rich in branched amino acids. The *Cytophaga* enzyme contained more Ile residues than the enzymes from mesophiles. The Ile content of *Cytophaga* aspartase was similar to that of the thermostable aspartase from *Bacillus* sp. YM55-1 (41 residues, 8.74%). The Ile/Ile + Val + Leu ratio of the *Cytophaga* enzymes was much higher than those of the enzyme from mesophiles. Generally, the thermal stability of protein is increased with an increase in hydrophobicity [10], helix stability [11,12], and tight packing [13] and ionic interactions of the enzymes [14,15]. Therefore, as compared with the mesophilic enzyme, the hydrophobic interactions of *Cytophaga* thermostable enzymes increase, and Ile residue is more effective in than Val and Leu residues to increase packing internal interactions of the enzyme [10].

The *Cytophaga* thermostable aldehyde dehydrogenase and aspartase show more markedly the structural differences in the C-terminal region of the enzymes from other microorganisms. The C-terminal region of aldehyde dehydrogenase is known to affect the subunit interaction, and to be involved in the thermal stability of aldehyde dehydrogenases [15]. In C-terminal region of human aldehyde dehydrogenase, Ser 500 of sub-

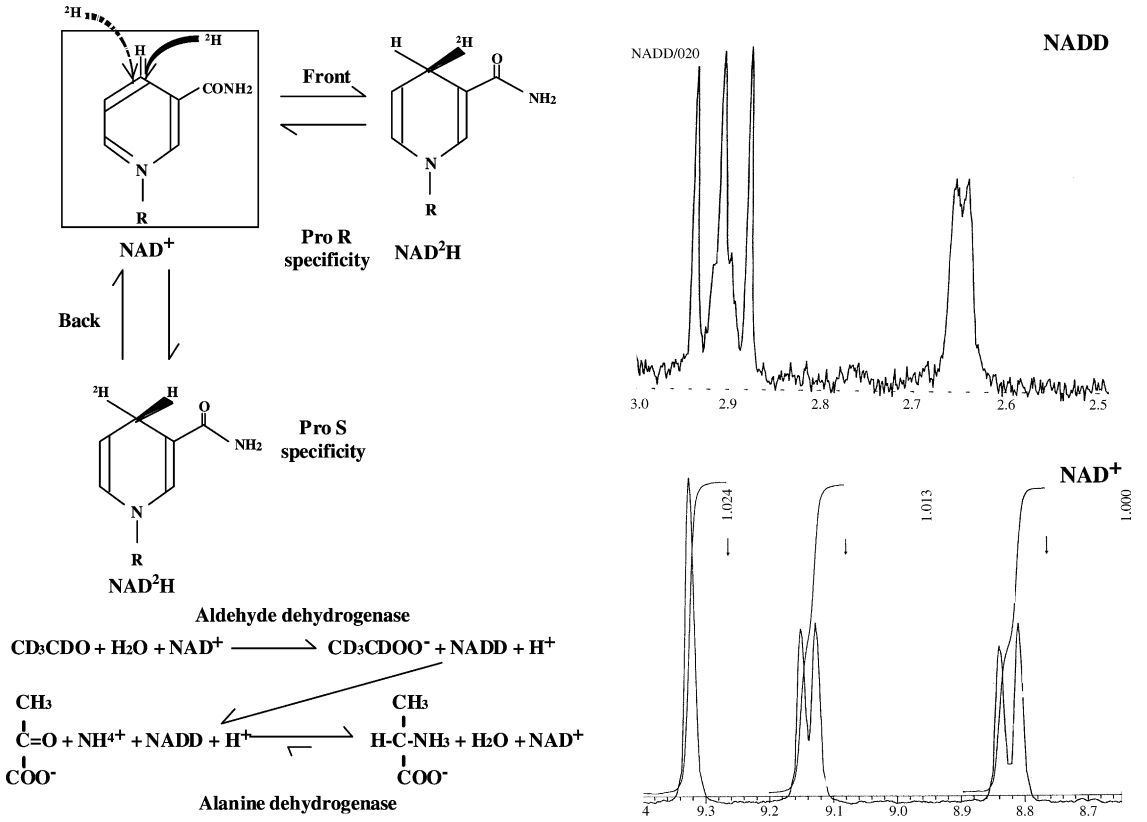


Fig. 2. Determination of hydride transfer stereospecificity of aldehyde dehydrogenase.

