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Thermostable Aspartase from a Marine Psychrophile, *Cytophaga* sp. KUC-1: Molecular Characterization and Primary Structure

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We found that a psychrophilic bacterium isolated from Antarctic seawater, *Cytophaga* sp. KUC-1, abundantly produces aspartase [EC4.3.1.1], and the enzyme was purified to homogeneity. The molecular weight of the enzyme was estimated to be 192,000, and that of the subunit was determined to be 51,000: the enzyme is a homotetramer. L-Aspartate was the exclusive substrate. The optimum pH in the absence and presence of magnesium ions was determined to be pH 7.5 and 8.5, respectively. The enzyme was activated cooperatively by the presence of L-aspartate and by magnesium ions at neutral and alkaline pHs. In the deamination reaction, the K_m value for L-aspartate was 1.09 mM at pH 7.0, and the $S_{1/2}$ value was 2.13 mM at pH 8.5. The V_{max} value were 99.2 U/mg at pH 7.0 and 326 U/mg at pH 8.5. In the amination reaction, the K_m values for fumarate and ammonium were 0.797 and 25.2 mM, respectively, and V_{max} was 604 U/mg. The optimum temperature of the enzyme was 55°C. The enzyme showed higher pH and thermal stabilities than that from mesophile: the enzyme was stable in the pH range of 4.5–10.5, and about 80% of its activity remained after incubation at 50°C for 60 min. The gene encoding the enzyme was cloned into *Escherichia coli*, and its nucleotides were sequenced. The gene consisted of an open reading frame of 1,410-bp encoding a protein of 469 amino acid residues. The amino acid sequence of the enzyme showed a high degree of identity to those of other aspartases, although these enzymes show different thermostabilities.

Key words: aspartase, *Cytophaga*, *Escherichia coli*, psychrophile, thermostable.

Abbreviations: MES, 2-morpholinoethanesulfonic acid; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid.

Aspartase (L-aspartate ammonia-lyase, EC4.3.1.1) catalyzes the reversible deamination of L-aspartate to fumarate and serves as a turning point between the amino acid metabolism and organic acid metabolism. Aspartase is found in various organisms and has been purified from mesophiles, *Escherichia coli* (*1–3*), *Pseudomonas fluorescens* (*4*), *Bacillus subtilis* (*5*), and *Pasteurella pestis* 17 (*6*), and a thermophile, *Bacillus* sp. YM55–1 (*7*). The genes encoding the enzymes have been cloned and sequenced (*5*, *8–10*). The *E. coli* enzyme was extensively studied: it has a molecular weight of about 200,000 and is composed of four identical subunits. It forms a complex cooperatively, which is affected by pH and divalent metal ion concentration (*3*). Various *E. coli* mutant enzymes were prepared to determine the amino acid residues participating in the enzyme catalysis (*11*, *12*) and show the structure–function relationship (*13–17*). Recently, the crystal structure of the *E. coli* enzyme was elucidated (*18*). It was postulated that its active site is located in a region that contains side chains from different subunits, and that Asp 10, Arg 29, Ser 143, and Lys 327 are involved in the catalysis and substrate binding. However, the catalytic mechanism and, in particular, the roles of the essential amino acid residues have not been elucidated.

Various psychrophilic and psychrotrophic microorganisms widely occur not only in natural environments but also in artificial environments such as cold rooms and refrigerated transport systems. They take part in the natural turnover of a variety of organic and inorganic compounds under cold conditions (*19*). Psychrophiles and psychrotrophs produce various psychrophilic enzymes to carry out metabolism efficiently under cold conditions, and some of these enzymes have recently been studied (*20–27*). They are characterized by a high catalytic efficiency at low temperature and by thermolability (*28*).

Recently, we discovered aspartase in a psychrophilic bacterium, namely, *Cytophaga* sp. KUC-1, isolated from Antarctic seawater. The enzyme shows a peculiar characteristic: its thermostability is higher than that of the *E. coli* enzyme. Here we describe the purification, characterization and determination of the primary structure of the *Cytophaga* aspartase, and compare it with other aspartases.

EXPERIMENTAL PROCEDURES

Materials—Amino acids and their derivatives were purchased from Wako Chemical. DEAE-Toyopearl 650M

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and Phenyl-Toyopearl 650M were products of Toso, and Red-Sepharose CL6B was purchased from Pharmacia. The plasmid purification kit and gel extraction kit were purchased from Bio-Rad, and LA PCR reaction reagents were products of Takara Shuzo.

Culture Conditions—The psychrophilic bacterium, *Cytophaga* sp. KUC-1, which was isolated from Antarctic seawater, is an atypical psychrophile because it grows optimally at 15°C and can even grow at 25°C, though slowly (*27*). This strain was grown aerobically at 15°C in a medium containing 2% polypepton and 1% yeast extract (pH 7.0). A seed culture (200 ml) of the cells grown at 15°C for 48 h (turbidity at 660 nm: about 10) was inoculated into 7.0 liters of medium in a jar fermenter (10 liters, Marubishi Bioeng., Tokyo) and cultured at 15°C for 48 h. The cells were harvested by centrifugation at 4°C, washed twice with chilled 10 mM potassium phosphate buffer (pH 7.0) containing 0.75% NaCl, and suspended in 10 mM potassium phosphate buffer (pH 7.0) (0.5 g wet-weight cells/ml). *E. coli* (NovaBlue) was obtained from Novagen and grown aerobically at 37°C in Luria-Bertani medium supplemented ampicillin (100 μg/ml).

Purification of Aspartase—All operations were carried out in the range of 0 to 4°C. After disruption of the cells (about 8 g wet-weight) by ultrasonication five times for 5 min with cooling intervals of 5 min, the suspension was centrifuged at 27,600 × *g* for 30 min. The supernatant solution was dialyzed against 10 mM potassium phosphate buffer (pH 7.0) containing 2% 2-mercaptoethanol (the standard buffer).

The enzyme solution was applied to a DEAE-Toyopearl column (ϕ2.5 × 13 cm) equilibrated with the standard buffer, and the column was washed with 500 ml of the standard buffer. The enzyme was eluted with a linear gradient of 10–150 mM potassium phosphate (pH 7.0) containing 2% 2-mercaptoethanol. Fractions containing the enzyme activity were pooled and dialyzed against the standard buffer containing 0.9 M ammonium sulfate.

