Studies on enzymatic properties and crystal structure of L-leucine dehydrogenase from a psychrophilic bacterium Sporosarcina psychrophila

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Studies on enzymatic properties and crystal structure of L-leucine dehydrogenase from a psychrophilic bacterium *Sporosarcina psychrophila*

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General introduction

1. Psychrophiles and psychrophilic enzymes

Psychrophile is a kind of extremophilic organism that is capable of growth and reproduction in cold temperatures, ranging from -15˚C to +10˚C. Most psychrophiles can grow at temperatures as low as 0˚C, but cannot thrive when the temperature is over 30˚C.

There are many ecosystems exposed to low temperatures on the earth. About 70% of the earth surface is covered by oceans, which have a constant temperature of about 5˚C below a depth of 1,000 m, and there are many other cold environments such as mountain and polar regions. Psychrophiles have colonized all the permanently cold environments, depending on their ability to thrive at temperatures close to or below the freezing point.

Many kinds of psychrophiles have been found in cold environments, including Gram-negative bacteria (e.g. *Moraxella*, *Moritella*, *Polaribacter*, *Polaromonas*, *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter*, *Psychroflexus* and *Vibrio* species), Gram-positive bacteria (e.g. *Arthrobacter*, *Bacillus* and *Micrococcus* species), archaea (e.g. *Haorubrum*, *Methanococcoides* and *Methanogenium* species), yeast (e.g. *Candida* and *Cryptococcus* species), fungi (e.g. *Cladosporium* and *Penicillium* species) and microalgae (e.g. *Chloromonas* species) [1-7].

Studies on the regulation of membrane fluidity [7-9], transcription and translation to maintain protein synthesis [10-12], production of cold-acclimation proteins

[13-16], antifreeze proteins [17] and psychrophilic enzymes have been carried out to understand molecular mechanisms for the cold-adaptation of psychrophiles. But mechanisim for the cellular adaptations to low temperatures and the essential molecular mechanisms of these psychrophiles are still not enough understood and are still remained to be investigated. In particular, much attention has been paid to psychrophilic key enzymes, which play important roles in keeping necessary reaction rates for biochemical procedures even in cold environments.

In general, enzyme activity increases with the increases of reaction temperature, but rapidly declines above a certain temperature due to enzyme denaturation. The enzymes from thermophiles, particularly hyperthermophiles, are highly stable under a variety of conditions and have high optimum reaction temperatures, but they show obvious reductions in catalytic activity with reductions in temperature [18]. In contrast, enzymes from psychrophiles exhibit high levels of activity at moderate or low temperatures, but are less stable than their counterparts from thermophiles and mesophiles.

Psychrophilic enzymes have many practical applications [19-21] such as large-scale processes, in which it can save the expensive heating of reactors. They can also be applied to domestic processes, as additives for detergents. In food industry, psychrophilic enzymes are useful for transformation or refinement of heat-sensitive products.

2. Amino acid dehydrogenases including leucine dehydrogense: researches and practical applications

Amino acid dehydrogenases (EC 1.4.1.-) are enzymes belonging to a family that catalyzes the deamination of L-amino acids to their corresponding keto acids, in the presence of NAD^+ or $NADP^+$ as a cofactor. The general reaction that amino acid dehydrogenase catalyzes is shown as follows.

$$\text{L-Amino acid} + NAD(P)^+ + H_2O \rightleftharpoons \text{Oxo acid} + NH_3 + NAD(P)H + H^+$$

More than 10 kinds of amino acid dehydrogenases have been so far found in various organisms and many of them have been purified and characterized. For example, alanine dehydrogenase (AlaDH, EC 1.4.1.1), glutamate dehydrogenase (GluDH, EC 1.4.1.2-1.4.1.4), serine dehydrogenase (SerDH, EC 1.4.1.7), valine dehydrogenase (ValDH, EC 1.4.1.8), leucine dehydrogenase (LeuDH, EC 1.4.1.9.), glycine dehydrogenase (GlyDH, EC 1.4.1.10), lysine dehydrogenase (LysDH, EC 1.4.1.15 and 1.4.1.18), tryptophan dehydrogenase (TryDH, EC 1.4.1.19), phenylalanine dehydrogenase (PheDH, EC 1.4.1.20) and asparate dehydrogenase (AspDH, EC 1.4.1.-) have been found in various kinds of organisms including archaea, bacteria, fungi, yeasts, plants and human organisms [22].

Amino acid dehydrogenases have been known to catalyze amino acid deaminations in the presence of NAD^+ or $NADP^+$ by the common mechanism: the oxidative deamination of amino acid catalyzed by amino acid dehydrogenase possesses sequential or random substrate-binding mechanisms. On the other hand,

with respective to hydrogen transfer from the C-4 position of nicotinamide moiety of NAD(P)H to oxo acids, the presence of two different stereospecificity, pro-R and pro-S, are known, just like other NAD(P)-dependent enzymes. GluDH, LeuDH, PheDH and ValDH are pro-S specific enzymes, and AlaDH, LysDH are pro-R specific enzymes.

Besides catalytic mechanisms, the detailed enzymological properties of amino acid dehydrogenases, especially AlaDH, GluDH, LeuDH and PheDH, have been studied by many researchers.

Among them, AlaDHs catalyze the deamination of L-alanine to pyruvate, using NAD^+ as a cofactor. AlaDH has been found in many kinds of microorganisms: bacteria, archaea and eukaryotes, but not in higher plants and animals. This enzyme has been purified and characterized from several microorganisms, such as *Lysinibacillus sphaericus* [23], *Geobacillus stearothermophilus* [24] and *Bradyrhizobium japonicum* [25].

Among the family enzymes, GluDHs catalyze the oxidative deamination of L-glutamate to 2-oxoglutarate, using NAD^+ or $NADP^+$, or either of them, as a cofactor. GluDHs can be classified into 3 groups according to its coenzyme specificity: NAD-specific GluDH (EC 1.4.1.2), NADP-specific GluDH (EC 1.4.1.4), NAD- and NADP- nonspecific GluDH (EC 1.4.1.3). GluDHs occur ubiquitously in almost all organisms, including animals, plants and microorganisms, expect for certain bacteria such as *Bacillus* species [26, 27]. Many *Bacillus* species lack GluDH but have AlaDH and LeuDH instead, although some of the *Bacillus* species also have GluDH. GluDHs have been purified and characterized from various organisms such as bovine, yeast, fungi and microorganisms, such as *Thermus*

thermophiles [28], *Bacillus cereus* [29] and *Sulfolobus solfataricus* [30].

LeuDHs catalyze the deamination of branched chain L-amino acids (L-leucine, L-valine and L-isoleucine) to their oxo analogs, using NAD^+ as a cofactor. The enzyme is found in limited species of bacteria, mainly in endospore-forming bacteria, such as *Bacillus* species [31]. It is known that the enzyme in *Bacillus* species cells to function physiologically for the formation of branched chain oxo acid, ammonia and NADH from L-branched amino acids and NAD^+ during spore germination [32]. The enzyme has been purified from several microorganisms, such as *Lysinibacillus sphaericus* [31], *B. cereus* [33], *G. stearothermophilus* [34], *B. licheniformis* [35] and *Thermoactinomyces intermedius* [36].

PheDH was found in some mesophilic species such as *Brevibacterium spec.*[37], *L. sphaericus* [38], *Sporosarcina ureae* [38], *B. badius* [39], *Rhodococcus* sp. [40], and also from a thermophilic strain *T. intermedius* [41].

Industrial uses of amino acid dehydrogenases have been developed on the stereospecific synthesis of amino acids, analyses of L-amino acids and oxo acids, and assay of enzymes which use amino acids as substrates or produce oxo acids as products. L-Amino acids, the substrates of oxidative deamination and products of reductive amination of amino acid dehydrogenases, are very important nutrients and starting materials in pharmaceutical compounds. Amino acid dehydrogenases have been used for the synthesis of L-amino acids from their analogs. For example, a membrane reactor system using LeuDH has been developed for the continuous production of L-leucine and L-*tert*-leucine [42]. Semipermeable nylon-polyethyleneimine artificial cells containing LeuDH, alcohol dehydrogenase and dextran-NAD^+ has also been developed, for the production of L-leucine,

L-valine and L-isoleucine [43]. AlaDH and PheDH are also used for the syntheses of L-alanine analog [44] and L-phenelalanine [45], respectively. The deaminations of amino acids catalyzed by amino acid dehydrogenases accompany the formation of NADH or NADPH, which has a maximum absorption at 340 nm. Thus, the amount of amino acids, oxo acids and ammonia can be determined spectrophotometrically by measuring the change of absorption at 340 nm, caused by the formation or disappearance of NAD(P)H during the reaction. As an important tool in clinical chemistry, bioprocess control and nutrition studies, analyses of amino acids, oxo acids or ammonia can be carried out using amino acid dehydrogenases instead of ion exchange high-performance liquid chromatography (HPLC) .

Hence, discovery and creation of novel type of amino acid dehydrogenases are very important, not only for elucidation of their new role in carbon and nitrogen metabolisms of cells, but also for new industrial applications such as syntheses and assays of amino acids and their analogs. Further investigation in biochemical and biotechnological aspects of novel amino acid dehydrogenases will have great significance.

3. Research background and aim of this study

As described in the prior section, L-leucine dehydrogenase (LeuDH, EC 1.4.1.9) is a kind of enzyme that catalyzes the deamination of L-leucine and other branched chain L-amino acids to their corresponding keto analogs, using NAD^+ as a cofactor. The reaction that LeuDH catalyzes is shown in Fig. i.

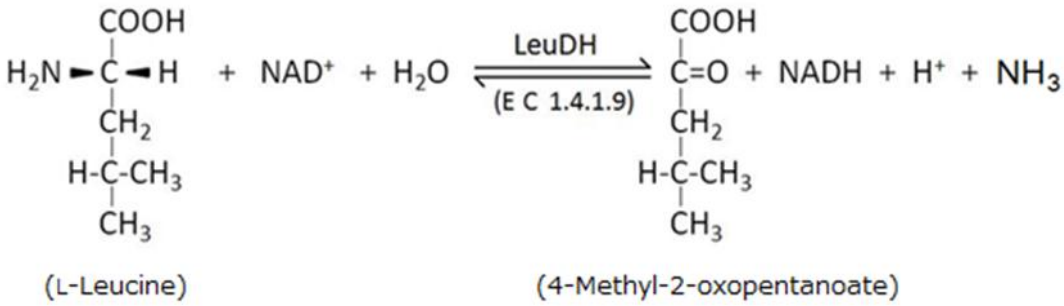

Fig. i. Reaction of L-leucine dehydrogenase

Till now, LeuDHs from mesophiles (e.g., *Lysinibacillus* [formerly: *Bacillus*] *sphaericus* [31], *B. cereus* [33], and *Corynebacterium pseudodiphtheriticum* [46]) and thermophiles (e.g., *Geobacillus* [formerly: *Bacillus*] s*tearothermophilus* [34, 47], *B. licheniformis* [35] and *Thermoactinomyces intermedius* [36]) have been purified and characterized. Moreover, the crystal structure of the LeuDH from *L. sphaericus* [48, 49] has been determined. By contrast, there are still no reports on LeuDHs from psychrophiles. And results of BLAST searches for sequence homology among psychrophiles using known LeuDH sequences as queries showed that no homologous genes were found among psychrophilic bacterial strains.

Again, as described in the prior section, LeuDHs have been used extensively in

the production of various compounds [50, 51] and in food and clinical analyses [52-55]. In general, two prerequisites for enzyme application in bioprocesses are high catalytic activity and stability. As described in section 1 “Psychrophiles and psychrophilic enzymes” in this general introduction, thermophilic enzymes have high thermostability but their activities decrease markedly when the temperature decreases. Thus, enzymes from thermophiles are not always practical under lower temperatures. On the other hand, psychrophilic enzymes have high catalytic efficiency at low temperatures, but are thermolabile. Therefore, an enzyme that exhibits high levels of both stability and catalytic activity even at low temperatures, would be highly desirable for practical use.

The relationship between the catalytic properties and the structure of highly thermostable amino acid dehydrogenases has been investigated using GluDH [28, 56-57], AspDH [58, 59] and AlaDH [60, 61]. Structural analysis of substrate specifity of mesophilic LeuDH has been carried out [49], but research about the relationship between structures and enzymatic characteristics related to temperatures of LeuDHs is still absent.

In this study, LeuDH activity was screened in several psychrophiles and strong activity was found in *Sporosarcina psychrophila* DSM 3, a psychrophile that survives only at temperatures below 30˚C [62, 63]. As a result, the *S. psychrophila* DSM 3 LeuDH is expected to be the counterpart of thermophilic and mesophilic LeuDHs. The aim of this study is to reveal the enzymatic and structural characterizations of psychrophilic LeuDH, by carrying out purification, characterization, gene sequencing and crystal structure analysis of the *S. psychrophila* LeuDH, for the purpose of gaining some insight into the basis of

psychrophilic properties,.

Chapter 1

Screening of L-leucine dehydrogenase among psychrophilic bacteria

1.1 Introduction

L-Leucine dehydrogenase isolated from psychrophile has not been reported before this work. To find candidate enzymes for the research of psychrophilic LeuDH, screening of LeuDH among psychrophiles are carried out in this chapter. As it is already known that LeuDH plays an important role in spore germination and exists mainly in mesophilic *Bacillus* species [31]. Thus, *Bacillus* strains and the closely related bacterial strains are selected as the source organisms for screening.

1.2 Materials and methods

1.2.1 Psychrophilic microorganisms

Microorganisms used for screening were: *B. psychrosaccharolyticus* DSM 6, *B. psychrosaccharolyticus* DSM 2270, *Jeotgalibacillus marinus* (synonyms: *Bacillus globisporus* subsp. *marinus*, *Bacillus marinus*, *Marinibacillus marinus*) DSM 1297, *J. marinus* DSM 1298, *Sporosacina psychrophila* (synonym: *Bacillus psychrophilus*) DSM 3 and *S. psychrophila* DSM 2274.

B. psychrosaccharolyticus DSM 6 and DSM 2270 are psychrophilic bacterial

strains with an optimum growth temperature of 20˚C, and the range of their growth temperature is 0˚C to 30˚C [62]. *J. marinus* DSM 1297 and DSM 1298 are psychrophilic bacterial strains isolated from Josephine Bank (North East Atlantic) sediments with an optimum growth temperature of 20˚C, and the range of their growth temperature is 5˚C to 30˚C [64]. *S. psychrophila* DSM 3 and DSM 2274 are psychrophilic bacterial strains isolated from soil and river water, with an optimum growth temperature of 25˚C, and the range of their growth temperature is 0˚C to 30˚C [62].