unit "D" interacts with Arg 84 of subunit "A" [16]. In case of *Cytophaga* aldehyde dehydrogenase, the Arg 84 of human aldehyde dehydrogenase was conserved at the position of 75, but the Ser 500 was replaced by Leu 498. This is probably attributed to the difference in subunit structure of these enzymes: human aldehyde dehydrogenase has a tetramatic structure, while *Cytophaga* aldehyde dehydrogenase is dimer. The C-terminal region of aspartase also plays an important for helix and thermal stability. The *Cytophaga* enzyme contains more α-helix structure than the *E. coli* enzyme. The C-terminal domain of the *E. coli* enzyme is composed of residues 397–459, and forms the smallest domain among three domains in the subunit [17]. It consists mainly of two helix-turn-helix motifs. An increase in the α-helix structure in the C-terminal domain of *Cytophaga* enzyme increases a helix stability, and this is probably regarded as a reason for atypical thermostability of the enzyme. Accordingly, the C-terminal region probably functions to the atypical thermostability of *Cytophaga* aldehyde dehydrogenase and aspartase. *Cytophaga* aspartase has also other strategies to increase the thermal stability. The Asp 29, which recognizes the β-carboxy group of the L-aspartate, is likely involved in a hydrogen-bonding network, and stabilizes the active site [17,18]. Additionally, Asn 217 in the *E. coli* enzyme is replaced by Arg 215 in the *Cytophaga* enzyme. The mutant enzyme of *E. coli* whose Asn 217 is replaced by Arg, increases its thermostability [19]. An Arg residue forms higher ionic interaction of the enzyme than Asn residue, and eventually increases thermostability [20,21].

3. Enzymological characteristics and potentiality for application

The stereospecificity of *Cytophaga* aldehyde dehydrogenase for the hydride transfer at C4 of nicoti-

Table 1
Substrate specificity of aldehyde dehydrogenase

Substrate	Relative activity(%)				
	Cytophaga sp. KUC-1 ALDH (EC 1.2.1.5)	Human liver ALDH (EC 1.2.1.1)	Rat cornea ALDH (EC 1.2.1.3)	*Acetobacter* ALDH (EC 1.2.1.4)	*A. calcoaceticus* ALDH (EC 1.2.1.7)
Formaldehyde	51.7	100	–	0.88	0
Acetaldehyde	51.2	0	0.30	100	0
Propionaldehyde	74.6	0	30.30	42	0
Butylaldehyde	23.1	–	–	58	0
Isobutylaldehyde	187.5	–	18.73	–	–
Valeraldehyde	16.3	–	–	–	–
Isovaleraldehyde	82.7	–	–	–	–
Hexanal	21.5	–	26.59	–	39.74
Heptanal	11.8	–	–	–	–
Octanal	9.5	–	26.59	–	37.18
Benzaldehyde	100	0	100	0.88	100
Cuminaldehyde (*p*-isopronyl benzaldebyde)	15.4	–	–	–	0
p-Chlorobenzaldehyde	35.4		76.74	–	–
m-Chlorobenzaldehyde	35.3	–	–	–	–
o-Chlorobenzaldehyde	0	–	–	–	0
p-Fluorobenzaldehyde	74.6	–	–	–	89.74
m-Fluorobenzaldehyde	40.6	–	–	–	56.41
o-Fluorobenzaldehyde	100.1	–	–	–	0
p-Bromobenzaldehyde	13.5	–	–	–	–
m-Bromobenzaldehyde	27.5	–	–	–	–
o-Bromobenzaldehyde	0	–	–	–	0
Anisaldehyde (4-methoxyhenzaldehyde)	18.0	–	–	–	67.95
DL-glyceraldehyde	13.8	–	–	–	0