The enzyme solution was centrifuged at 27,600 × *g* for 30 min, and the supernatant was applied to a Phenyl-Toyopearl column (ϕ2.5 × 5 cm) equilibrated with the standard buffer containing 0.9 M ammonium sulfate. The column was washed with 300 ml of the standard buffer containing 0.75 M ammonium sulfate, then the adsorbed protein was eluted with 300 ml of the standard buffer containing 0.55 M ammonium sulfate. The active fractions were combined and concentrated by ultrafiltration (Advantec ultrafilter, PO200 membrane).

The enzyme solution was dialyzed against the standard buffer and applied to a Red-Sepharose 4B column (ϕ2.5 × 5 cm) equilibrated with the standard buffer. The column was washed sequentially with 50 ml of the standard buffer, 175 ml of the standard buffer containing 1 mM NAD^+, and 120 ml of potassium phosphate buffer (pH 7.2), then the enzyme was eluted with 50 ml of potassium phosphate buffer (pH 7.4). After dialysis against the standard buffer, the enzyme was concentrated and stored at –20°C.

Enzyme Assay—The enzyme was assayed spectrophotometrically at 240 nm by determination of fumarate formed in a reaction mixture (3 ml) consisting of 200 mM L-aspartate, 2 mM $MgCl_2$ in 50 mM Tris-HCl, pH 8.5, and enzyme at 30°C (the standard assay of deamination reaction). In the amination reaction, the progress curve exhibited an initial lag followed by a linear phase as reported by Ida *et al.* (*29*). Accordingly, the enzyme was assayed by determination of fumarate consumption at 293 nm in a reaction mixture (3 ml) consisting of 10 mM fumarate, 100 mM ammonium chloride, 1 mM L-aspartate, and 2 mM $MgCl_2$ in 50 mM Tris-HCl, pH 9.5, and enzyme at 30°C. The molar absorption coefficients of fumarate at 240 and 293 nm were 2,530 and 82.0 $M^{-1}\cdot cm^{-1}$, respectively. The reactions were initiated by addition of the enzyme. Initial steady-state rates were determined from the initial linear portions of reaction progress curves. One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 μmol of fumarate per min.

Effect of pH on Enzyme Stability—The effect of pH on enzyme stability was examined by measuring the remaining activity under the standard conditions of the deamination reaction after incubation of 0.05 mg/ml enzyme at various pHs and at 30°C for 30 min. The following buffers were used: MES-NaOH (pH 6.0–7.0), HEPES-NaOH (pH 7.0–8.0), TAPS-NaOH (pH 8.0–9.5), and glycine-NaOH buffer (pH 9.5–11.5).

Circular Dichroism—Circular dichroism of the aspartase (0.06 mg/ml) in 10 mM potassium phosphate buffer was measured at 222 nm in a 1.0-cm light path quartz glass cuvette at various temperatures ranging from 5 to 82.5°C with a spectropolarimeter, JASCO J-720WI.

Effect of Inhibitors—The effect of the following compounds was examined: thiol reagents, 0.1 mM *p*-(chloromercuri)benzoic acid, 1 mM *N*-ethylmaleimide and 1 mM iodoacetate, and carbonyl reagent, 1.0 mM semicarbazide hydrochloride. The remaining activity was determined under the standard conditions of the deamination reaction except for the addition of each compound.

Steady-State Kinetics—In the deamination reaction, kinetic constants were determined at pH 7.0 and 8.5 by measurement of initial velocities at various concentrations (0.3–20 mM) of L-aspartate. In the amination reaction, kinetic constants were determined at various concentrations (1.5–4 mM) of fumarate at several fixed levels (10–40 mM) of ammonium. Apparent maximum velocities (V'_{max}) and apparent Michaelis constants (K'_m) were determined by Lineweaver-Burk double reciprocal plots. The maximum velocity (V_{max}) and the Michaelis constant (K_m) were determined with the plots of $1/V'_{max}$ against the reciprocal of counterpart concentrations.

N-Terminal Amino Acid Sequence Determination—The N-terminal amino acid sequence was determined by Edman degradation with an automated sequencer (model 477A: Applied Biosystems). For N-terminal sequence analysis, approximately 180 pmol of protein was transferred to a polyvinylidene difluoride membrane.

Cloning and Sequence Analysis of the Gene Encoding Aspartase—On the basis of the highly conserved regions of aspartases from *Bacillus* sp. YM55–1, *E. coli* and *P. fluorescens*, KMGRTQ/HLQDA and MPA/GKVNP, the degenerated oligonucleotides, 5′-AARATGGGIMGHACICAIYTDCARGAYGC-3′ and 5′-GGRTTHACYTTIWCHGGCAT-3′ were designed and used as forward and reverse PCR primers, respectively. PCR was performed with 100 pmol of each of the above primers and 70 ng of chromosomal DNA isolated from the cells of *Cytophaga* sp.

Table 1. **Purification of aspartase of *Cytophaga* sp. KUC-1.**

	Total activity (U)	Total prptein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	1,310	506	2.60	100	1
DEAE Toyopearl	1,130	90.4	12.5	85.7	4.80
Phenyl Toyopearl	635	5.90	108	48.3	41.4
Red Sepharose	331	1.10	301	25.2	116

KUC-1. The thermal profile involved 30 cycles of denaturation at 94°C for 60 s, annealing at 57°C for 120 s, and extension at 72°C for 60 s. The resulting 430-bp DNA fragment was ligated with pT7 Blue T-Vector (Novagene) and sequenced. The Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit and a model 377A DNA Sequencing System gel apparatus (Applied Biosystems) were used according to the manufacturer's instructions. Based on the partial 430-bp sequence determined, two primers CYASP1 (5′-TTACATCCTCGCCTATAGTTG-3′) and CYASP2 (5′-GCACGTCAGCCAGGATCTTC-3′) were designed, and genome-walking PCR was performed with the LA PCR™ *in vitro* cloning kit (Takara). Chromosomal DNA extracted from *Cytophaga* sp. KUC-1 cells was digested with *Hin*dIII or *Eco*RI, and ligated to the *Hin*dIII or *Eco*RI cassette, respectively. The DNA fragments obtained were used as the template for amplification by PCR. PCR were carried out successively with CYASP1 and C1, and CYASP2 and C2 (C1: primer for *Hin*dIII cassette; C2: primer for *Eco*RI cassette). The thermal profile involved 30 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 90 s, and extension at 72°C for 60 s. The PCR fragments obtained were sequenced as described above.