Psychrophilic bacterial strains mentioned above were all purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, GERMANY).

1.2.2 Microorganisms cultivation

Microorganisms mentioned above were aerobically cultivated under their optimum growth conditions. Details of cultivations are shown in the following tables.

Table 1-1. Medium and cultivation temperature for *B. psychrosaccharolyticus* DSM 6

Peptone	5 g
Meat extract	3 g
dH_2O	1000 ml
pH	7.0
Cultivation temp.	20˚C

Table 1-2. Medium and cultivation temperature for *S. psychrophila* DSM 3, DSM 2274 and *B. psychrosaccharolyticus* DSM 2270

Peptone from casein	15 g
Peptone from soymeal	5 g
NaCl	5 g
dH_2O	1000 ml
pH	7.2
Cultivation temp.	20˚C

Table 1-3. Medium and cultivation temperature for *J. marinus* DSM 1297 and DSM 1298

NaCl	24.00 g	SrCl2·6H2O	0.04 g
$MgCl_2·6H_2O$	11.00 g	$NaSiO_3·9H_2O$	5 mg
Na_2SO_4	4.00 g	NaF	3 mg
$CaCl_2$·6H2O	2.00 g	NH_4NO_3	2 mg
KCl	0.70 g	Fe_3PO_4·4H2O	2 mg
KBr	0.10 g	Peptone	5.00 g
H_3BO_3	0.03 g	Yeast extract	1.00 g
dH_2O	1000 ml	Cultivation temp.	20˚C

The cells were cultivated until the optical density of the culture reaches 0.6 and harvested by centrifugation at 5,000×g for 15 min at 4˚C. The harvested cells were washed by 0.85% NaCl once, and 10 mM potassium phosphate buffer containing

0.02% 2-mercaptoethanol (pH 7.0) twice. The washed cells were stored at -20˚C until use.

1.2.3 Preparation of crude extract

Washed cells were suspended in 10 mM potassium phosphate buffer containing 0.02% 2-mercaptoethanol (pH 7.0), whose volume (ml) is 3 fold of the cells' wet weight (g). The suspended cells were disrupted by sonication for 10 min 3 times, with a 5 min break every time at 4˚C. Then the mixture was centrifuged at 5,000×g for 20 min. The supernatant solution was used as crude extract.

1.2.4 Enzyme activity assay

Activity of LeuDH deamination was assayed in a spectrophotometer with a glass cubette thermostatically maintained at 25˚C. The standard reaction mixture for the oxidative deamination reaction contains 200 μmol of glycine-KOH buffer (pH 10.5), 10 μmol of L-leucine, 1.25 μmol of NAD^+ and enzyme in a final volume of 1.0 ml. Reaction was started by addition of enzyme into the reaction mixture. The components of reaction mixture are shown in Table 1-4.

Table 1-4. Reaction mixture for LeuDH deamination activity assay

Glycine-KOH buffer (pH 10.5)	200 μmol
L-leucine	10 μmol
NAD^+	1.25 μmol
dH_2O	
Enzyme	
Total volume	1.0 ml

1.2.5 Definition of LeuDH activity unit

One unit of LeuDH activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of NADH per minute under the reaction conditions mentioned above. Number of LeuDH activity unit can be calculated according to the following formulation.

$$\text{activity } (\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1} \text{ or unit} \cdot \text{ml}^{-1}) = \frac{\Delta A_{340}(\text{min}^{-1}) \times \text{volume}_{\text{Reaction Mixture}}(1.0 \text{ ml})}{\varepsilon\ (6.22\ \text{mM}^{-1} \cdot \text{cm}^{-1}) \times \text{volume}_{\text{enzyme solution}}(\text{ml}) \times \text{length}(1.0 \text{ cm})}$$

Activity: enzyme activity per ml enzyme solution (μmol·min^{-1}·ml^{-1} or unit·ml^{-1})

ΔA340: Change of 340nm absorption per minute during reaction (min^{-1})

Length: optical path length of the cubette (1.0 cm)

ε: Molecular absorption coefficient of NADH (6.22 $\text{mM}^{-1} \cdot \text{cm}^{-1}$)

Specific activity is defined as enzyme activity per mg protein (unit/mg protein) and can be calculated according to the following formulation.

$$\text{specific activity } (\text{unit} \cdot \text{mg}^{-1} \text{ protein}) = \frac{\text{activity } (\text{unit} \cdot \text{ml}^{-1})}{\text{protein concentration } (\text{mg} \cdot \text{ml}^{-1})}$$

1.2.6 Protein assay

Protein concentration was determined by Bradford method [65].

Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, US) was diluted by water into 5 folds of its original volume and BSA was used as

standard protein. Various volume (0, 2, 4, 6, 8 μl) of BSA solution (1.162 mg/ml) was added into 1.0 ml diluted Protein Assay Dye Reagent Concentrate, and the solution was completely mixed. Then absorption at 595 nm (A_{595}) was measured after incubation at room temperature for 5 min; a calibration curve of relationship between protein concentration and A_{595} was made. Proper amount of protein sample was added to 1.0 ml diluted Protein Assay Dye Reagent Concentrate and A_{595} was measured after 5 min incubation. Then protein concentration of the sample was calculated according to its A_{595} value and the calibration curve.

1.3 Results

Activity of LeuDH in the cell extracts of several psychrophilic microorganisms was detected (Table 1-5).

Table 1-5. LeuDH activity distribution in psychrophilic bacteria

Strains		Specific activity (unit/mg protein)
Bacillus psychrosaccharolyticus	DSM 6	0.0068
	DSM 2270	0.0100
Jeotgalibacillus marinus	DSM 1297	0.0627
	DSM 2398	0.0498
Sporosarcina psychrophila	DSM 3	0.0740
	DSM 2274	0.0441

1.4 Discussion

LeuDH activity was detected in all the psychrophilic bacterial strains used for screening. The specific activities were detected mainly in a range between 0.01 and 0.08 units/mg protein. As the purpose of this screening step is to find the candidate enzyme for purification and characterization, *Sporosarcina psychrophila* DSM 3, which showed the highest activity of LeuDH in its cell extract, was selected for purification and characterization of LeuDH.

1.5 Summary

In this chapter, screening of LeuDH was carried out among 6 psychrophilic bacterial strains.

1) LeuDH activity was detected in crude extract of all the 6 psychrophilic bacterial strains.
2) *Sporosarcina psychrophila* DSM 3, a psychrophilic bacterial strain which has an optimum growth temperature of 25˚C, and cannot survive at temperatures over 30˚C, showed a highest specific activity in its crude extract.

Chapter 2

Purification of L-leucine dehydrogenase from psychrophilic bacterium *Sporosarcina psychrophila*

2.1 Introduction

In general, highly purified preparation of enzyme is necessary for enzymatic characterization, because undesirable side reactions may occur when impure enzymes are used. To examine the enzymatic properties of LeuDH from *S. psychrophila* DSM 3, the purification of the enzyme was tried from the cell extract, using various separation methods.

As described in general introduction, although some mesophilic and thermophilic LeuDHs have been purified, there is still no report on psychrophilic LeuDH purification. In this chapter, various methods were used to highly and efficiently purify the *S. psychrophila* DSM 3 LeuDH from the cell extract.

2.2 Materials and methods

Potassium phosphate (10 mM, pH 7.0) buffer with 0.02% 2-mercaptoethanol was used as the standard buffer in the purification procedure, and the operation temperature was below room temperature.

2.2.1 Microorganism and cultivation conditions

As described in Chapter 1, the psychrophilic bacterium *S. psychrophila* DSM 3 was selected as the LeuDH production strain.

S. psychrophila DSM 3 was grown in a medium containing 1.5% peptone from casein, 0.5% peptone from soymeal and 0.5% NaCl. The pH was adjusted to 7.2 using 10 M NaOH. The growth temperature (20˚C) recommended by DSMZ was used for the cell cultivation. The cells were aerobically cultivated in a glass test tube (Φ2 cm × 18 cm) containing 5 ml medium at 20˚C for 3 days using shaking culture (70 rpm). Then the previously cultivated mixture was added to a 2 L conical flask containing 500 ml medium, cultivated at 20˚C for 48 h by shaking (70 rpm). The cells were harvested by centrifugation at 5,000×g under 4˚C for 15 min. The cells harvested were twice washed by 0.85% NaCl and once washed by the standard buffer. The washed cells were stored at -20˚C until use.

2.2.2 Preparation of crude extract

Washed cells were suspended with about 3-fold volume of 10 mM the standard buffer and disrupted by sonication for 10 min 3 times, with a 5 min break every time at 4˚C. Then the mixture was centrifuged at 5,000×g for 10 min. The supernatant solution was used as the cell extract (crude extract).

2.2.3 Ammonium sulfate precipitation

Ammonium sulfate (17.6 g) was added to the crude extract (100 ml) to 30% saturation, and then the mixture was centrifuged at 10,000×g for 10 min. The supernatant was collected, and the ammonium sulfate (19.8 g) was added to the

supernatant to make the ammonium sulfate concentration 60% saturation. And then the mixture was again centrifuged at 10,000×g for 10 min. The precipitate was collected, dissolved in standard buffer, and dialyzed against the standard buffer (more than 100 volumes of the enzyme one) overnight. The dialyzed solution was used as enzyme solution for the next step.

2.2.4 Ion exchange chromatography

For the further purification of the enzyme, a DEAE-Toyopearl ion exchange chromatography was used. The enzyme solution (91 ml) was applied to a DEAE-Toyopearl column (Φ2.5 cm × 10 cm) previously equilibrated with the standard buffer. After the column was washed with the same buffer (250 ml), the enzyme was eluted with a linear gradient between two buffer solutions (200 ml each) containing 0 and 0.5 M NaCl. The active fractions were pooled to be enzyme solution used for the next step.

2.2.5 Hydrophobic chromatography

Butyl Sepharose hydrophobic chromatography was used in the next purification step. Solid ammonium sulfate (30% saturation) was added to the enzyme solution and the enzyme solution (50 ml) was applied to a butyl Sepharose column (Φ1.0 cm × 5 cm) previously equilibrated with standard buffer containing 30% saturation of ammonium sulfate. After the column was washed with the same buffer (75 ml), the enzyme was eluted with a linear gradient between two buffer solutions (60 ml each) containing 30% and 0% saturation of ammonium sulfate. The active fractions were collected.

2.2.6 Size exclusion chromatography

Superdex 200 pg column (GE Healthcare, Waukesha, WI, USA) was used for further purification as the size exclusion chromatography. The enzyme solution was concentrated to a volume of 12 ml using centrifugal units (Millipore) and centrifugation at 4˚C for 10 min and dialyzed against the standard buffer containing 0.15 M NaCl (1 l volume). Then the enzyme solution was centrifuged and filtered to remove the precipitate formed. All the buffers and solutions in this step were previously filtered and degassed before use.

The enzyme solution was applied on a column of Superdex 200 pg (Φ2.6 cm × 60 cm) previously equilibrated with the standard buffer containing 0.15 M NaCl, and then eluted with the same buffer. The active fractions were collected and the purity was checked by SDS-PAGE.

2.2.7 Enzymatic and protein assays

The activity of LeuDH in all the purification steps was determined by the method described in section 1.2.4 “Enzymatic activity assay” and the definition of unit is the same as described in section 1.2.5 “Definition of LeuDH activity unit”.

Protein concentration was determined by Bradford method, which has been described in section 1.2.6 “Protein assay”.

2.2.8 Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the method described as follows. Protein sample (10 μl) for SDS-PAGE was mixed with 10 μl SDS treatment solution and 1 μl

2-mercaptoethanol, and was heat treated at 100˚C for 4 min. Treated sample (20 μl) was applied to 12.5% polyacrylamide gel and electrophoresed together with protein markers (Precision Plus Protein All Blue Standards, Bio-Rad Laboratories) with an electric power of 20 mA. Electrophoresis was stopped when the BPB marker band reached near the bottom of gel, and the gel was stained with CBB staining reagent for 2 min and destained with 10% methanol and 10% acetic acid containing solution (destaining reagent) until protein bands appeared clearly. Components of reagents mentioned above are showed in the following tables.

Table 2-1. SDS treatment solution

BPB	0.01%
SDS	4%
Glycerol	20% (w/v)
0.125 M Tris-HCl buffer	(pH 6.9)

Table 2-2. Polyacrylamide gel - stacking gel

0.5 M His-Tris buffer (pH 6.8) + 0.4% SDS	1.25 ml
30% polyacrylamide + 0.8% Bis-Tris	0.67 ml
dH_2O	2.58 ml
TEMED	5 μl
1.5% APS	0.50 ml

Table 2-3. Polyacrylamide gel - separating gel

1.5 M His-Tris buffer (pH 8.9) + 0.4% SDS	2.50 ml
30% polyacrylamide + 0.8% Bis-Tris	4.17 ml
dH_2O	2.83 ml
TEMED	5 µl
1.5% APS	0.50 ml

Table 2-4. SDS-PAGE running buffer

Tris	3.0 g
Glycine	14.4 g
SDS	1.0 g
dH_2O	
Total volume	1000 ml

Table 2-5. CBB staining reagent

CBB - R250	0.5 g
Methanol	100 ml
Acetic acid	10 ml
dH_2O	90 ml
Total Volume	200 ml

Table 2-6. Destaining reagent

Methanol	100 ml
Acetic acid	100 ml
dH_2O	800 ml

2.3 Results

2.3.1 Summary of S. psychrophila LeuDH purification

The cells (wet weight: 29.5 g) were obtained by cultivation of 2 L culture medium. The enzyme was purified by the procedure described in Table 2-7.

Table 2-7. Purification of LeuDH from *S. psychrophila* DSM 3

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (folds)
Crude extract	712	801	1.12	100	1.00
Ammonium sulfate precipitation	118	694	5.88	86.6	5.25
DEAE-Toyopearl	46.6	541	11.6	67.5	10.4
Butyl Sepharose	18.7	416	22.2	51.9	19.8
Size exclusion chromatography	2.90	156	53.8	19.5	48.0

2.3.2 Ion-exchange chromatography

The elution pattern on DEAE cellulose column chromatography is shown in Fig. 2-1. Protein concentrations in the fractions were monitored by measuring absorbance at 280nm (A_{280}), and LeuDH activity of every fraction was determined by the method described in section 2.2.7 "Enzymatic activity and protein concentration assay". Only one activity peak was observed and the active fractions were collected.