Table 2
Substrate specificity of aspartase

Substrates	Relative activity (%)	Substrates		Relative activity (%)
20 mM		10 mM	100 mM	
(A) Deamination		(B) Amination		
L-Aspartate	100	Fumarate	NH_4Cl	100
D-Aspartate	0	Fumarate	NH_2OH	64
α-Methyl-DL-aspartate	0	Fumarate	NH_2CH_3	0
DL-*threo*-Hydroxy-aspartate	0	Mesaconate	NH_4Cl	0
L-Asparagine	0	Maleate	NH_4Cl	0
L-Alanine	0			
L-Glutamate	0			
L-Cysteine sulfonate	0			
L-Cysteine sulfinate	0			

namide moiety of NAD^+ was shown in Fig. 2 [*C*4-^{2}H] NADH was produced from NAD^+ and C^2H_3 C^2HO by catalysis of *Cytophaga* aldehyde dehydrogenase, and the resonance was found at a chemical shift of δ 2.65 ppm. Furthermore, the [*C*4-^{2}H] NADH produced was oxidized by *B. sphaericus* L-alanine dehydrogenase with *pro-R* specificity [8], and the NAD^+ produced was also detected. These data suggest that the *Cytophaga* enzyme shows the *pro-R* stereospecificity [22–24] and this reaction system can be applicable to other dehydrogenases to determine the stereospecificity for the hydride transfer at the C4 site of nicotinamide moiety of NAD^+.

The *Cytophaga* aldehyde dehydrogenase shows a low substrate specificity. The *Cytophaga* aldehyde dehydrogenase shows a similar substrate specificity to the *S. cerevisiae* enzyme [9]: both acts on various aliphatic and aromatic aldehydes (Table 1). However, the *S. cerevisiae* enzyme preferably acts on short and straight chain aliphatic aldehydes and shows low activity on aromatic aldehydes, in contrast the *Cytophaga* enzyme effectively reacts with aldehydes with bulky side chains such as isobutylaldehyde and benzaldehyde. The *Cytophaga* enzyme uses either NAD^+ or $NADP^+$ as a coenzyme. These characteristics of the *Cytophaga* enzyme are at an greater advantage than the other aldehyde dehydrogenases to synthesize various kinds of organic acids from the carbonyl counterparts. In contrast to the low substrate specificity of *Cytophaga* enzyme aldehyde dehydrogenase, *Cytophaga* aspartase only acts on L-aspartate: D-aspartate, α-methyl-DL-aspartate, DL-*threo*-β-hydroxyaspartate, L-asparagine, L-alanine, L-glutamate, L-cysteine, L-cysteine sulfinate were inert (Table 2). When fumarate was an amino acceptor, ammonium chloride and hydroxylamine served as an amino donor, but methylamine was not active, and fumarate was exclusively used as an amino acceptor (Table 2). This high substrate specificity of *Cytophaga* aspartase is useful for specific determination of aspartate and fumarate in various biological systems under low and even high temperature conditions.

4. Conclusion

The wide temperature range of catalytic ability shown by thermostable *Cytophaga* enzymes is useful for various biotechnological applications, since they have both psychrophilicity and thermostability. Most enzymes from psychrophile are only active under low temperature conditions, and are not suitable at room temperatures for long time operation in industry. In this study, we have shown that psychrophilic microorganism produces not only psychrophilic enzyme but thermostable enzyme with psychrophilicity. Therefore, the psychrophilic microorganism is regarded as one of hopeful candidates for a source of new type of biocatalysts, which are applicable for various industrial purposes.

The *Cytophaga* aldehyde dehydrogenase and aspartase were recently crystallized to undergo X-ray crystallographic study. The solution of three-dimensional structure of *Cytophaga* enzymes is very interest-

ing to understand the relationship between structure and paradoxical thermostability of the psychrophilic enzymes.