Other Methods—Protein concentrations were measured by the method of Bradford (*30*) with bovine serum albumin as a standard. During column chromatographies, proteins were determined by measurement of A_{280}. The molecular weight of the enzyme was estimated by gel filtration with a Superdex 200 column (Pharmacia) at 4°C with the following proteins as molecular weight standards: chymotypsinogen A (25,000), ovalbumin (43,000), albumin (67,000), catalase (231,000), ferritin (440,000), and thyroglobulin (669,000). PAGE and SDS-PAGE were performed by the methods of Tulchin *et al.* (*31*) and Laemmli (*32*), respectively. The LMN Marker kit (Pharmacia) was used as molecular weight markers in SDS-PAGE.

RESULTS

Enzyme Purification—The *Cytophaga* aspartase was purified to homogeneity with a yield of 25.2% (Table 1): a single band was detected both on PAGE and SDS-PAGE. The specific activity for deamination was 301 U/mg. The enzyme was stored at –20°C in the standard buffer without loss of activity for several months. The specific activity of the *Cytophaga* enzyme (301 U/mg at 30°C) was higher than that of the *E. coli* enzyme (167 U/mg at 30°C) but lower than that of the *Bacillus* YM55–1 enzyme (700 U/mg at 30°C) (*7*).

Molecular Weight and Subunit Structure—The apparent molecular weight of the enzyme was determined to be about 192,000 by Superdex 200 gel chromatography. SDS-PAGE showed a subunit molecular weight of 51,000. These findings suggest that the enzyme has a homotetrameric structure.

Substrate Specificity—L-Aspartate was the exclusive substrate of the enzyme: D-aspartate, α-methyl-D,L-aspartate, D,L-*threo*-β-hydroxyaspartate, L-asparagine, L-alanine, L-glutamate, L-cysteine, L-cystein sulfinate were inert. When fumarate was an amino acceptor, ammonium chloride (100%) and hydroxylamine (64%) served as an amino donor, but methylamine was not active. Fumarate was the sole amino acceptor: mesaconate and maleate were inert. The *Cytophaga* enzyme was closely similar to the *E. coli* enzyme in substrate specificity (*3*).

Effect of Temperature—The enzyme showed the maximum activity at 55°C. From the linear parts of the Arrhenius plots, the activation energy was calculated as 42.1 kJ/mol (Fig. 1A). The thermostability of enzyme was examined in the range of 15 to 60°C. When the enzyme was incubated at various temperatures for 60 min, about more than 95% of initial activity was retained in the temperature range of 15 to 45°C, and about 80% was retained at 50°C. Its half-life was estimated to be 5.1 min at 55°C, and the enzyme was rapidly inactivated at 60°C (Fig. 1B).

Effect of pH—The activity was determined in the presence or absence of magnesium ions at various pH values (Fig. 2). The pH optimum in the absence of magnesium ions was pH 7.5, and about 70 and 28% of the activity were found at pH 6.5 and 8.5, respectively. In the presence of magnesium ions, the enzyme showed the maximum activity at pH 8.5, and 71 and 72% of the activity were found at pH 7.5 and 9.5, respectively. Magnesium ions did not affect the activity at pH 6.0. Although the activity of the *E. coli* enzyme is not affected by magnesium ions up to pH 7.5 (*3*), the activity of *Cytophaga* enzyme was activated at the neutral pH in the presence of magnesium ions: the activities in the presence of magnesium ions were 1.18, 1.69, and 2.22 times higher than those in the absence of magnesium ions at pH 6.5, 7.0, and 7.5, respectively. The *Cytophaga* enzyme was much more stable than the *E. coli* enzyme: the *Cytophaga* and *E. coli* enzymes were stable between pH 4.5 and 10.5 and between pH 7.0 and 8.0, respectively (*15*).

Circular Dichroism—The negative ellipticity profile at 222 nm suggests that the α-helix structure of the enzyme unfolded between 52.5 and 60°C (Fig. 3). The T_m of the enzyme was calculated to be 56°C. The α-helix structure was probably unfolded completely at 60°C, because the mol ellipticity values were not increased in the temperature region of 60–82.5°C, which is close to the temperature range in which the enzyme was rapidly inactivated.

Effect of Metal Ions and Compounds—We next examined the effects of metal chlorides, thiol reagents, and a carbonyl reagent. Various divalent metal ions and Na^+

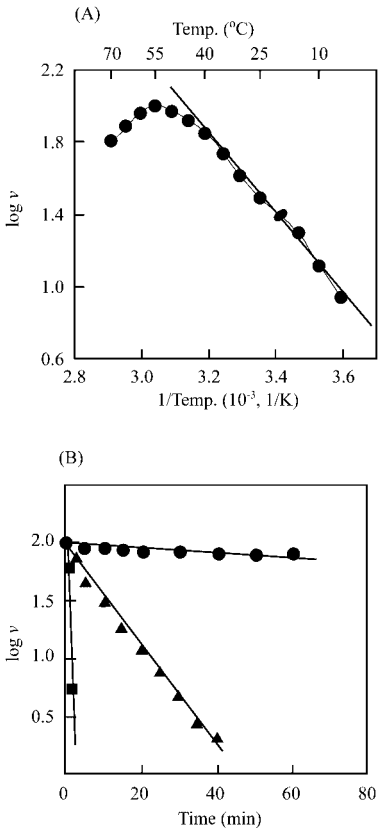

Fig. 1. **Effect of temperature on the enzyme activity and stability.** (A) Arrhenius plot. The effect of temperature on enzyme activity was examined under the standard assay conditions of the deamination reaction except for temperature, which ranged from 5 to 70°C. The activation energy for the deamination of L-aspartate was calculated from the Arrhenius plot. (B) The effect of temperature on the stability. The enzyme was incubated in 10 mM potassium phosphate buffer, pH 7.0 at various temperatures. At intervals, 3 μl of aliquots of the incubated 0.05 mg/ml enzyme solution were withdrawn and used for the enzyme assay under the standard conditions for deamination. Solid circle, solid triangle, and solid square represent the remaining activities after incubation at 50, 55, and 60°C, respectively.