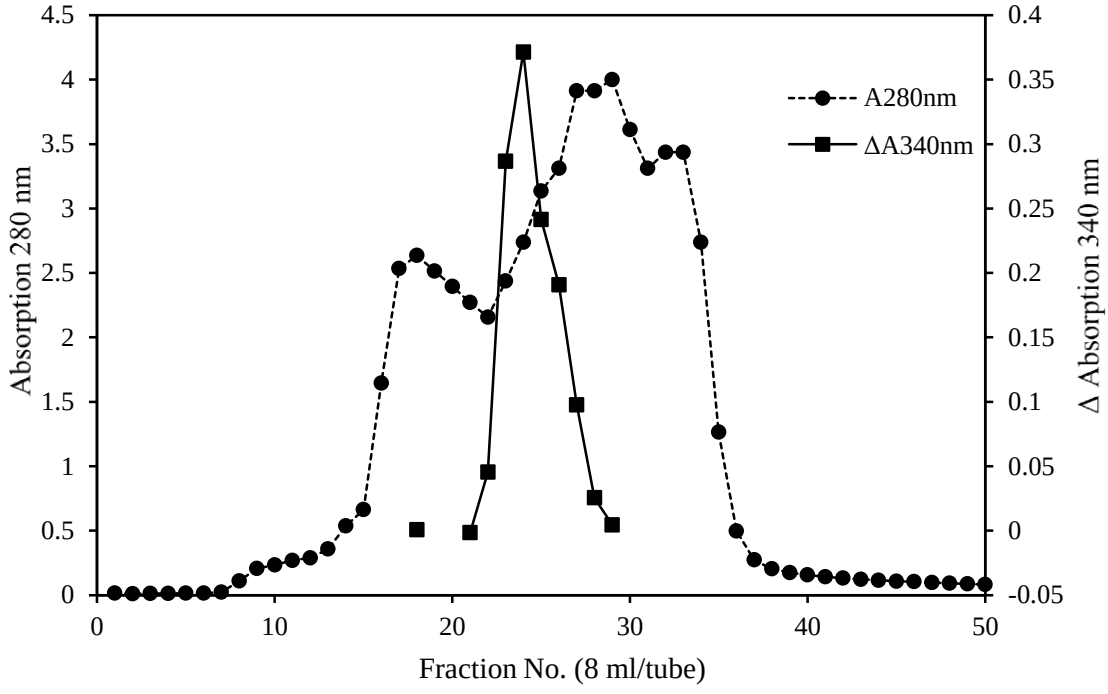

Fig. 2-1. Protein concentration and LeuDH activity of fractions after DEAE-Toyopearl column chromatography: (●) absorption at 280 nm; (■) increase of absorption at 340 nm per min (5 μl enzyme).

2.3.3 Hydrophobic chromatography

Butyl Sepharose column chromatography was carried out as described in section 2.2.5 “Hydrophobic chromatography”, and a typical elution pattern is shown in Fig. 2-2. Only one activity peak was observed in the elution profile and active fractions of LeuDH were collected.

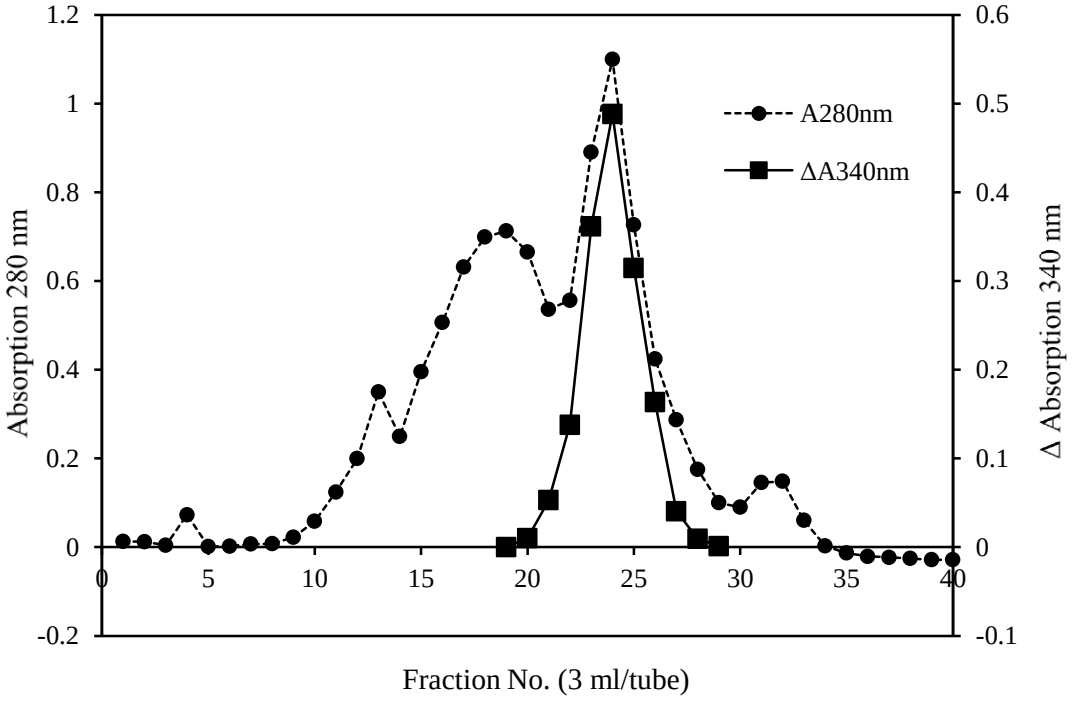

Fig. 2-2. Protein concentration and LeuDH activity of fractions after butyl Sepharose column chromatography: (●) absorption at 280 nm; (■) increase of absorption at 340 nm per min when 5 μl enzyme of every fraction was used for enzyme assay.

2.3.4 Size exclusion chromatography

Protein concentration and enzyme activity of fractions after gel filtration were determined. Two different protein peaks in fractions of gel filtration were obtained and the one which had a smaller elution volume showed LeuDH activity. The active fractions were collected and the solution was concentrated.

The concentrated solution after gel filtration was detected by SDS-PAGE, and the result is shown in Fig. 2-3. The pooled active fractions after size exclusion chromatography have been purified to homogeneity judging from the single band on the polyacrylamide gel.

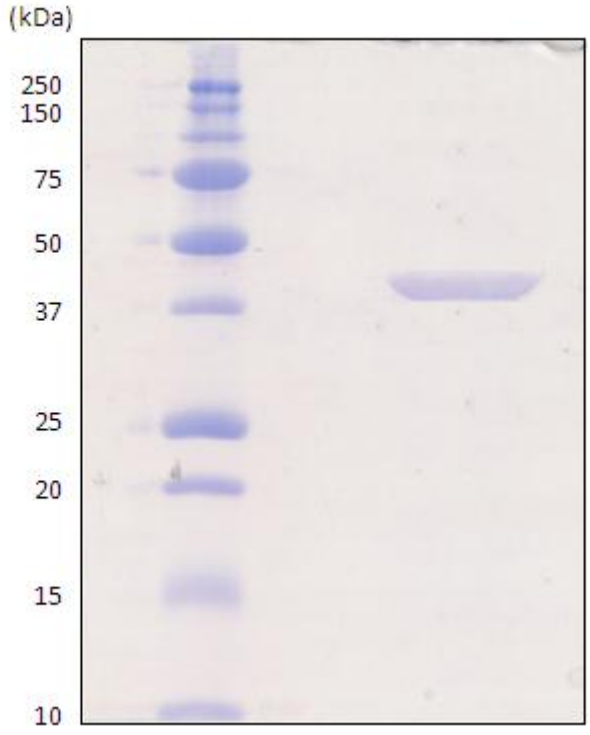

Fig. 2-3. SDS-PAGE of purified *S. psychrophila* DSM 3 LeuDH after size exclusion chromatography. The left lane is standard protein marker, and the right lane is the purified LeuDH.

2.4 Discussion

According to the results described in section 2.3 “Results”, the effective purification of *S. psychrophila* LeuDH was established in this chapter. Three different chromatographies were used to purify the enzyme, and the result of SDS-PAGE showed that the enzyme was purified to homogeneity. The yield of enzyme was 19.5%, and the specific activity of purified enzyme was 48-fold higher compared with that of crude extract, which means that this procedure is efficient to purify the native *S. psychrophila* LeuDH. Enzyme amount that can be obtained from every 2 L culture was 2.9 mg, which is efficient to get enough protein for enzymatic characterization.

The purified *S. psychrophila* LeuDH has a specific activity of 53.8 units/mg protein, which is almost as high as the mesophilic and thermophilic ones. As there was no report about psychrophilic LeuDH, this is the first LeuDH purified from psychrophiles.

2.5 Summary

Purification of L-leucine dehydrogenase from psychrophilic bacterium *S. psychrophila* was carried out in this chapter.

1) The enzyme is purified to homogeneity using ammonium sulfate precipitation and 3 kinds of chromatography.
2) This is the first LeuDH purified from psychrophile.
3) The specific activity of the purified enzyme is 53.8 units/mg protein.

Chapter 3

Enzymatic Characterization of L-leucine dehydrogenase from psychrophilic bacterium *Sporosarcina psychrophila*

3.1 Introduction

Enzymes from psychrophiles are generally considered to have high catalytic activity at even low temperatures, but thermolabile. As *S. psychrophila* DSM 3 is a psychrophilic bacterium whose maximum growth temperature is 30˚C, LeuDH from this psychrophile is also expected to have general psychrophilic properties, at least the enough high catalytic efficiency to support its growth at low temperatures. Otherwise it would not be functional enough *in vivo*.

In this chapter, characteristics of LeuDH purified from *S. psychrophila*, as described in Chapter 2, were determined: including molecular mass, effects of pH and temperature on enzyme activity and stability, kinetics parameters and N-terminal amino acid sequence.

3.2 Methods and materials

3.2.1 Molecular mass determination

Subunit molecular mass was determined by SDS-PAGE using protein markers

mentioned in section 2.2.8 “Gel electrophoresis”. Subunit molecular mass of the sample was calculated by comparing its mobility with protein markers.

Native molecular mass was determined by Superdex 200 pg (Φ2.6 cm × 60 cm) column chromatography equilibrated with the standard buffer used in purification procedure containing 0.15 M NaCl. The following standard proteins were used to make a calibration curve: thyroglobulin (M_r. 669,000), ferritin (M_r. 440,000), aldolase (M_r. 158,000), conalbumin (M_r. 75,000), and ovalbumin (M_r. 43,000).

3.2.2 Effect of pH on enzyme activity

Various buffers were used to examine the effect of pH on the activity of LeuDH from *S. psychrophila* DSM 3. For oxidative deamination, the reaction mixture contained 200 μmol of different buffers, 10 μmol L-leucine, 1.25 μmol NAD^+, enzyme and distilled water in a total volume of 1.0 ml. Reaction mixture except enzyme was incubated in glass cubette at 25˚C for 2 min and the reaction was started by addition of enzyme. Increase of absorption at 340 nm (ΔA_{340}) during the first one minute after reaction start was measured to calculate enzyme activity. The pH of reaction mixture after the assay was checked. Buffers used are listed in Table 3-1.

Table 3-1. Buffers used for measurement of optimum pH

Buffer	Buffer pH	pH after assay
MES Buffer	6.0	6.13
	6.5	6.69
Phosphate-KOH buffer	6.5	6.83
	7.0	7.11
	7.5	7.49
	8.0	8.00
Glycine-KOH buffer	8.0	8.12
	8.5	8.71
	9.0	9.25
	9.5	9.42
	10.0	10.17
Carbonate buffer	10.0	9.98
	10.5	10.47
	11.0	10.64
	11.5	10.96
Phosphate-KOH buffer	10.5	10.74
	11.0	11.28
	11.5	11.74
Na_2HPO_4-NaOH Buffer	11.5	11.72
	12.0	12.01

For reductive amination, the reaction mixture contained 750 μmol NH_4Cl-NH_4OH buffer at various pHs, 10 μmol ketoleucine-Na, 0.1 μmol NADH, distilled water and enzyme solution in a final volume of 1.0 ml. Reaction mixture except NADH was incubated in a glass cubette at 25˚C for 2 min and the reaction was started by addition of NADH. Decrease of absorbance at 340 nm for the first one minute after start of the reaction was monitored to determine enzyme activity. Then pH of the reaction mixture was also checked after each reaction. The pHs of original buffer and pHs after reaction are shown in Table 3-2.

Table 3-2. pHs of various NH_4Cl-NH_4OH buffer used for measurement of amination optimum pH

Buffer pH	pH after assay
8.1	7.95
8.5	8.26
9.0	8.75
9.5	9.22
10.0	9.68

3.2.3 Effect of pH on enzyme stability

Enzyme was incubated in various buffers (final concentration 100 mM) for 30 min at 25˚C and then the activity of the aliquot was measured under the standard assay conditions. Buffers used are shown in Table 3-3.

Table 3-3. Buffers used for measurement of pH stability

pH	Buffer
4~5	HAc-NaAc buffer
6	MES buffer
7	MOPS-NaOH buffer
8	Tris-HCl buffer
9~11	Glycine-KOH buffer
11.5~12	Na_2HPO_4-NaOH buffer

3.2.4 Effect of temperature on enzyme activity

Reaction mixture containing 200 μmol glycine-KOH buffer (pH 11.0), 10 μmol L-leucine and 1.25 μmol NAD^+ was incubated at various temperatures for 5 min. Reaction was started by addition of enzyme. Initial increase of absorbance at 340 nm was measured.

3.2.5 Effect of temperature on enzyme stability

Enzyme was incubated at different temperatures between 25˚C and 65˚C for 10 min, and then the remained activity was measured. The enzyme was also incubated at 45˚C, 50˚C and 55˚C for 2 h. An aliquot of enzyme was taken out every 10 min to check the remained activity.

3.2.6 Steady state kinetics analyses

The basic incubation mixture used for kinetic analyses was similar to that described in section 1.2.4 “Enzyme activity assay”; other conditions are specified in each figure in the results section (section 3.3.6 “Enzymatic kinetics”).

The initial velocity was determined by varying the concentration of one substrate at different fixed concentrations of the other substrate in the reaction. Analysis of the kinetic data was carried out according to the methods proposed by Cleland [66-68]. The K_m and k_{cat} values were determined from the secondary plots of intercepts versus reciprocal concentrations of the substrates.

3.2.7 Protein electrotransfer and N-terminal sequence determination

Purified LeuDH from *S. psychrophila* was electrotransfered from an SDS-PAGE gel to a PVDF membrane under a voltage of 15 V for 1 h, in transfer buffer. The PVDF membrane was washed by distilled water and washing buffer, and then stained by Poncceau S staining reagent for 5 min. The PVDF membrane decolorized by the destaining reagent was dried, and protein bands were cut to be used for determination of the N-terminal sequence.

The solutions used in blotting are shown in Table 3-4.