Acknowledgements

This work was supported in part by the Research Grant from Japan Foundation of Applied Enzymology, by the Research Grant from Kansai University High Technology Research Center and by the Science Research Promotion Fund from the Japan Private School Promotion Foundation.

References

[1] F. Rentier-Delrue, S.C. Manned, S. Moyens, P. Terpstra, V. Mainfroid, K. Goraj, M. Lion, W.G. Hol, J.A. Martial, J. Mol. Biol. 229 (1993) 85.
[2] K. Yokoigawa, H. Kawai, K. Endo, Y.H. Lim, N. Esaki, K. Soda, Biosci. Biotechnol. Biochem. 57 (1993) 93.
[3] I. Tsigos, K. Velonia, I. Smonou, V. Bouriotis, Eur. J. Biochem. 254 (1998) 356.
[4] U. Gerike, M.J. Danson, N.J. Russell, D.W. Hough, Eur. J. Biochem. 248 (1997) 49.
[5] H. Tsuruta, S.T. Tsuneta, Y. Ishida, K. Watanabe, T. Uno, Y. Aizono, J. Biochem. (Tokyo) 123 (1998) 219.
[6] G. Feller, F. Payan, F. Theys, M. Qian, R. Haser, C. Gerday, Eur. J. Biochem. 222 (1994) 441.
[7] T. Oikawa, K. Yamanaka, T. Kazuoka, N. Kanzawa, K. Soda, Eur. J. Biochem. 268 (2001) 4375.
[8] T. Ohshima, K. Soda, Eur. J. Biochem. 100 (1979) 29.
[9] N. Tamaki, M. Nakamura, K. Kimura, T. Hama, J. Biochem. (Tokyo) 82 (1977) 73.
[10] K. Yutani, K. Ogasahara, T. Tsujita, Y. Sugino, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 4441.
[11] T. Imanaka, M. Shibazaki, M. Takagi, Nature 324 (1986) 695.
[12] S. Dao-Pin, W.A. Baase, B.W. Matthews, Proteins 7 (1990) 198.
[13] P. Argos, M.G. Rossman, U.M. Grau, H. Zuber, G. Frank, J.D. Tratschin, Biochemistry 18 (1979) 5698.
[14] C. Vetriani, D.L. Maeder, N. Tolliday, K.S. Yip, T.J. Stillman, K.L. Britton, D.W. Rice, H.H. Klump, F.T. Robb, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 12300.
[15] J.H. Lim, K.Y. Hwang, J. Choi, D.Y. Lee, B.Y. Ahn, Y. Cho, K.S. Kim, Y.S. Han, Biochem. Biophys. Res. Commun. 288 (2001) 263.
[16] J.S. Rodriguez-Zavala, H. Weiner, Biochemistry 41 (2002) 8229.
[17] W. Shi, J. Dunbar, M.M. Jayasekera, R.E. Viola, G.K. Farber, Biochemistry 36 (1997) 9136.
[18] R.E. Viola, Adv. Enzymol. Relat. Areas Mol. Biol. 74 (2000) 295.
[19] L.J. Wang, X.D. Kong, H.Y. Zhang, X.P. Wang, J. Zhang, Biochem. Biophys. Res. Commun. 276 (2000) 346.
[20] D.J. Merkler, G.K. Farrington, F.C. Wedler, Int. J. Pept. Protein Res. 18 (1981) 430.
[21] F.S. Qaw, J.M. Brewer, Mol. Cell. Biochem. 71 (1986) 121.
[22] K.H. Jones, R. Lindahl, D.C. Baker, R. Timkovich, J. Biol. Chem. 262 (1987) 10911.
[23] N. Nakajima, K. Nakamura, N. Esaki, H. Tanaka, K. Soda, J. Biochem. 106 (1989) 515.
[24] N. Esaki, H. Shimoi, N. Nakajima, T. Ohshima, H. Tanaka, K. Soda, J. Biol. Chem. 264 (1989) 9750.