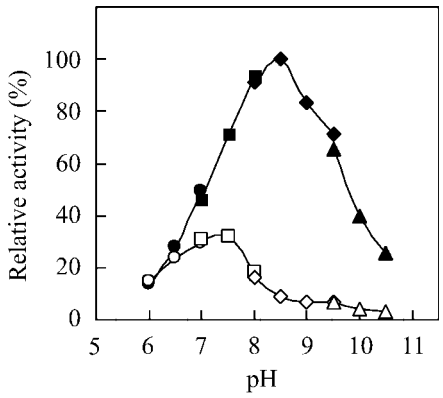

Fig. 2. **Effect of pH on the enzyme activity**. The effect of pH on the enzyme activity was examined under the standard assay conditions of the deamination reaction except that the following buffers (100 mM) were used in the presence or absence of magnesium ions: MES-NaOH (pH 6.0–7.0, circles), HEPES-NaOH (pH 7.0–8.0, squares), TAPS-NaOH (pH 8.0–9.5, diamonds), and glycine-NaOH buffer (pH 9.5–11.5, triangles). Filled and unfilled symbols represent the activities in the presence and absence of magnesium ions, respectively.

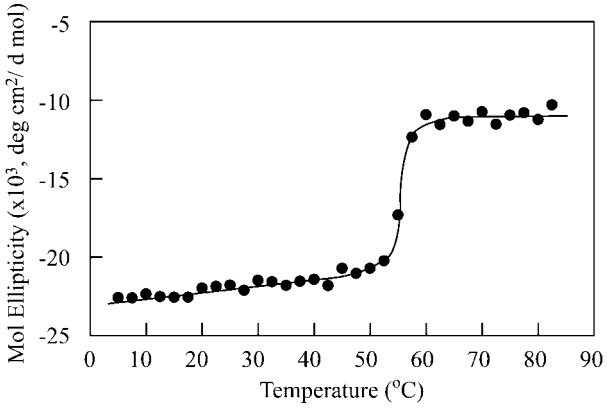

Fig. 3. **Temperature dependence of molecular ellipticity at 222 nm**. The data are the averages of duplicate measurements.

activated the enzyme, whereas K^+ and Hg^{2+} inhibited the enzyme (Table 2). The response of the *Cytophaga* enzyme to monovalent metal ions was different from that of the *E. coli* enzyme: the *E. coli* enzyme was activated by K^+ but inhibited by Na^+ (*3*). The enzyme was strongly inhibited by thiol reagents: 0.1 mM *p*-(chloromercuri)benzoic acid (inhibition, 100%), 1 mM iodoacetate (94%), and 1 mM *N*-ethylmaleimide (100%). The carbonyl reagent, 1 mM semicarbazide hydrochloride, decreased the enzyme activity by about 30%.

Steady-State Kinetics—In the deamination reaction, the saturation curve was sigmoidal at pH 8.5 and hyperbolic at pH 7.0 (Fig. 4, A and B). From the Hill coefficient analysis, a Hill constant of 1.0 was obtained (Fig. 4b), indicating that the enzyme obeyed Michaelis-Menten rules of kinetics at pH 7.0. At pH 8.5, the Hill constant was 1.0 at concentrations of L-aspartate higher than 1.5 mM and 2.7 at concentrations lower than 1.5 mM (Fig. 4a). This suggests that the enzyme is activated cooperatively by the presence of L-aspartate. The K_m value for L-aspartate was 1.09 mM at pH 7.0, and the $S_{1/2}$ value was 2.13 mM at pH 8.5. The V_{max} values were 99.2 U/mg at pH 7.0 and 326 U/mg at pH 8.5. In the amination reaction, double reciprocal plots of the initial velocity against the concentrations of substrate and cosubstrate in the presence of various fixed concentrations of cosubstrate and substrate, respectively, gave sets of straight lines. The results show that the reaction proceeds *via* formation of a ternary complex of the enzyme with a substrate and a cosubstrate in the amination reaction. The kinetic parameters for fumarate and ammonium were calculated from the secondary plots of intercepts *versus* reciprocal concentrations of a counterpart substrate. The K_m values for fumarate and ammonium were 0.797 and 25.2 mM, respectively, and V_{max} value was 604 U/mg. The *Cytophaga* enzyme was activated cooperatively by the presence of L-aspartate at pH 8.5, and it was activated by magnesium ions at neutral and alkaline pHs. These properties are similar to those of the *E. coli* enzyme (*3*), but different from those from the *Bacillus* YM55-1 enzyme, which is

Table 2. **Effect of metal ions.**

Meal ion (2 mM)	Relative activity (%)
None	0.832
Mg^{2+}	100
Mn^{2+}	108
Ca^{2+}	102
Zn^{2+}	90.0
Co^{2+}	82.6
Ni^{2+}	1.91
Ba^{2+}	1.01
Hg^{2+}	0
K^{+}	0.567
Na^{+}	1.05

After dialysis against 10 mM Tris-HCl, pH 8.0, containing 10 mM EDTA, the enzyme was dialyzed against 10 mM Tris-Cl, pH 8.0. The effect of metal ions was examined under the standard conditions of the deamination reaction except for the addition of 2 mM metal chloride.

not activated by either L-aspartate or magnesium ions at alkaline pH (*7*).

Sequencing of Amino Terminal Region—Sequencing of amino terminal region was carried out for 11 cycles yielding identifiable residues. The amino acid sequence of the N-terminal region was MGSTRKEHDFL.

Cloning and Sequence Analysis—The amplified DNA fragment (about 430 bp) was cloned into a pT7 Blue T-Vector and nucleotides were sequenced. The results showed an open reading frame (ORF) lacking initiation and termination codons. The overlapping segments, about 1.2 kb for upstream and 0.3 kb for downstream regions, were amplified by a genome-walking PCR method, and nucleotides were sequenced to obtain the entire gene. The entire ORF is accordingly composed of 1,410 bases and codes for a protein of 469 amino acid residues with an estimated molecular weight of 51,400. The deduced amino acid sequence was used for searching in GenBank and protein databases with the BLAST program. Significant sequence identities were found with amino acid sequences of aspartases from *P. fluorescens* (58%), *E. coli* (55%), and *Bacillus* sp. YM55–1 (49%). Alignment of amino acid sequences of various aspartases is summarized in Fig. 5. Important residues are fully conserved in the enzymes. The key residues include Arg 29 and Lys 327 (the residue number of the *Cytophaga* enzyme), which recognize the β- and α-carboxy groups of the L-aspartate, respectively; Asp 9, which is probably involved in a hydrogen-bonding network that is required to stabilize the active site; and Ser 141, which serves as an acid catalyst (*12*, *18*).