Table 3-4. Buffers used in protein electrotransfer

1. Washing buffer		2. Transfer buffer		3. 10×CAPS buffer	
H_3BO_3	0.618 g	10×CAPS buffer	10 ml	CAPS	2.21 g
NaCl	1.461 g	Methanol	10 ml	NaOH	0.32 g
dH_2O		dH_2O	80 ml	dH_2O	
Total vol.	1000 ml	Total vol.	100 ml	Total vol.	100 ml
pH adjusted to 8.0 by NaOH				pH adjusted to 11.0	

4. Poncceau S staining reagent		5. Destaining reagent	
Poncceau S	0.1 g	50% methanol	100 ml
acetic acid	1 ml	10% acetic acid	20 ml
dH_2O	99 ml	dH_2O	80 ml

A protein band was excised and subjected to automated Edman degradation using a Shimadzu Model PPSQ-10 protein sequencer (Shimadzu Cooperate, Kyoto, Japan).

BLAST search was carried out using the database of National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

3.3 Results

3.3.1 Molecular mass and subunit structure

SDS-PAGE of purified *S. psychrophila* LeuDH was carried out using a 12.5% polyacrylamide gel. Subunit molecular mass was determined to be about 43 kDa by comparing its mobility with protein markers (Fig. 3-1).

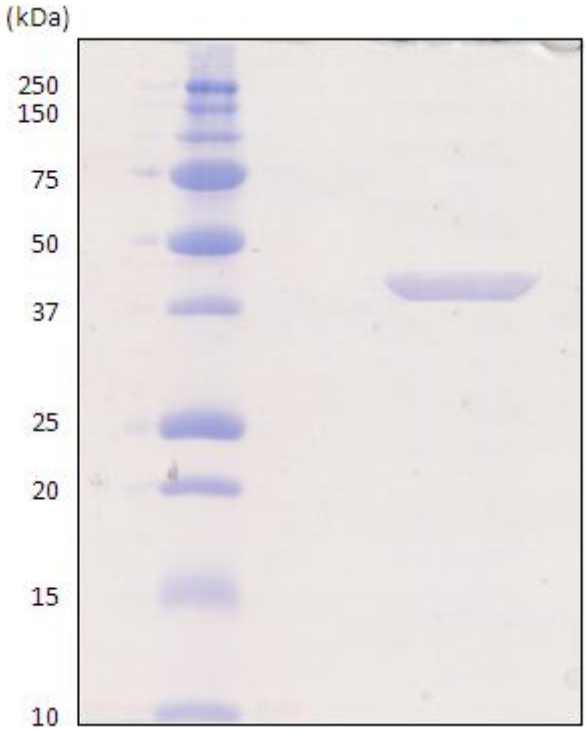

Fig. 3-1. SDS-PAGE of purified *S. psychrophila* LeuDH after size exclusion chromatography. The left lane is standard protein marker, the right lane is the purified LeuDH.

After carrying out the Superdex 200 pg column chromatography, calibration curve was made using elution volumes and molecular masses of the standard proteins mentioned in section 3.2.1 “Molecular mass determination” (Fig. 3-2). The elution volume of *S. psychrophila* LeuDH was measured, and its native molecular mass was determined to be about 340 kDa.

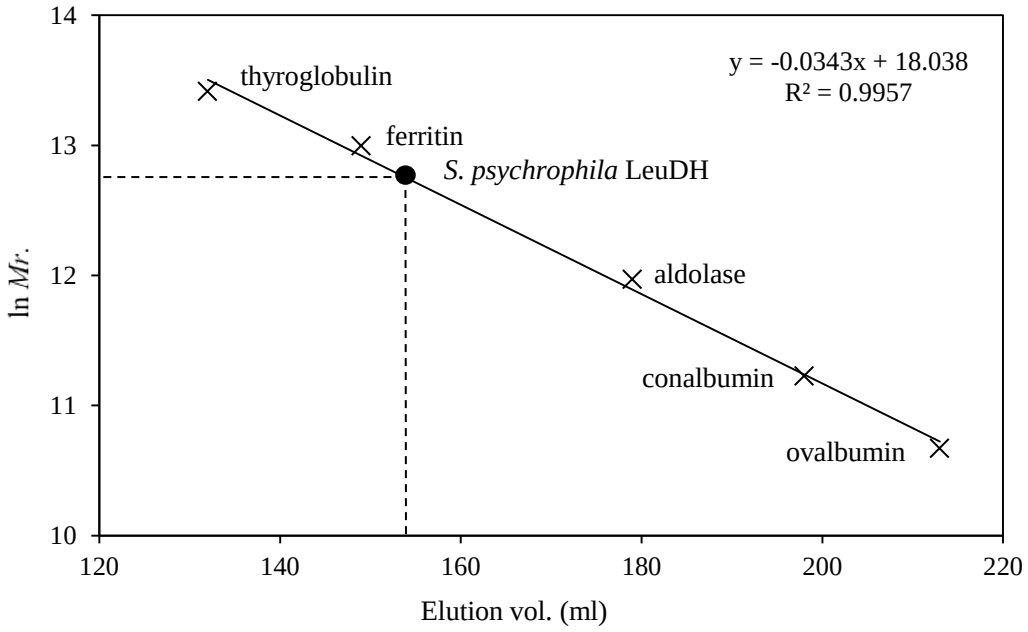

Fig. 3-2. Determination of native molecular mass by gel filtration

According to the molecular mass of subunit (about 43 kDa) and native molecule (about 340 kDa), we can conclude that *S. psychrophila* LeuDH exists as a homooctamer.

3.3.2 Effect of pH on enzyme activity

The optimum pH of oxidative deamination and reductive animation were determined to be around 11 and 9, respectively (Fig. 3-3).

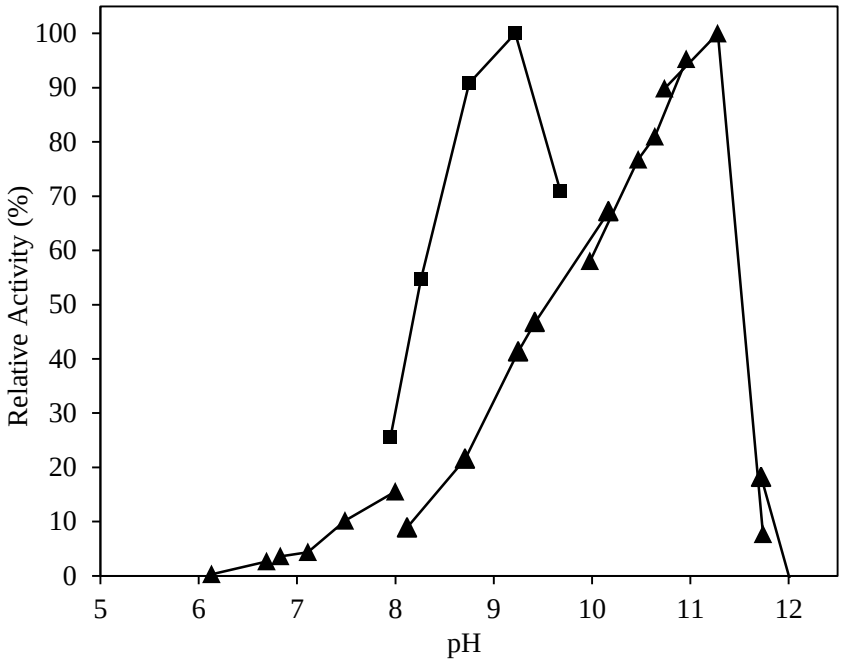

Fig. 3-3. Effect of pH on enzyme activity (▲: deamination and ■: amination). The buffers used for deamination were: MES-NaOH (pH6~6.7), glycine-KOH (pH 6.8~10.2), carbonate-bicarbonate (pH 8~10.8) and Na_2HPO4-NaOH (pH 11~12). NH_4Cl-NH_4OH buffer was used for amination. The pHs of buffers were adjusted at 25˚C.

3.3.3 Effect of pH on enzyme stability

Almost full activity (over 90%) of enzyme was retained after incubation at a pH range of 5~11 at 25˚C (Fig. 3-4), indicating that the enzyme is stable at a wide range of pH.

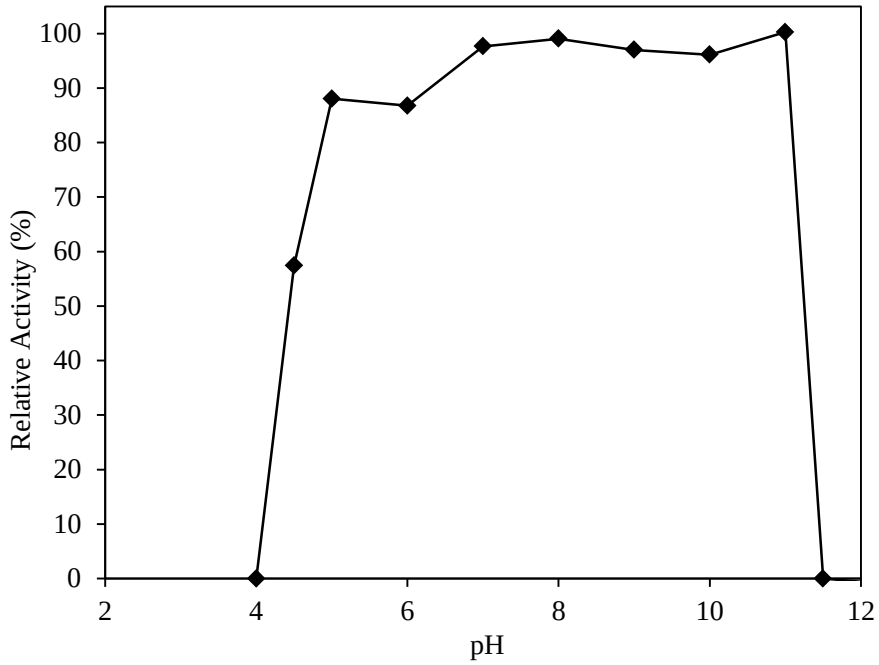

Fig. 3-4. Effect of pH on enzyme stability. The enzyme in buffer of various pHs was incubated at 25˚C for 30 min, and the residual activity was assayed for the oxidative deamination of L-leucine at 25˚C. The buffers used were described in section 3.2.3 "Effect of pH on enzyme stability". The pHs of all buffers were adjusted at 25˚C.

3.3.4 Effect of temperature on enzyme activity

The maximum activity was observed at 50˚C, and the relative activities were 30% at 25˚C and 9% at 0˚C. Even at temperatures as low as 0˚C, *S. psychrophila* LeuDH had a specific activity of about 10 units/mg. But steep decrease of activity was observed above 55˚C, and the activity was completely lost at 70˚C (Fig. 3-5).

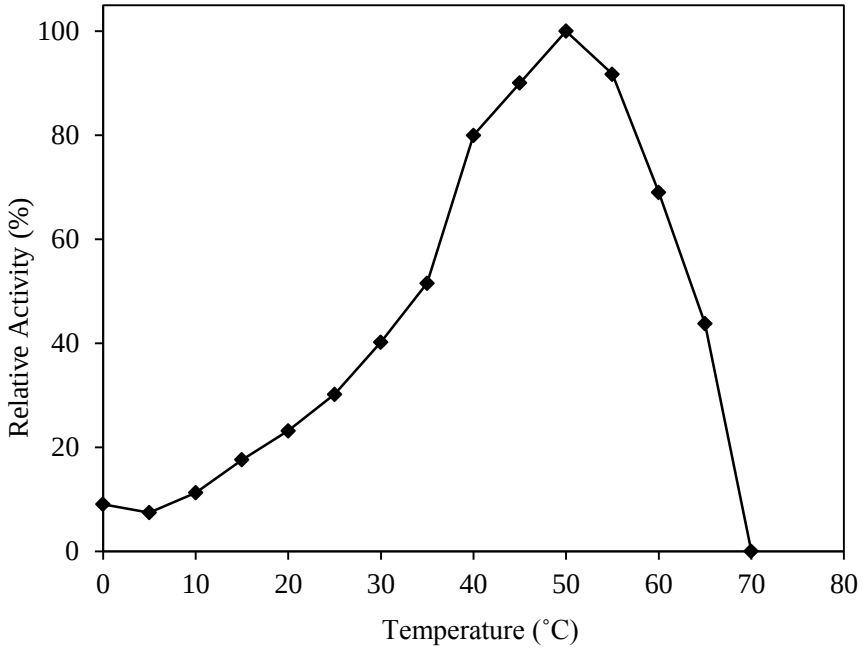

Fig. 3-5. Effect of temperature on enzyme activity. The reaction was assayed at various temperatures as described in section 3.2.4 “Effect of temperature on enzyme activity”.

3.3.5 Effect of temperature on enzyme stability

After the enzyme was incubated at various temperatures for 10 min, the remaining activity was measured. The full activity was retained after incubation below 50˚C for 10 min, and the activity disappeared completely after 10 min incubation at 65˚C (Fig. 3-6. A). During 2 h continuous incubation, *S. psychrophila* LeuDH lost half of its activity after about 30 min and 10 min at 50˚C and 55˚C, respectively (Fig. 3-6. B).

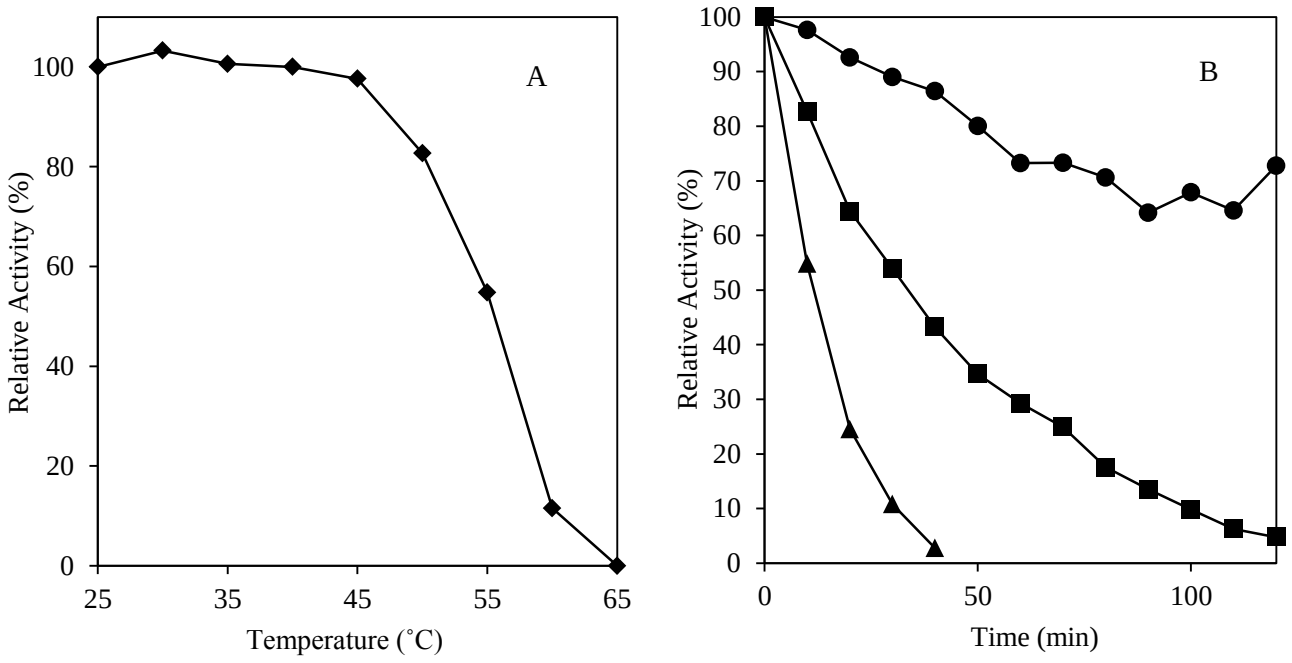

Fig. 3-6. Thermostability. (A) Residual activity at various temperatures. Enzyme solution was incubated at various temperatures for 10 min and cooled down on ice. (B) Residual activity during 2 h incubation under three different temperatures: (▲) 55˚C, (■) 50˚C and (●) 45˚C.

3.3.6 Steady state kinetics

To assess enzyme kinetics, the initial velocity analyses on the oxidative deamination of L-leucine at 20˚C was initially conducted. Double reciprocal plots of the initial velocity against various concentrations of L-leucine in the presence of several fixed concentrations of NAD^+ yielded intersecting straight lines (Fig. 3-7). This shows that the reaction proceeds in sequential steps via the formation of a ternary complex that includes the enzyme, NAD^+ and L-leucine [66-68]. From the secondary plots of the intercepts against the reciprocal of the fixed NAD^+ concentration, the K_m value for L-leucine was determined to be 0.65 mM. The K_m for NAD^+ was calculated to be 0.015 mM using the same method, according to secondary plots of the intercepts against the reciprocal of the fixed L-leucine concentration. The k_{cat} value was 10.0 s^{-1} at 20˚C, and the k_{cat}/K_m for L-leucine and NAD^+ were calculated to be 15.4 $mM^{-1}•s^{-1}$ and 667 $mM^{-1}•s^{-1}$, repectively.

Because *S. psychrophila* LeuDH was also active at temperatures as low as 0˚C, we also analyzed the apparent turnover number at 0˚C. The apparent k_{cat}/K_m for L-leucine and NAD^+ were calculated to be 4.96 $mM^{-1}•s^{-1}$ and 35.5 $mM^{-1}•s^{-1}$ at 0˚C, respectively, which indicates that this LeuDH is an effective catalyst at temperatures as low as 0˚C.

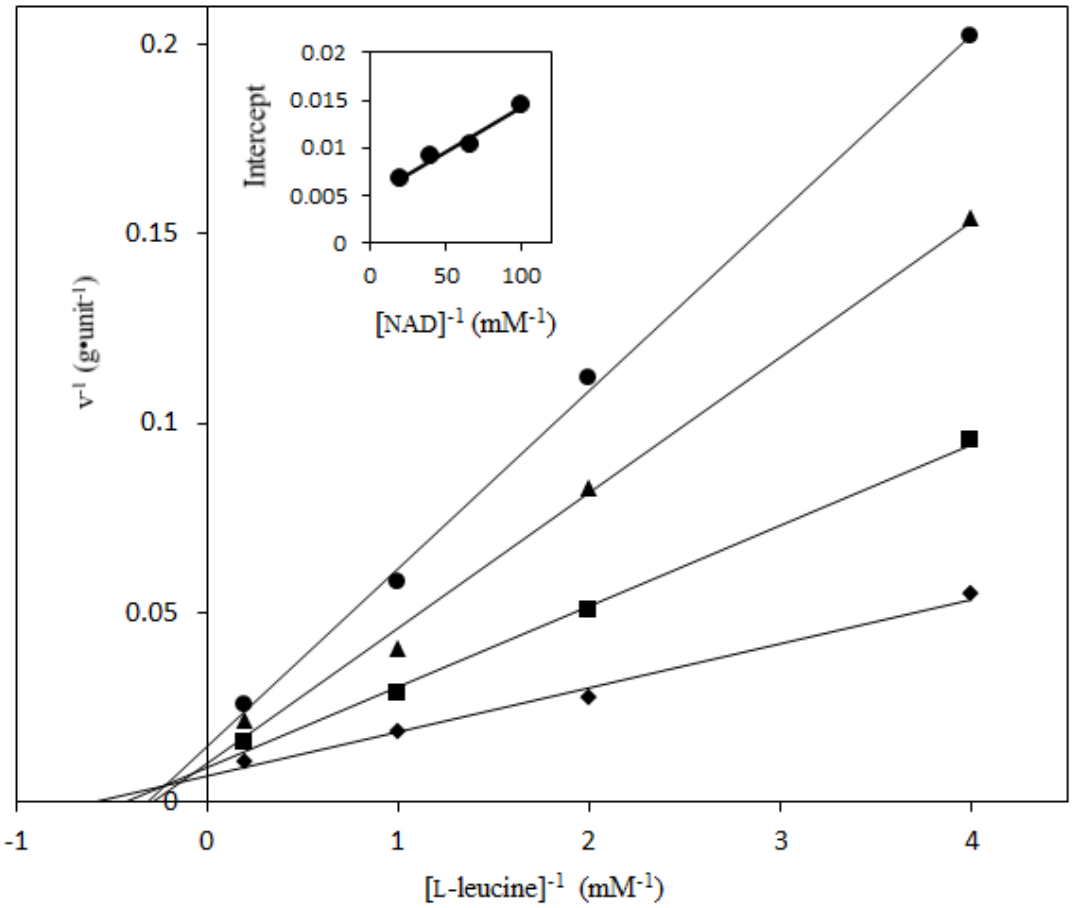

Fig. 3-7. Double reciprocal plots of initial velocity against L-leucine concentration at a series of fixed concentrations of NAD^+ (●-0.01 mM, ▲-0.015 mM, ■-0.025 mM, ◆-0.05 mM). *Inset*: secondary plots of the intercepts against the fixed NAD^+ concentrations.

3.3.7 N-terminal amino acid sequence

N-terminal amino acid sequence of LeuDH from *S. psychrophila* DSM 3 was determined to be **MEIFKYME**.

Protein-protein BLAST showed that similar N-terminal sequences were present in those of many other *Bacillus* species LeuDHs. Although some of them have a Leu3 from the N-terminal, instead of the Ile3 in *S. psychrophila* LeuDH. The sequences of some *Bacillus* LeuDHs are shown in Table 3-5.

Table 3-5. Comparison of N-terminal amino acid sequence of *S. psychrophila* LeuDH and some other *Bacillus* species LeuDHs

Strain	N-terminal amino acid sequence of LeuDH
Sporosarcina psychrophila	MEIFKYME----------
Geobacillus stearothermophilus	MELFKYMETYDYEQVLFCQR----------
Bacillus licheniformis	MELFRYMEQYDYEQLVFCQD----------
Geobacillus thermodenitrificans	MELFKYMETYDYEQVLFCQDKESGLKAIIA----
Bacillus amyloliquefaciens	MEIFKYMETYDYEQLVFCQDEQSGLKAIIA-----

3.4 Discussion

In this chapter, the enzymatic properties of *S. psychrophila* LeuDH were determined. In order to make clear the characteristics of psychrophilic LeuDH, the mesophilic and thermophilic LeuDHs from a mesophile *L. sphaericus* and a thermophile *G. stearothermophilus* are chosen for the comparison.

The N-terminal amino acid sequence of *S. psychrophila* LeuDH showed a high similarity with LeuDHs from other *Bacillus* species. This sequence is used as a reference for forward primer design in Chapter 4.

The molecular mass of *S. psychrophila* LeuDH native molecule is about 340 kDa, and molecular mass of subunit is about 43 kDa. According to the molecular mass, we can know that this enzyme exists as a homooctamer. LeuDH from the mesophile *L. sphaericus* is also homooctamer, with a native molecular mass of about 340 kDa [69]. LeuDH from the thermophile *G. stearothermophilus* has a native molecular mass of about 300 kDa [32], and its subunit molecular mass was calculated to be 46,903 Da from nucleotide sequence [47], which indicates that *G. stearothermophilus* LeuDH is also homooctamer. The subunit structure of *S. psychrophila* LeuDH is very similar to its mesophilic and thermophilic counterparts.

The pH optima of *S. psychrophila* LeuDH were around 11.0 for oxidative deamination and around 9.0 for reductive amination, similar to those of its mesophilic and thermophilic counterparts. And the enzyme is stable over a wide range of pHs (around 5 to 11, at 25˚C), just like the cases of the mesophilic and thermophilic counterparts.

S. psychrophila LeuDH has an optimum reaction temperature of 50˚C, showing

that the temperature is much lower than those of thermophilic *G. stearothermophilus* LeuDH and mesophilic *L. sphaericus* LeuDH (70˚C and 60˚C, respectively). Activation energy was also calculated according to the data of relative activities at various temperatures. The activation energy of *S. psychrophila* LeuDH was calculated to be 45.6 kJ/mol, which is quite similar to those of mesophilic *B. cereus* LeuDH (44.4 kJ/mol) [70] and thermophilic *G. stearothermophilus* LeuDH (47.8 kJ/mol) [34].

S. psychrophila LeuDH is stable up to 45˚C after 10 min incubation, while the *L. sphaericus* one is stable up to 55˚C [47], and the *G. stearothermophilus* one is more stable, up to 70˚C even after 30 min incubation [71]. The psychrophilic *S. psychrophila* LeuDH is less stable to heat compared with mesophilic and thermophilic counterparts.

Kinetics parameters of *S. psychrophila* LeuDH were also determined and they have no significant difference compared with those of LeuDHs from *L. sphaericus* and *G. stearothermophilus*.

To sum up, *S. psychrophila* LeuDH is similar to its thermophilic and mesophilic counterparts, except that it has a lower optimum reaction temperature and is less thermostable. But the high specific activity and k_{cat}/K_m values at 0˚C indicated that the enzyme has high catalytic efficiency at temperatures lower than room temperature. Comparison of enzymatic properties is shown in Table 3-6.

Table 3-6. Characteristics of LeuDHs from *S. psychrophila*, *L. sphaericus* and *G. stearothermophilus*

	S. psychrophila	*L. sphaericus* [31]	*G. stearothermophilus* [34]
Optimum reaction pH (deamination)	11	10.5	11
Optimum reaction pH (amination)	9	9-9.6	8.8-9.7
pH stability	5-11	6.5-9	5-11
Optimum reaction temperature (˚C)	50	60	70
Thermostability	stable up to 45˚C	stable up to 55 ˚C	stable up to 70˚C
Activation energy (kJ/mol)	45.6	44.4 (*B. cereus*) [a]	47.8 [b]
K_m for L-leucine (mM)	0.65	1.0	4.4
K_m for NAD^+ (mM)	0.015	0.39	0.49
k_{cat} of L-Leu (s^{-1})	10.0 (20 ˚C)	10.83 [c]	50 [69]

[a]: Activation energy of *B. cereus* LeuDH calculated from published data [70].

[b]: Activation energy of *G. stearothermophilus* LeuDH calculated from published data [34].

[a]: k_{cat} of *L. sphaericus* LeuDH with a His-tag [72].

3.5 Summary

Characterization of *S. psychrophila* LeuDH was carried out in this chapter.

1) Molecular mass of the native enzyme molecule was determined to be about 340 kDa, and molecular mass of the subunit was determined to be about 43 kDa. The enzyme is a homooctamer.
2) pH optima for oxidative deamination and reductive amination were determined to be around 11 and 9, respectively.
3) Optimum reaction temperature was determined to be 50˚C.
4) The enzyme is stable up to 45˚C and is stable at a wide range of pH 5~11.
5) K_m values for L-leucine and NAD^+ were determined to be 0.65 mM and 0.015 mM respectively, and the k_{cat} value was 10.0 s^{-1} at 20˚C. Apparent k_{cat}/K_m for L-leucine and NAD^+ at 0˚C indicated that this enzyme is an effective catalyst at temperatures as low as 0˚C.
6) N-terminal amino acid sequence was determined to be "MEIFKYME".
7) This enzyme is similar to its thermophilic and mesophilic counterparts, except that it has a lower optimum reaction temperature and is shows less thermostability.

Chapter 4

Gene sequence determination and expression of *Sporosarcina psychrophila* L-leucine dehydrogenase

4.1 Introduction

The results of prior chapter showed that *S. psychrophila* LeuDH has the maximum reaction rate at lower temperature and has less thermostability, compared with its mesophilic counterpart *L. sphaericus* LeuDH and thermophilic counterpart *G. stearothermophilus* LeuDH, and the enzyme is active enough at moderate and low temperatures. Analyses of primary and crystal structures may give us useful molecular information to explain these characteristics, but the primary and crystal structures of *S. psychrophila* LeuDH are both unknown so far.

Although the N-terminal amino acid sequence of *S. psychrophila* LeuDH has been determined in the above chapter, we can still not get the corresponding gene sequence simply from genome information, because the complete genome of *S. psychrophila* has not been analyzed yet.

Comparison of primary structure is impossible without full nucleotide / amino acid sequence. In addition, molecule replacement and model building of protein crystal structure are also difficult without amino acid sequence information. In this chapter, full sequence of *S. psychrophila* LeuDH gene was determined, in order to obtain more information about the functional and structural characteristics of the psychrophilic *S. psychrophila* LeuDH.

4.2 Methods and materials

4.2.1 Strains and cultivation conditions

S. psychrophila DSM 3 cells were cultivated as described in section 2.2.1 "Microorganism and cultivation conditions".

Escherichia coli DH5α and *E. coli* BL21 Rosetta (DE3) used in this study were purchased from Merck Millipore (Darmstadt, Germany). All the *E. coli* cells were grown on solid Luria-Bertani medium (LB medium. 0.5% yeast extract, 1% tryptone, 1% NaCl, 1.5% agar, pH 7.2~7.5) at 37˚C. Ampicillin (50 μg/ml), isopropyl-β-dithiogalactopyranoside (IPTG, 100 μg/ml) and 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal, 75 μg/ml) were added if needed. Cultivation in liquid medium was carried out in LB medium at 37˚C, and 50 μg/ml of ampicillin was added if needed.