Amino Acid Composition—The amino acid composition of the *Cytophaga* enzyme was compared with those of the enzymes from mesophiles and a thermophile. The *Cytophaga* enzyme shows the characteristic amino acid composition. It contained more Ile residues (43 residues, 9.15%) than the enzymes from mesophiles, *E. coli* (32 residues, 6.69%) and *P. fluorescens* (31 residues, 6.49%), and had a similar Ile content to the thermostable aspartase from *Bacillus* sp. YM55–1 (41 residues, 8.74%). The Ile/Ile + Val + Leu ratio of the *Cytophaga* enzyme (0.361) was much higher than those of the enzyme from mesophiles

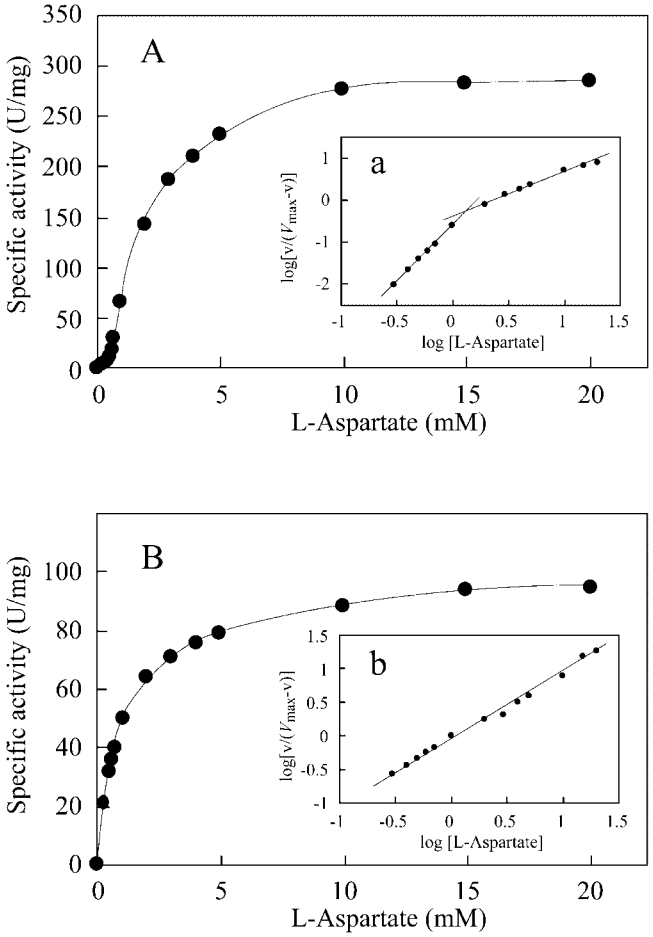

Fig. 4. **Effect of pH on substrate saturation curve.** (A) Substrate saturation curve for *Cytophaga* aspartase at pH 8.5. The enzyme activities were determined under the standard deamination conditions except for the buffer, 50 mM HEPES, pH 8.5, and L-aspartate concentrations (0–20 mM). (a) Hill plot at pH 8.5. The Hill constants were calculated from the Hill plot. (B) Substrate saturation curve for *Cytophaga* aspartase at pH 7.0. The enzyme activities were determined under the standard deamination conditions except for the buffer, 50 mM TAPS, pH 8.5, and L-aspartate concentrations (0–20 mM). (b) Hill plot at pH 8.5. The Hill constant was calculated from the Hill plot.

(*E. coli*, 0.269 and *P. fluorescens*, 0.252) and similar to that of the *Bacillus* enzyme (0.360).

DISCUSSION

We have found that a psychrophilic bacterium, *Cytophaga* sp. KUC-1 isolated from Antarctic seawater, abundantly produces aspartase. The enzyme accounted for about 0.86% of the total soluble protein produced irrespective of whether the substrate L-aspartate was added to the growth medium.

The specific activity of the *Cytophaga* enzyme was about twice that of the *E. coli* enzyme, and the ratio of the activity at 25°C to that at 55°C of the *Cytophaga* enzyme (30.1%) was also about twice that of the *E. coli* enzyme (16.1%). This property is characteristic of psychrophilic enzymes. The activation energy of the *Cytophaga* enzyme

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Cytophaga sp. KUC-1       1:MG----ST---RKEHDFLGELDIPNHLYYGIQTFRAVENFNITGIPISKEPLFIKALGYV 53
Bacillus sp. YM55-1       1:MNTDV------RIEKDFLGEKEIPKDAYYGVQTIRATENFPITGYRIH--PELIKSLGIV 52
Escherichia coli          1:MSNNI------RIEEDLLGTREVPADAYYGVHTLRAIENFYISNNKISDIPEFVRGMVMV 54
Pseudomonas fluorescens   1:MISVMSSAASFRTEKDLLGVLEVPAQAYYGIQTLRAVNNFRLSGVPISHYPKLVVGLAMV 60
                            *          * * * **  . *  .*** .* ** .** . .  *.  *     .  *

Cytophaga sp. KUC-1      54:KKAAALANKDCGRLDPKIAEAICYGSDQVIAGKFDQE-FVSDLIQGGAGTSVNMNANEVI 112
Bacillus sp. YM55-1       53:KKSAALANMEVGLLDKEVGQYIVKAADEVIEG-KWNDQFIVDPIQGGAGTSINMNANEVI 111
Escherichia coli          55:KKAAAMANKELQTIPKSVANAIIAACDEVLNNGKCMDQFPVDVYQGGAGTSVNMNTNEVL 114
Pseudomonas fluorescens   61:KQAAADANRELGQLSERKHAAISEACARLIRGDFHEE-FVVDMIQGGAGTSTNMNANEVI 119
                            *..** ** . . .      .*  . . .. .      * .* .******* ***.***.