4.2.2 Genomic and plasmid DNA extraction

S. psychrophila DSM 3 cells were harvested and suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. The suspension was incubated with 0.1% lysozyme for 20 min at 37˚C, after which 1% SDS, Proteinase K (50 μg/ml) and RNaseA (20 μg/ml) were added, and the mixture was incubated at 37˚C until it turned clear. Genomic DNA was isolated from the solution using phenol/chloroform extraction.

Plasmid DNA was extracted using HiYield Plasmid Mini Kit (RBC Bioscience, Taipei, Taiwan).

4.2.3 DNA assay

Concentration of genomic or plasmid DNA was determined by measuring A_{260} or using Quant-iT dsDNA Broad-Range Assay Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). Purity and length of DNA fragments were checked by 1.5% or 0.8% agarose gel electrophoresis, using standard DNA ladders (Bioneer, Alameda, CA, USA) as markers.

4.2.4 Cloning and sequencing of S. psychrophila LeuDH gene

A protein-protein BLAST search was carried out using the N-terminal amino acid sequence of *S. psychrophila* LeuDH, which was determined in Chapter 3, as a query sequence. The N-terminal amino acid sequence of *S. psychrophila* LeuDH was highly similar to those of *Anoxybacillus flavithermus* WK-1, *B. amyloliquefaciens* FZB42, *B. pumilus* SAFR-032, *G. thermodenitrificans* NG80-2 and *B. subtilis* subsp. *subtilis* str.168. The forward primer was then designed based on the determined N-terminal amino acid sequence; the reverse primer was then designed based on a conserved sequence in the butyrate kinase gene, which is situated just downstream of the LeuDH gene in the bacterial genome picked up above (Fig. 4-1). Two primers (FWD 5'-ATGGAGATTTTTAAATATATGGA-3' and REV 5'-GTTGANGTNGANCCNGGATT-3') were applied to amplify the DNA fragment (presumed length ~ 1.1 kbp) which is considered to contain the LeuDH gene. Polynucleotides synthesis was ordered through Hokkaido System Science Co., Ltd (Sapporo, Hokkaido, Japan).

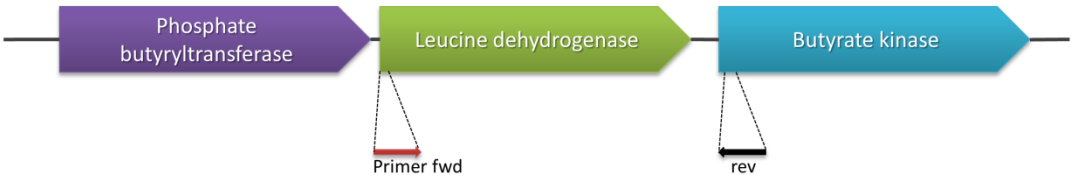

Fig. 4-1. Conserved genes upstream and downstream LeuDH gene. In the picked up strains (*Anoxybacillus flavithermus* WK-1, *B. amyloliquefaciens* FZB42, *B. pumilus* SAFR-032, *G. thermodenitrificans* NG80-2 and *B. subtilis* subsp. *subtilis* str.168), phosphate butyryltransferase gene locates upstream the LeuDH gene, and butyrate kinase gene locates downstream it.

Polymerase chain reaction (PCR) was carried out using *TaKaRa Ex Taq* polymerase (Takara Bio Inc., Otsu, Japan) and the following protocol was used: the reaction was started at 94˚C (2 min) and thermo-cycled for 35 cycles of 94˚C (30 s), 47˚C (30 s) and 72˚C (1 min), with a final extension at 72˚C (10 min). The reaction was carried out in a 100 µl reaction mixture containing 500 ng genomic DNA, 40 pmol primer FWD and 40 pmol primer REV.

The amplified DNA fragments were cloned into pTA2 vector using TArget Clone (TOYOBO Life Science, Osaka, Japan).

E. coli DH5α cells were transformed with the resulting plasmid and cultured. The plasmid was then extracted and sequenced using an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Forest City, CA, USA) with BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems) and BigDye XTerminator Purification Kits (Applied Biosystems).

4.2.5 Construction of plasmids for expression of recombinant S. psychrophila LeuDH

The gene of *S. psychrophila* LeuDH was amplified by PCR, using primers 5'-CATATGGAGATTTTTAAATATATGGA-3' (underline: *Nde* I site) and 5'-CTCGAGAGCGTCTTGAAAGTACGC-3' (underline: *Xho* I site), which are designed according to the nucleotide sequence of *S. psychrophila* LeuDH. Polynucleotides synthesis was also ordered through Hokkaido System Science Co., Ltd. PCR was carried out using *TaKaRa Ex Taq* polymerase (Takara Bio Inc.) and the following protocol: the reaction was started at 94˚C (2 min) and thermo-cycled for 35 cycles of 94˚C (30 s), 45˚C (30 s) and 72˚C (2 min), with a final extension at 72˚C (10 min). The reaction was carried out in a 100 μl reaction mixture containing 500 ng genomic DNA and 40 pmol of each primer.

Amplified fragments were inserted into pTA2 vector and the constructed pTA2 vector was transformed into *E. coli* DH5α. The transformed *E. coli* DH5α cells were cultivated, and the constructed pTA2 plasmids were extracted. The plasmids extracted from *E. coli* DH5α were digested by *Nde* I and *Xho* I at 37˚C for 4 h, and then the fragment with a length of about 1 kbp was extracted and purified from agarose gel, using QIAquick Gel Extraction Kit (QIAGEN, Venlo, Netherlands). Sequencing of the purified fragment was carried out to ensure that it has the same sequence with the original *S. psychrophila* DSM 3 LeuDH gene. Then this fragment was cloned into pET21a vector (Merck) and the constructed pET21a plasmid was named as *Sp*LeuDH-pET21a.

4.2.6 Abundant expression of recombinant S. psychrophila LeuDH

*Sp*LeuDH-pET21a plasmid was transformed into *E. coli* BL21 Rosetta (DE3), and the *E. coli* cells harboring *Sp*LeuDH-pET21a were grown aerobically at 37˚C in 5 ml LB medium containing ampicillin until A_{660} reached 0.5, and then IPTG was added to the medium to a final concentration of 1 mM. After further cultivation for 3 h, cells were harvested by centrifugation and resuspended in 10 mM potassium phosphate buffer (pH 7.0) containing 0.02% 2-mercaptoethanol. The harvested cells were disrupted by sonication, and the mixture was centrifuged at 5,000×g for 10 min. The supernatant solution was used to check the expression of *S. psychrophila* LeuDH, by 12.5% SDS-PAGE.

4.3 Results

4.3.1 Sequence of S. psychrophila LeuDH gene

The *S. psychrophilia* LeuDH gene was cloned into *E. coli* DH5α cells and the entire nucleotide sequence of the gene was analyzed as described in section 4.2.4 "Sequencing of *Sporosarcina psychrophila* DSM 3 LeuDH gene". The nucleotide sequence of the analyzed fragment (1,095 bp) contained one open reading frame encoding the enzyme. The complete sequence is shown in Fig. 4-2, and its Genbank accession No. is AB706401. The gene encoded a polypeptide consisting of 364 amino acid residues, and the N-terminal amino acid sequence was identical to that determined through Edman degradation. The sequence pattern GXGXXG, which indicates the presence of dinucleotied-binding motif, was found at residues 139-144. The calculated molecular mass of this peptide was 39,345 Da, and the

amino acid sequence showed 80% identity with the sequences of *L. sphaericus* LeuDH and *G. stearothermophilus* LeuDH (Fig. 4-4).

```
ATGGAGATTTTTAAATATATGGAACATCAGGATTATGAACAACTTGTTATTTGTCAGGAT   60
 M  E  I  F  K  Y  M  E  H  Q  D  Y  E  Q  L  V  I  C  Q  D    20

AAAGCTTCAGGACTAAAAGCGATCATCGCTATTCATGATACGACACTCGGCCCGGCGCTT  120
 K  A  S  G  L  K  A  I  I  A  I  H  D  T  T  L  G  P  A  L    40

GGTGGAACACGTATGTGGACGTATGCAAGTGAAGAAGAAGCAATTGAAGACGCACTTCGC  180
 G  G  T  R  M  W  T  Y  A  S  E  E  E  A  I  E  D  A  L  R    60

CTTGCGCGCGGTATGACTTACAAAAACGCGGCTGCTGGATTGAATCTTGGTGGCGGAAAA  240
 L  A  R  G  M  T  Y  K  N  A  A  A  G  L  N  L  G  G  G  K    80

ACAGTTATTATAGGGAATCCAAAAACAGACAAAAACGATGAAATGTTCCGTGCTTTCGGT  300
 T  V  I  I  G  N  P  K  T  D  K  N  D  E  M  F  R  A  F  G   100

CGTTATATTGAAGGATTGAACGGTCGTTATATCACTGCAGAAGATGTAGGAACAACTGAA  360
 R  Y  I  E  G  L  N  G  R  Y  I  T  A  E  D  V  G  T  T  E   120

GCGGATATGGACTTAATTAATCTTGAAACAGATTATGTTACAGGTACTTCTGCTGGAGCG  420
 A  D  M  D  L  I  N  L  E  T  D  Y  V  T  G  T  S  A  G  A   140

GGTTCATCTGGTAATCCTTCGCCTGTTACTGCTTATGGTATTTATTACGGTATGAAGGCA  480
 G  S  S  G  N  P  S  P  V  T  A  Y  G  I  Y  Y  G  M  K  A   160

GCAGCAAAAGAAGCATTTGGAGACGACTCGCTAGCGGGCAAAACAGTAGCGGTACAAGGT  540
 A  A  K  E  A  F  G  D  D  S  L  A  G  K  T  V  A  V  Q  G   180

GTCGGAAACGTTGCTTATGCACTTTGTGAGTATTTGCACGAAGAAGGTGCAAAACTAATC  600
 V  G  N  V  A  Y  A  L  C  E  Y  L  H  E  E  G  A  K  L  I   200

ATCACGGATATCAACGAAGAAGCAGTTCAGCGTGCAGTTGATGCATTCGGTGCAACAGCA  660
 I  T  D  I  N  E  E  A  V  Q  R  A  V  D  A  F  G  A  T  A   220

GTCGGCATCAATGAAATTTACTCTCAAGAAGCAGATATCTTCGCACCATGTGCACTAGGT  720
 V  G  I  N  E  I  Y  S  Q  E  A  D  I  F  A  P  C  A  L  G   240

GCAATCATAAACGATGAGACGATTCCACAATTGAAAGCAAAAGTTATTGCAGGATCTGCT  780
 A  I  I  N  D  E  T  I  P  Q  L  K  A  K  V  I  A  G  S  A   260

AACAATCAATTGAAAGAAACGCGTCACGGCGATTTAATCCATGAAATGGGTATTGTTTAC  840
 N  N  Q  L  K  E  T  R  H  G  D  L  I  H  E  M  G  I  V  Y   280

GCACCTGACTACGTTATCAACTCGGGCGGCGTTATCAACGTTGCAGATGAGCTTGATGGT  900
 A  P  D  Y  V  I  N  S  G  G  V  I  N  V  A  D  E  L  D  G   300

TATAACCGTGAACGTGCACTTAAACGCGTAGAAGGAATCTATGACGTAATCGGGAAAATT  960
 Y  N  R  E  R  A  L  K  R  V  E  G  I  Y  D  V  I  G  K  I   320

TTCGCAATCTCAAAACGTGATAATATCCCAACGTATGTTGCAGCTGACCGCATGGCGGAA 1020
 F  A  I  S  K  R  D  N  I  P  T  Y  V  A  A  D  R  M  A  E   340

GAGCGTATTGCACGTGTTGCTAATACAAGAAGCACTTTCCTACAAAACGAAAAAAGCGTA 1080
 E  R  I  A  R  V  A  N  T  R  S  T  F  L  Q  N  E  K  S  V   360

CTTTCAAGACGCTAA                                               1095
 L  S  R  R  -                                                 364
```

Fig. 4-2. Complete nucleotide sequence of *S. psychrophila* LeuDH gene and its deduced amino acid sequence. The N-terminal amino acid sequence identical with experimental result is underlined.

4.3.2 Abundant expression of recombinant LeuDH

The result of SDS-PAGE is shown in Fig. 4-3. Abundantly expressed protein band was detected in the supernatant of *E. coli* cell extract. The subunit molecular mass of the abundantly expressed protein was almost the same with native *S. psychrophila* LeuDH and the LeuDH activity was confirmed by the spectrophotometric method described in Section 1.2.4 “Enzyme activity assay”.

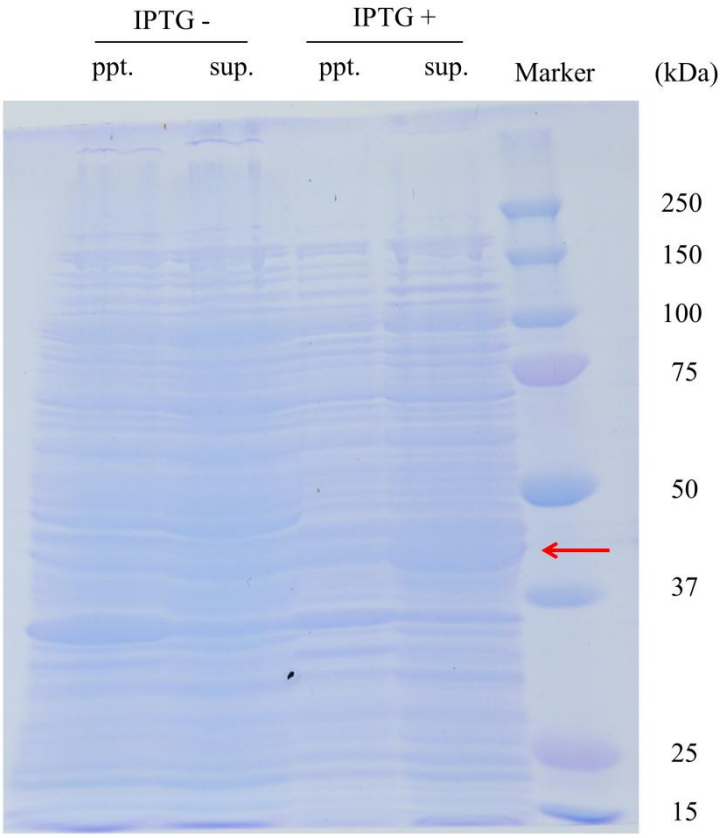

Fig. 4-3. SDS-PAGE of recombinant *S. psychrophila* LeuDH expressed abundantly. Sample lanes are: precipitate and supernatant obtained from culture without IPTG, precipitate and supernatant obtained from culture with IPTG, standard protein marker from the left. Expressed protein is pointed out with a red arrow.

4.4 Discussion

In this chapter, the complete nucleotide sequence of *S. psychrophila* LeuDH gene was determined. The deduced amino acid sequence showed high similarity with the sequences of mesophilic *L. sphaericus* LeuDH and thermophilic *G. stearothermophilus* one, but there are still some differences.

Alignment of LeuDH amino acid sequences is shown in Fig. 4-4. Five glycine residues (Gly41, Gly42, Gly77, Gly78 and Gly290) thought to contribute to the shape of the active site [49, 73] are conserved in the sequence of *S. psychrophila* LeuDH, as they are in *L. sphaericus* and *G. stearothermophilus* LeuDHs. As important residues for the catalysis, Lys68 is known to bind to the 1-carboxyl group of the substrate [74], and Lys80 has an unusually low pK_a that assists the nucleophilic attack by a water molecule on the α-carbon atom of the substrate [75, 76]. Both of the two Lys residues are well conserved in the sequence of *S. psychrophila* LeuDH, just like they are in the sequences of other LeuDHs. In contrast, about 20% of the amino acid residues of the *S. psychrophila* LeuDH are not identical with those of the mesophilic *L. sphaericus* and thermophilic *G. stearothermophilus* LeuDHs. Thus, it is thought that these different amino acids may be responsible to the specific characteristics of *S. psychrophila* LeuDH. But no more distinct information to explain the high catalytic efficiency at low temperatures and poor thermostability of *S. psychrophila* LeuDH can be obtained just from the sequence information and further structural analysis is necessary.

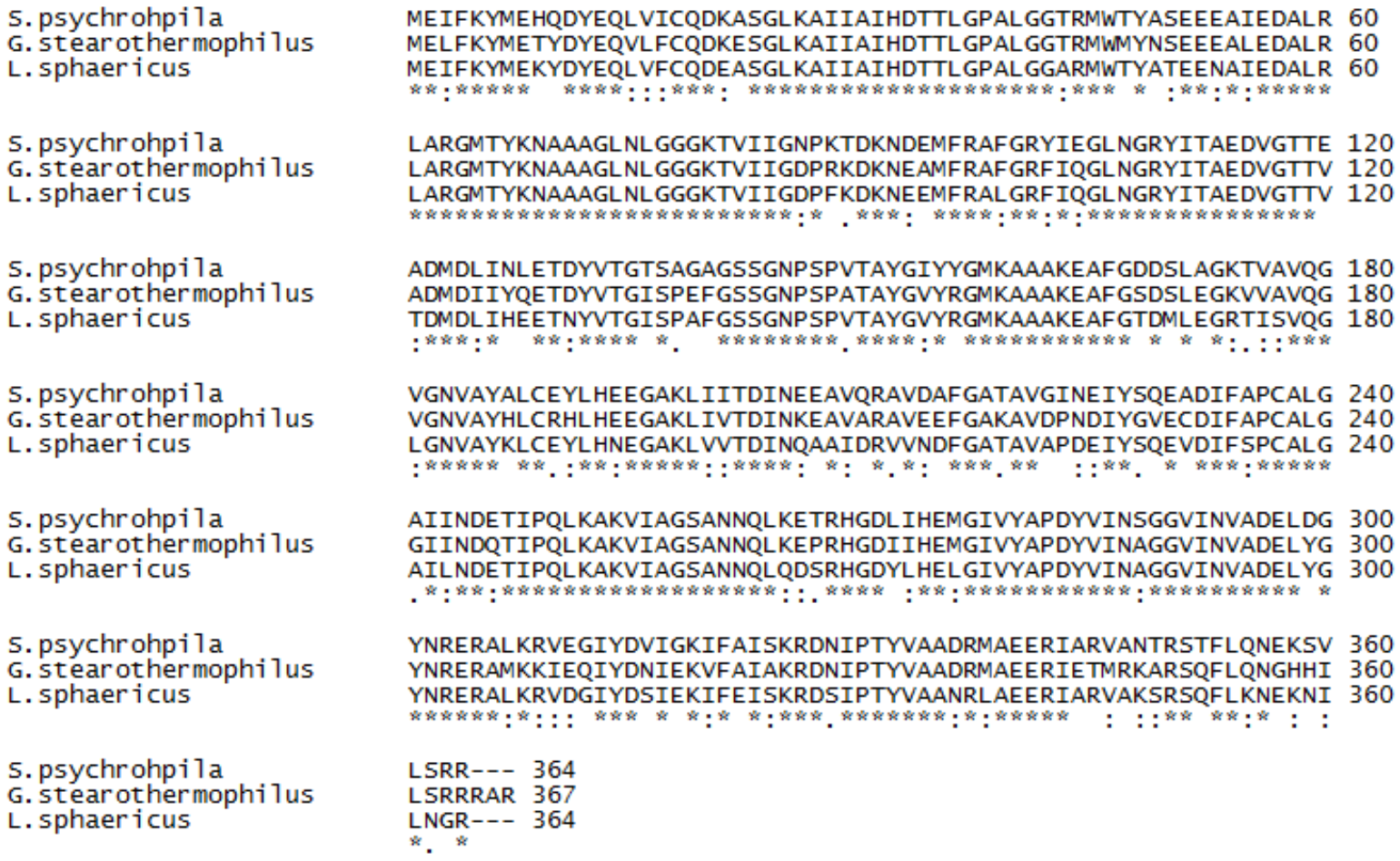

Fig. 4-4. Alignment of amino acid sequences: *S. psychrophila*, *L. sphaericus* and *G. stearothermophilus* LeuDHs.

4.5 Summary

1) Nucleotide sequence of *S. psychrophila* LeuDH gene (1,095 bp) was determined.
2) The deduced amino acid sequence of *S. psychrophila* LeuDH was very similar (>80% identities) with its mesophilic and thermophilic counterparts.
3) Success in expression of recombinant *S. psychrophila* LeuDH indicated that this protein can be produced in *E. coli* cells and efficient preparation of recombinant protein is possible.
4) Further structural analysis is needed to find explanations for the catalytic efficiency and poor thermostability of *S. psychrophila* LeuDH.

Chapter 5

Crystallization and structural analysis of *Sporosarcina psychrophila* L-leucine dehydrogenase

5.1 Introduction

As described in the prior chapter, significant difference between the psychrophilic *S. psychrophila* LeuDH and the mesophilic or thermophilic counterparts was not found from comparison of the primary structure. Analysis of crystal structures may be needed to find out the structural differences between these LeuDHs.

Although there are various methods to predict protein 3-dimensional structure from amino acid sequence information, rigorous structural analysis is still necessary for solid structural descriptions and comparison. In this chapter, *S. psychrophila* LeuDH was crystallized and the 3-dimensional structure was analyzed. With respect to 3-dimensional structure of LeuDH, only mesophilic LeuDH has been analysed so far [49]. Comparison between the structures of psychrophilic and mesophilic LeuDHs is expected to give us important information on inherent characteristics of psychrophilic enzyme.

5.2 Methods and materials

5.2.1 Screening for S. psychrophila LeuDH crystallization conditions

Crystallization of *S. psychrophila* LeuDH was carried out by sitting drop vapor diffusion method, and crystal screen kits Wizard (Emerald Biosystems, Bainbridge Island, WA, USA), Cryo (Emerald Biosystems) and Crystal Screen (Hampton Research, Aliso Viejo, CA, USA) were used for primary screening. Drops (1 μl) of 14 mg/ml protein solution in 10 mM potassium phosphate (pH 7.0) were mixed with 1 μl of crystallization solution and equilibrated against 0.1 ml of the reservoir solution at 20˚C. Growth of crystals was observed with a stereomicroscope OLYMPUS SZX-1LLD2-100 (Olympus Corporation, Tokyo).

5.2.2 Optimum conditions of S. psychrophila LeuDH crystallization

The following crystallization conditions were used for obtaining large crystals of *S. psychrophila* LeuDH.

1) 0.1 M tri-sodium citrate dihydrate pH 5.6 / 0.5 M ammonium sulfate / 0.1 M lithium sulfate monohydrate

2) 0.1 M CHES pH 9.5 / 1.26 M ammonium sulfate / 0.2 M sodium chloride

Buffers that have the sample components, but different pHs or different salt concentrations of the 2 buffers mentioned above were prepared, and crystals of *S. psychrophila* LeuDH were grown under these conditions, using the same method described in section 5.2.1 “Screening for *S. psychrophila* LeuDH crystallization conditions”. Growth of crystals was observed with a stereomicroscope.

5.2.3 X-ray diffraction and data collection

Crystals of *S. psychrophila* LeuDH were grown under its optimum crystallization conditions, and were soaked in a cryoprotective solution containing 30% glycerol and flash-cooled in a nitrogen stream (-173 ˚C). Data were collected under cryo conditions at beamline AR-NW12 at the Photon Factory (Tsukuba, Japan). Diffraction images were processed using the program HKL2000 [77].

5.2.4 Molecular replacement, model building and refinement

Assuming that an asymmetric unit contained two subunits, the V_M and V_{sol} values [78] were approximately 3.58 $Å^3Da^{-1}$ and 0.66, respectively. The crystal structure of LeuDH from *L. sphaericus* (PDB ID: 1LEH, [48]) was applied as a search model, and the program MOLREP [79] was used for molecular replacement phase determination. The model was refined using the programs Coot [80] and CNS [81]. The final model was validated using the program PROCHECK in the CCP4 suite [82]. Figures were made using the program PyMOL (DeLano Scientific; http://www.pymol.org).

5.3 Results

5.3.1 Screening for S. psychrophila LeuDH crystallization conditions

Crystals were formed in several different buffers and the scale of the crystals, as well as corresponding buffer components, are shown in Table 5-1.

The *S. psychrophila* LeuDH crystals were obtained mainly in crystallization

buffers containing ammonium sulfate. In most buffers, multiple tiny protein crystals were observed; large single crystals were observed only under two crystallization conditions. The two conditions were chosen as the initial optimization conditions for crystallization (Table 5-1).

Table 5-1. Primary screening for crystallization

Components of crystal growth buffer	Crystal scale
0.1 M tri-sodium citrate dihydrate pH 5.6 / 0.5 M ammonium sulfate / 0.1 M lithium sulfate monohydrate	Large
0.1 M CHES pH 9.5 / 1.26 M ammonium sulfate / 0.2 M sodium chloride	Large
0.1 M Tris pH 8.5 / 1.5 M ammonium sulfate / 12% (v/v) glycerol anhydrous	Medium
0.1 M Tris pH 8.5 / 1.26 M ammonium sulfate / 0.2 M lithium sulfate	Medium
0.1 M Tis hydrochloride pH 8.5 / 2.0 M ammonium sulfate	Small
0.1 M HEPES-Na pH 7.5 / 2% (v/v) polyethylene glycerol 400 / 2.0 M ammonium sulfate	Small
0.1 M MES pH 6.5 / 1.6 M ammonium sulfate / 10% (v/v) dioxane	Small
0.1 M MES pH 6.5 / 0.01 M cobaltous chloride hexahydrate / 1.8 M ammonium sulfate	Small
0.1 M MES pH 6.5 / 0.2 M ammonium sulfate / 30% (v/v) polyethylene glycol monomethyl ether 5000	Small
0.1 M HEPES pH 7.5 / 0.1 M sodium chloride / 1.6 M ammonium sulfate	Small
0.1 M CAPS pH 10.5 / 2.0 M Na/K phosphate / 0.2 M lithium sulfate	Small
0.1 M CHES pH 9.5 / 1.0 M Na/K tartrate / 0.2 M lithium sulfate	Small
0.1 M CHES pH 9.5 / 1.0 M sodium citrate	Small
0.1 M Tris pH 7.0 / 2.0 M ammonium sulfate / 0.2 M lithium sulfate	Small
0.1 M phosphate-citrate pH 4.2 / 35% (v/v) 2-propanol	Small

5.3.2 Optimum crystallization conditions of S. psychrophila LeuDH

The following conditions were used for the optimization of crystallization.

1) 0.1 M tri-sodium citrate dihydrate pH 4.5 / 5.0 / 5.6 / 6.2
   0 M / 0.5 M / 1.0 M / 1.5 M / 2.0M ammonium sulfate
   0.1 M lithium sulfate monohydrate

2) 0.1 M CHES pH 8.5 / 9.0 / 9.5
   0 M / 0.5 M / 1.26 M / 1.7 M / 2.5 M ammonium sulfate
   0.2 M sodium chloride

Observation of crystal growth showed that *S. psychrophila* LeuDH crystals grew best under the conditions of: 0.1 M tri-sodium citrate dihydrate pH 5.6 / 1.5 M ammonium sulfate / 0.1 M lithium sulfate. This condition was considered to be the optimum crystallization condition and was used for the production of *S. psychrophila* LeuDH protein crystals in the following experiments.

Crystal of *S. psychrophila* LeuDH obtained had cube-like shape and exhibited a maximum dimension of about 0.2 mm (Fig. 5-1).

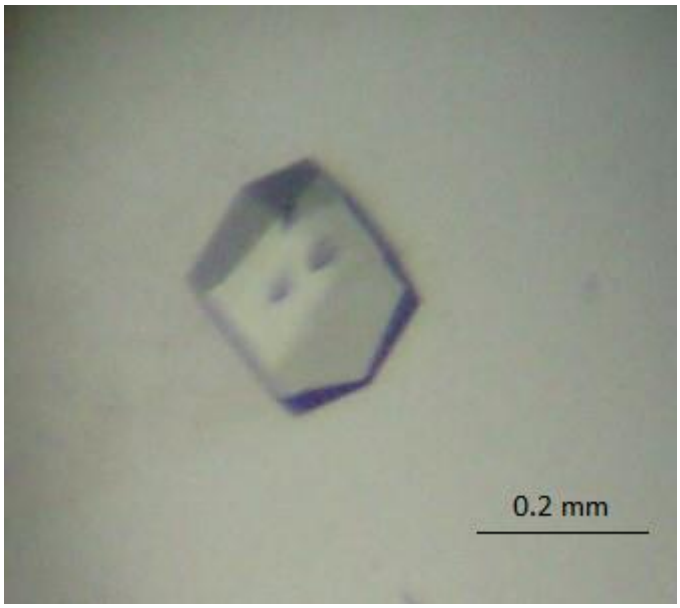

Fig. 5-1. Crystal of *S. psychrophila* DSM 3 LeuDH.