Cytophaga sp. KUC-1     113:ANIGLEYLGHKKGDYNFLHPNNHVNCSQSTNDAYPSAFRIALYLKMESFIKTLEGLEVAF 172
Bacillus sp. YM55-1      112:ANRALELMGEEKGNYSKISPNSHVNMSQSTNDAFPTATHIAVLSLLNQLIETTKYMQQEF 171
Escherichia coli         115:ANIGLELMGHQKGEYQYLNPNDHVNKCQSTNDAYPTGFRIAVYSSLIKLVDAINQLREGF 174
Pseudomonas fluorescens  120:ANIALEAMGHQKGEYQYLHPNNDVNMAQSTNDAYPTAIRLGLLLGHDALLASLDSLIQAF 179
                            **. ** .*. ** *  . ** .**  ******.*.. ...      .     .   *

Cytophaga sp. KUC-1      173:VANGEEFKSVLKMGRTQLQDAVPMTLGQEFRSYATTIGEDVRRLKEAQS-LVL-EINMGA 230
Bacillus sp. YM55-1      172:MKKADEFAGVIKMGRTHLQDAVPILLGQEFEAYARVIARDIERIANTRNNLYDINM--GA 229
Escherichia coli         175:ERKAVEFQDILKMGRTQLQDAVPMTLGQEFRAFSILLKEEVKNIQRTAELLLEVNL--GA 232
Pseudomonas fluorescens  180:AAKGAEFSHVLKMGRTQLQDAVPMTLGQEFRAFATTLGEDLARLKTLAPEL-LTEVNLGG 238
                               .  **  ..*****.******..*****.. .    ..  .       *       *.

Cytophaga sp. KUC-1      231:TAIGTRVNAPEGYPEICVNYLAKEVGIPLTLSPDLIEATVDTGAYVQIMGTLKRTAVKIS 290
Bacillus sp. YM55-1      230:TAVGTGLNADPEYISIVTEHLAKFSGHPLRSAQHLVDATQNTDCYTEVSSALKVCMINMS 289
Escherichia coli         233:TAIGTGLNTPKEYSPLAVKKLAEVTGFPCVPAEDLIEATSDCGAYVMVHGALKRLAVKMS 292
Pseudomonas fluorescens  239:TAIGTGINADPRYQALAVQRLATISGQPLVPAADLIEATSDMGAFVLFSGMLKRTAVKLS 298
                            **.**. *.   *    .  **   * *.  . .*..** . ....   . **. ... *

Cytophaga sp. KUC-1      291:KICNDLRLLSSGPRTGFNEINLPARQPGSSIMPGKVNPVIPEVVNQTCFYVIGQDLTVTM 350
Bacillus sp. YM55-1      290:KIANDLRLMASGPRAGLSEIVLPARQPGSSIMPGKVNPVMPEVMNQVAFQVFGNDLTITS 349
Escherichia coli         293:KICNDLRLLSSGPRAGLNEINLPELQAGSSIMPAKVNPVVPEVVNQVCFKVIGNDTTVTM 352
Pseudomonas fluorescens  299:KICNDLRLLSSGPRTGINEINLPARQPGSSIMPGKVNPVIPEAVNQVAFQVIGNDLALTM 358
                            **.*****..**** * .**.**..*.******.***** **..**. * *.*.*.. *.

Cytophaga sp. KUC-1      351:AAEAGQLQLNVMEPVIAFAMFTSLDYLSNAIQTLIDK-CIIGITANVDHCYNMVMNSIGI 409
Bacillus sp. YM55-1      350:ASEAGQFELNVMEPVLFFNLIQSISIMTNV-FKSFTENCLKGIKANEERMKEYVEKSIGI 408
Escherichia coli         353:AAEAGQLQLNVMEPVIGQAMFESVHILTNACYNLL-EKCINGITANKEVCEGYVYNSIGI 411
Pseudomonas fluorescens  359:AAEGGQLQLNVMEPLIAFKIFDSIRLLQRAMDMLREH-CIVGITANEARCRELVEHSIGL 417
                            *.*.**..******.. .  . *   . ..   .    *. **.**   .   *  ***.

Cytophaga sp. KUC-1      410:VTQLNPILGYEISASIAGEALKMNKSVHEIVVVERK-----LITQEKWDEIYSLDNLINP 464
Bacillus sp. YM55-1      409:ITAINPHVGYETAAKLAREAYLTGESIRELCIKYGVLTEEQLNEILNPYEMTHPGIAGRK 468
Escherichia coli         412:VTYLNPFIGHHNGDIVGKICAETGKSVREVVLERGLLTEAELDDIFSVQNLMHPAYKAKR 471
Pseudomonas fluorescens  418:VTALNPYIGYENATRIARIALESGRGVLELVREEGL-----LD-DAMLDDILRPENMIAP 471
                            .* .**  *..     .  .   . .. * .   .      *          .

Cytophaga sp. KUC-1      465:KFITK--                                                  469
Bacillus sp. YM55-1      469:-------                                                  468
Escherichia coli         472:YTDESEQ                                                 478
Pseudomonas fluorescens  472:RLVPLKA                                                 478
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Fig. 5. **Alignment of amino acid sequences of *Cytophaga* aspartase and other aspartases.** Amino acid sequences of aspartases from *Cytophaga* sp. KUC-1 (this study), *Bacillus* sp. YM55–1 (accession number, AB028242), *E. coli* (X02307), and *P. fluorescens* (X04441) were aligned. Asterisks (*) and dots (·) show the residues conserved in all four sequences and in three sequences, respectively. Gaps (-) are introduced to obtain maximal matching. Key residues, Asp 9, Arg 29, Ser 141, and Lys 327 (the number of the *Cytophaga* enzyme), are shadowed in gray.

(42.1 kJ/mol) was lower than that of the *E. coli* enzyme (54.0 kJ/mol) (Kawata, Y., personal communication). The low activation energy of the enzyme is advantageous for catalysis the reaction at low temperature.

The optimum temperatures of the *Cytophaga* (55°C at pH 8.5) and *E. coli* (55°C at pH 8.0) enzymes were closely similar, but the *Cytophaga* enzyme was much more thermostable than the enzyme from the mesophile, although L-valine dehydrogenase from *Cytophaga* sp. KUC-1 is quite thermolabile and psychrophilic (*27*): the *E. coli* enzyme lost about 83% of its activity after incubation at 50°C for 45 min, and the *Cytophaga* enzyme retained about 80% of its activity after incubation at 50°C for 60 min (*7*, *15*). The T_m of enzyme was calculated to be 56°C, which is similar to that of the thermostable aldehyde dehydrogenase (58°C) from *Cytophaga* sp. KUC-1. However, it is impossible to compare the T_m value of enzyme with those of aspartase from other sources, which have been reported. The amino acid sequence of the *Cytophaga* aspartase shows a high level of identity to the *E. coli* enzyme (55%) and the thermostable *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). This high level of similarity indicates that the three enzymes have similar structures and that the difference in their thermostabilities are the result of a few changes in amino acid sequence. Several structures have been considered to increase the thermostability of a protein: increases of hydrophobicity in the interior of a molecule (*33*), helix stability (*34*, *35*), tight packing interactions inside the molecule (*36*) and ionic interactions (*37*, *38*). A detailed discussion is not possible, because the crystal structure of the *Cytophaga* enzyme is still unknown.

However, study of the amino acid compositions revealed that the number of Ile residue of the *Cytophaga* enzyme was higher than that of the *E. coli* enzyme and similar to that of the *Bacillus* YM55-1 enzyme, and that the Ile/Val + Leu ratio of the *Cytophaga* enzyme was also higher than that of the *E. coli* enzyme and similar to that of the *Bacillus* YM55-1 enzyme. These findings suggest that an increase in hydrophobic branched amino acid residues such as Ile leads to more hydrophobic interactions than in the mesophilic enzyme, and that the Ile residue is more favorable from the standpoint of packing interactions in the interior the enzyme than Val and Leu residues (*33*). In terms of α-helicity, the α-helicity of the C-terminal region of the *Cytophaga* enzyme is higher than that of the *E. coli* enzyme (data not shown). The C-terminal domain of the *E. coli* enzyme includes residues 397–459 and is the smallest of the three domains in the subunit (*18*). It consists mainly of two helix-turn-helix motifs. The higher α-helicity in the C-terminal domain of the *Cytophaga* enzyme leads to helix stability, and this may be one of the reasons fot the higher thermostability (*34*, *35*). Furthermore, Asn 217 in the *E. coli* enzyme is replaced by Arg 215 in the *Cytophaga* enzyme. The mutant enzyme of *E. coli* in which Asn 217 is replaced by Arg has increased thermostability (*16*). The Arg residue forms stronger ionic interaction in the enzyme than Asn residue and may lead to an increased thermostability (*39*, *40*). The various factors described above may additively increase the thermostability of the *Cytophaga* enzyme.

The enzyme gene was inserted into the multicloning site of pET17b and expressed in the cells of *E. coli* BL21(DE3). The enzyme accounted for about 30% of soluble cellular protein without addition of IPTG. We are now studying by site-directed mutagenesis, differential microcalorimetry and other methods why the *Cytophaga* enzyme is thermostable.

The nucleotide sequence of the gene encoding aspartase has been submitted to the DDBJ/EMBL/GenBank Data Bank with accession number AB091344. This work was supported in part by a Research Grant from the Japan Foundation of Applied Enzymology, by a Research Grant from Kansai University, by the Science Research Promotion Fund from the Japan Private School Promotion Foundation, and by the Research Grant from Japan Society for the Promotion of Science.

REFERENCES

1. Karsten, W.E., Hunsley, J.R., and Viola, R.E. (1985) Purification of aspartase and aspartokinase-homoserine dehydrogenase I from *Escherichia coli* by dye-ligand chromatography. *Anal. Biochem.* **147**, 336–341
2. Rudolph, F.B. and Fromm, H.J. (1971) The purification and properties of aspartase from *Escherichia coli*. *Arch. Biochem. Biophys.* **147**, 92–98
3. Suzuki, S., Yamaguchi, J., and Tokushige, M. (1973) Studies on aspartase. I. Purification and molecular properties of aspartase from *Escherichia coli*. *Biochim. Biophys Acta* **321**, 369–381
4. Takagi, J.S., Fukunaga, R., Tokushige, M., and Katsuki, H. (1984) Purification, crystallization, and molecular properties of aspartase from *Pseudomonas fluorescens*. *J. Biochem.* **96**, 545–552
5. Sun, D.X. and Setlow, P. (1991) Cloning, nucleotide sequence, and expression of the *Bacillus subtilis* ans operon, which codes for L-asparaginase and L-aspartase. *J. Bacteriol.* **173**, 3831–3845
6. Korobeinik, N.V. and Domaradskii, I.V. (1968) Isolation, purification and catalytic properties of aspartase from *Pasteurella pestis*. *Biokhimiia* **33**, 1128–1134
7. Kawata, Y., Tamura, K., Yano, S., Mizobata, T., Nagai, J., Esaki, N., Soda, K., Tokushige, M., and Yumoto, N. (1999) Purification and characterization of thermostable aspartase from *Bacillus* sp. YM55–1. *Arch. Biochem. Biophys.* **366**, 40–46
8. Takagi, J.S., Ida, N., Tokushige, M., Sakamoto, H., and Shimura, Y. (1985) Cloning and nucleotide sequence of the aspartase gene of *Escherichia coli* W. *Nucleic Acids Res.* **13**, 2063–2074
9. Takagi, J.S., Tokushige, M., and Shimura, Y. (1986) Cloning and nucleotide sequence of the aspartase gene of *Pseudomonas fluorescens*. *J. Biochem.* **100**, 697–705
10. Kawata, Y., Tamura, K., Kawamura, M., Ikei, K., Mizobata, T., Nagai, J., Fujita, M., Yano, S., Tokushige, M., and Yumoto, N. (2000) Cloning and over-expression of thermostable *Bacillus* sp. YM55–1 aspartase and site-directed mutagenesis for probing a catalytic residue. *Eur. J. Biochem.* **267**, 1847–1857
11. Saribas, A.S., Schindler, J.F., and Viola, R.E. Mutagenic investigation of conserved functional amino acids in *Escherichia coli* L-aspartase. *J. Biol. Chem.* **269**, 6313–6319
12. Viola, R.E. (2000) L-Aspartase: new tricks from an old enzyme. *Adv. Enzymol. Relat. Areas Mol. Biol.* **74**, 295–341
13. Murase, S., Takagi, J.S., Higashi, Y., Imaishi, H., Yumoto, N., and Tokushige, M. (1991) Activation of aspartase by site-directed mutagenesis. *Biochem. Biophys. Res. Commun.* **177**, 414–419
14. Murase, S. and Yumoto, N. (1993) Characterization of three types of aspartase activated by site-directed mutagenesis, limited proteolysis, and acetylation. *J. Biochem.* **114**, 735–739
15. Zhang, H.Y., Zhang, J., Lin, L., Du, W.Y., and Lu, J. (1993) Enhancement of the stability and activity of aspartase by random and site-directed mutagenesis. *Biochem. Biophys. Res. Commun.* **192**, 15–21
16. Wang, L.J., Kong, X.D., Zhang, H.Y., Wang, X.P., and Zhang, J. (2000) Enhancement of the activity of L-aspartase from *Escherichia coli* W by directed evolution. *Biochem. Biophys. Res. Commun.* **276**, 346–349
17. Jayasekera, M.M., Shi, W., Farber, G.K., and Viola, R.E. (1997) Evaluation of functionally important amino acids in L-aspartate ammonia-lyase from *Escherichia coli*. *Biochemistry* **36**, 9145–9150
18. Shi, W., Dunbar, J., Jayasekera, M.M., Viola, R.E., and Farber, G.K. (1997) The structure of L-aspartate ammonia-lyase from *Escherichia coli*. *Biochemistry* **36**, 9136–9144
19. Maresin, R. and F. Schinner. (1999) *Biotechnological Applications of Cold-Adapted Organisms*. Springer-Verlag, Berlin Heidelberg, New York
20. Rentier-Delrue, F., Mande, S.C., Moyens, S., Terpstra, P., Mainfroid, V., Goraj, K., Lion, M., Hol, W.G., and Martial, J.A. (1993) Cloning and overexpression of the triosephosphate isomerase genes from psychrophilic and thermophilic bacteria. Structural comparison of the predicted protein sequences. *J. Mol. Biol.* **229**, 85–93
21. Yokoigawa, K., Kawai, H., Endo, K., Lim, Y.H., Esaki, N., and Soda, K. (1993) Thermolabile alanine racemase from a psychrotroph, *Pseudomonas fluorescens*: purification and properties. *Biosci. Biotechnol. Biochem.* **57**, 93–97
22. Tsigos, I., Velonia, K., Smonou, I., and Bouriotis, V. (1998) Purification and characterization of an alcohol dehydrogenase from the Antarctic psychrophile *Moraxella* sp. TAE123. *Eur. J. Biochem.* **254**, 356–362
23. Gerike, U., Danson, M.J., Russell, N.J., and Hough, D.W. (1997) Sequencing and expression of the gene encoding a cold-active citrate synthase from an Antarctic bacterium, strain DS2-3R. *Eur. J. Biochem.* **248**, 49–57
24. Tsuruta, H., Tsuneta, S.T., Ishida, Y., Watanabe, K., Uno, T., and Aizono, Y. (1998) Purification and some characteristics of phosphatase of a psychrophile. *J. Biochem.* **123**, 219–225

25. Feller, G., Payan, F., Theys, F., Qian, M., Haser, R., and Gerday, C. (1994) Stability and structural analysis of alpha-amylase from the Antarctic psychrophile *Alteromonas haloplanctis* A23. *Eur. J. Biochem.* **222**, 441–447
26. Shing, Y.W., Akagi, J.M., and Himes, R.H. (1975) Psychrophilic, mesophilic, and thermophilic triosephosphate isomerases from three clostridial species. *J. Bacteriol.* **122**, 177–184
27. Oikawa, T., Yamanaka, K., Kazuoka, T., Kanzawa, N., and Soda, K. (2001) Psychrophilic valine dehydrogenase of the Antarctic psychrophile, *Cytophaga* sp. KUC-1: purification, molecular characterization and expression. *Eur. J. Biochem.* **268**, 4375–4383
28. Gianese, G., Bossa, F., and Pascarella, S. (2002) Comparative structural analysis of psychrophilic and meso- and thermophilic enzymes. *Proteins* **47**, 236–249
29. Ida, N. and Tokushige, M. (1985) L-Aspartate-induced activation of aspartase. *J. Biochem.* **98**, 35–39
30. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
31. Tulchin, N., Ornstein, L., and Davis, B.J. (1976) A microgel system for disc electrophoresis. *Anal. Biochem.* **72**, 485–490
32. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
33. Yutani, K., Ogasahara, K., Tsujita, T., and Sugino, Y. (1987) Dependence of conformational stability on hydrophobicity of the amino acid residue in a series of variant proteins substituted at a unique position of tryptophan synthase alpha subunit. *Proc. Natl. Acad. Sci. USA* **84**, 4441–4444
34. Imanaka, T., Shibazaki, M., and Takagi, M. (1986) A new way of enhancing the thermostability of proteases. *Nature* **324**, 695–697
35. Dao-Pin, S., Baase, W.A., and Matthews, B.W. (1990) A mutant T4 lysozyme (Val 131→Ala) designed to increase thermostability by the reduction of strain within an alpha-helix. *Proteins* **7**, 198–204
36. Argos, P., Rossman, M.G, Grau, U.M., Zuber, H., Frank, G., and Tratschin, J.D. (1979) Thermal stability and protein structure. *Biochemistry* **18**, 5698–5703
37. Vetriani, C., Maeder, D.L., Tolliday, N., Yip, K.S., Stillman, T.J., Britton, K.L., Rice, D.W., Klump, H.H., and Robb, F.T. (1998) Protein thermostability above 100 degreesC: a key role for ionic interactions. *Proc. Natl Acad. Sci. USA* **95**, 12300–12305
38. Lim, J.H., Hwang, K.Y., Choi, J., Lee, D.Y., Ahn, B.Y., Cho, Y., Kim, K.S., and Han, Y.S. (2001) Mutational effects on thermostable superoxide dismutase from *Aquifex pyrophilus*: understanding the molecular basis of protein thermostability. *Biochem. Biophys. Res. Commun.* **288**, 263–268
39. Merkler, D.J., Farrington, G.K., and Wedler, F.C. (1981) Protein thermostability. Correlations between calculated macroscopic parameters and growth temperature for closely related thermophilic and mesophilic bacilli. *Int. J. Pept. Protein Res.* **18**, 430–442
40. Qaw, F.S. and Brewer, J.M. (1986) Arginyl residues and thermal stability in proteins. *Mol. Cell. Biochem.* **71**, 121–127