5.3.3 Crystal structure of S. psychrophila LeuDH

The crystal structure of *S. psychrophila* LeuDH was determined using molecular replacement with *L. sphaericus* LeuDH as an initial search model. The crystal structure was deposited in Protein Data Bank, and its PDB code is 3VPX.

There were two subunits in the asymmetric unit (A and B). The regions containing residues 138-144 (A), 364 (A), 141-144 (B) and 364 (B) were disordered. The data collection and refinement statistics are shown in Table 5-2.

Table 5-2. Data collection and refinement statistics

Data collection	
Space group	*I*4
Cell dimensions	
a, *b*, *c* (Å)	135.589, 135.589, 122.553
α, β, γ (°)	90, 90, 90
Wavelength (Å)	1.0000
Resolution (Å)[a]	50–2.55 (2.59–2.55)
R_{merge} (%)[a]	4.4 (30.8)
Average $I/\sigma(I)$ [a]	49.3 (7.5)
Completeness (%)[a]	99.2 (98.3)
Redundancy[a]	6.9 (6.0)
Refinement	
Resolution (Å)	31.8–2.55
No. reflections	31,963
R_{work}/R_{free}[b]	0.243/0.290
No. atom	
Protein	715
Water	67
B-factors	
Protein	53.5
Water	37.9
Rmsds[c]	
Bond lengths (Å)	0.007
Bond angles (°)	1.3
Ramachandran plot	
Favored regions (%)	82.2
Allowed regions (%)	17.9
Disallowed regions (%)	0.0
Protein Data Bank Code	3VPX

[a] Values in parentheses are for the outermost shell.

[b] R_{free} is monitored with 10% of the reflection data excluded from refinement.

[c] Root mean square deviations

The *S. psychrophila* LeuDH subunit is constructed from 12 α-helices and 11 β-strands (Figs. 5-2 and 5-3A). As shown in Fig. 5-3A, the secondary structural elements were folded into two domains, with a deep cleft between the two domains. Five glycine residues (Gly41, Gly42, Gly77, Gly78 and Gly290) thought to contribute to the shape of the active site [49, 73] are conserved in both the primary and crystal structures of *S. psychrophila* LeuDH, like *L. sphaericus* LeuDH. In the case of *L. sphaericus* LeuDH, it is known that the Lys68 binds to the 1-carboxyl group of the substrate [74], and Lys80 has an unusually low pK_a that assists the nucleophilic attack by a water molecule on the substrate α-carbon atom [75, 76]. The two lysine residues are also conserved in both the primary and crystal structures of *S. psychrophila* LeuDH. The sequence pattern GXGXXG, which indicates the presence of a dinucleotide-binding motif [83], is also conserved at residues 139-144. With respect to the quaternary structure, the eight subunits fold into an octamer like that of *L. sphaericus* LeuDH (Fig. 5-3A and B). Through logical operation in the crystallographic analysis, we determined that the enzyme exists as an octamer similar to *L. sphaericus* LeuDH (Fig. 5-3B and C) [49]. This is consistent with the size exclusion chromatography, which showed *S. psychrophila* LeuDH to be in a homooctameric state in solution.

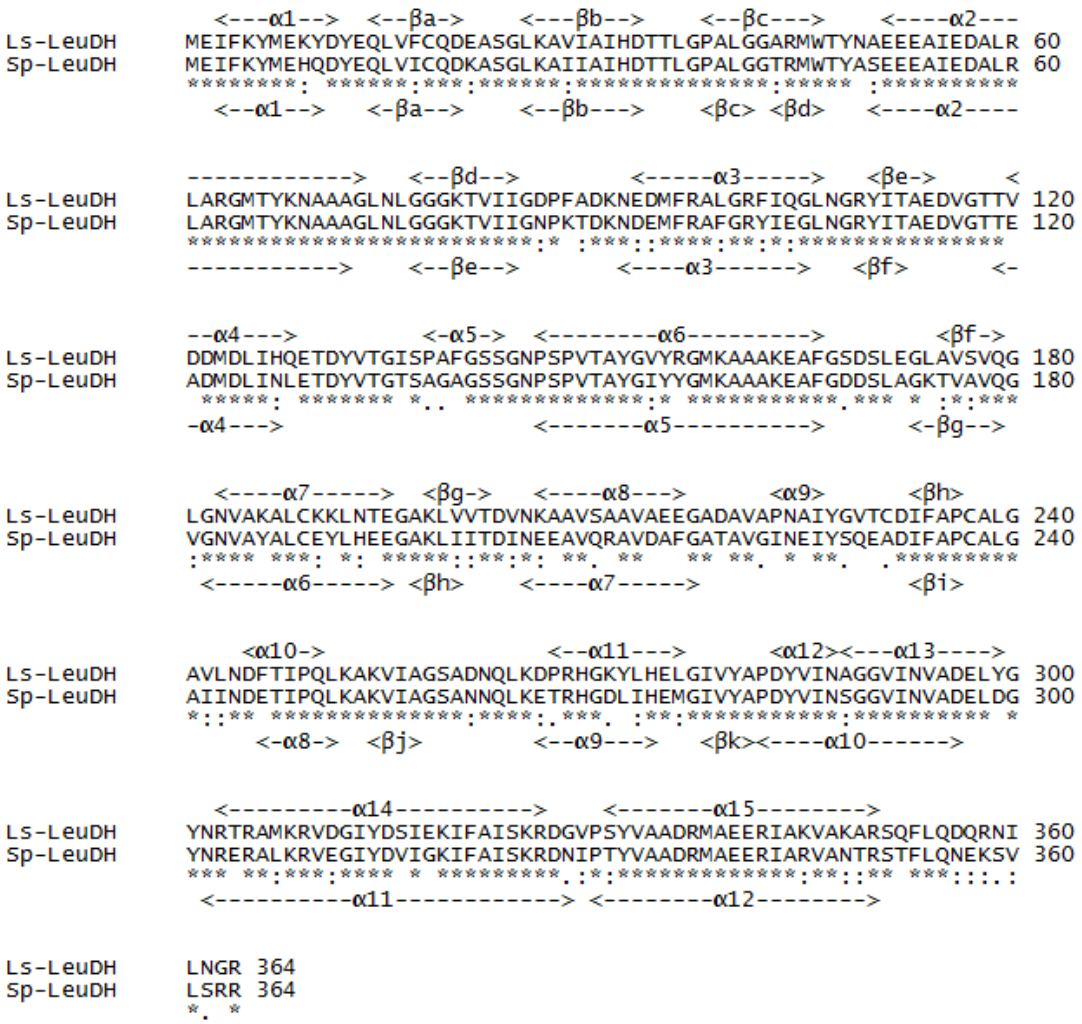

Fig. 5-2. Amino acid sequence alignment of *S. psychrophila* LeuDH (Sp-LeuDH) and *L. sphaericus* LeuDH (Ls-LeuDH). The amino acid sequence alignment was prepared with ClustalW [84, 85] and manually modulated, taking into account results of the structural information shown by MATRAS [86, 87].

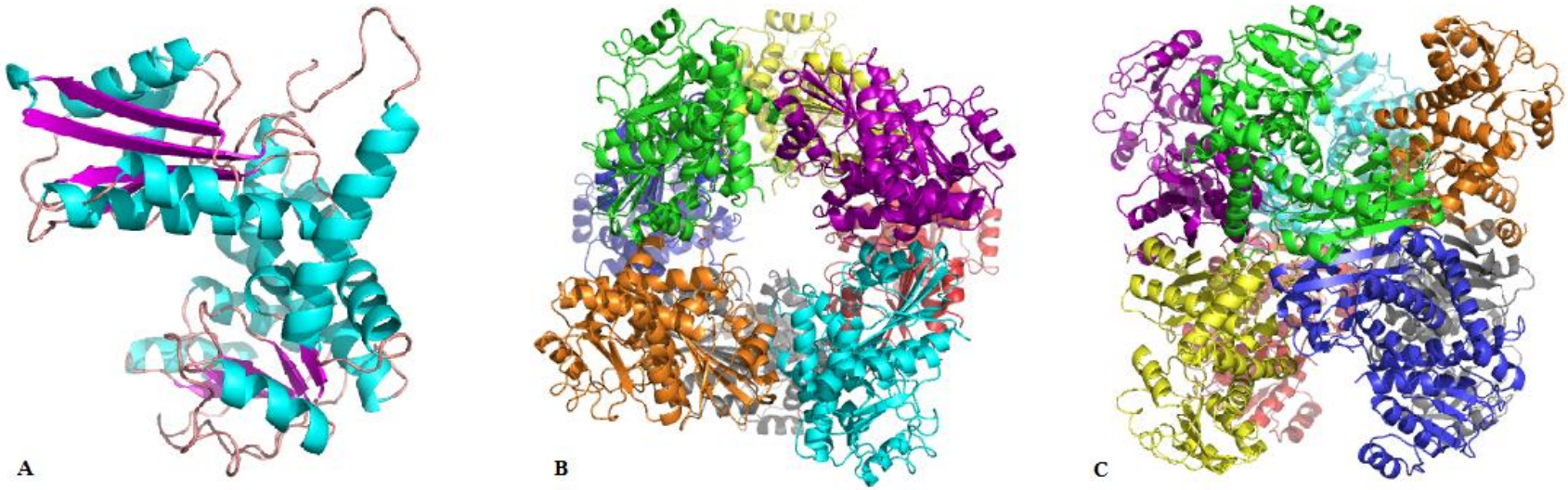

Fig. 5-3. Crystal structure of *S. psychrophila* LeuDH. Single single subunit structure (**A**), overall octamer structure view down the four-fold axis (**B**), and view down the non-crystallographic two-fold axis (**C**). Chains A~H of an octamer are colored green, blue, yellow, purple, cyan, pink, gray and orange, respectively.

5.4 Discussion

The structure of *S. psychrophila* LeuDH shows high homology to that of the *L. sphaericus* one at both the monomer and octamer levels. The structure of *S. psychrophila* LeuDH is nearly identical to that of the *L. sphaericus* one, with a RMSD of 0.9 Å for equivalent Cα atoms. Nonetheless, *S. psychrophila* LeuDH shows less thermostability than its *L. sphaericus* counterpart (Table 3-6). Using amino acid sequence alignment (Fig. 5-2) and three-dimensional structures, we identified a number of differences in the amino acid sequences that likely contribute to the diminished thermostability of the *S. psychrophila* LeuDH.

For example, the *L. sphaericus* LeuDH has several hydrophobic residues (Phe88, Phe102, Ala207, Ala211, Val229, Gly328, Ala349 and Gly363) on its surface, whereas *S. psychrophila* LeuDH has hydrophilic residues (Lys88, Tyr102, Glu207, Arg211, Gln229, Asn328, Thr349, and Arg363) at the corresponding positions. Some differences of intrasubunit interactions between amino acid residues in these two LeuDHs were observed. In the *L. sphaericus* LeuDH, for example, the distance between the side chains of Ile248 and Tyr272 is 3-4 Å; in the *S. psychrophila* LeuDH, Tyr272 is changed to a leucine, and the distance between the side chains of Ile248 and Leu272 is about 6 Å (Fig. 5-4). In addition, Gln128A in chain A of the *L. sphaericus* LeuDH can interact with Tyr299C in chain C. In the *S. psychrophila* LeuDH, these two residues are changed to Leu128A and Asp299C, which do not interact with one another (Fig. 5-5). These amino acid changes reduce the interaction within and between subunits, which together with the hydrophilicity of the molecule's surface may explain the lower thermostability of *S.*

psychrophila LeuDH.

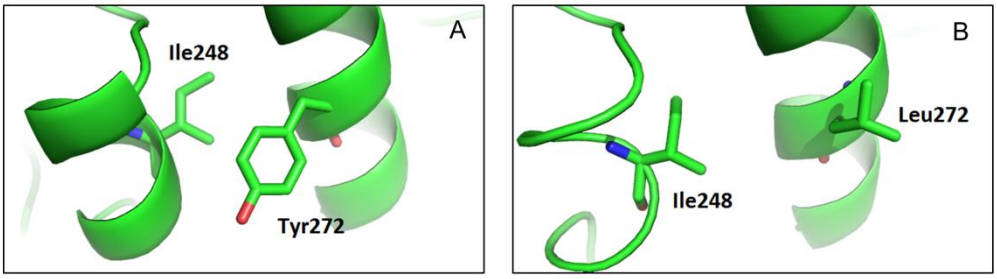

Fig. 5-4. Close up of No.248 and No.272 residues in *L. sphaericus* LeuDH (A) and *S. psychrophila* LeuDH (B). Distances between side chains of two residues are different.

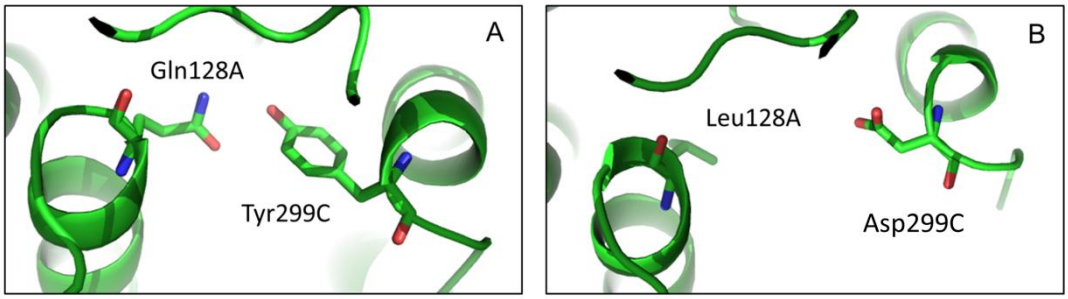

Fig. 5-5. Representation of interaction difference among subunits. **A** shows the interaction between Gln128 in chain A and Tyr299 in another chain C in *L. sphaericus* LeuDH. **B** shows that there is no such interaction in *S. psychrophila* LeuDH.

5.5 Summary

1) Primary screening of crystallization conditions was carried out, and *S. psychrophila* LeuDH crystals were found in 15 kinds of conditions.
2) Optimum crystallization condition of *S. psychrophila* LeuDH was determined.
3) Crystal structure of *S. psychrophila* LeuDH was determined by X-ray diffraction analysis.
4) The structure of *S. psychrophila* LeuDH shows similarities to the structure of *L. sphaericus* one at both the single subunit and octamer levels. But some amino acid changes that reduce the interaction within and between subunits, and the hydrophilicity of the molecule's surface may explain the lower thermostability of the enzyme.

General conclusions

In this study, LeuDH was screened in six psychrophilic bacteria, and the highest level of enzyme activity was found in *S. psychrophila* DSM 3. LeuDH was purified from *S. psychrophila* cell extract to homogeneity with several different chromatographies. This is the first LeuDH isolated from a psychrophile, and the enzymatic and kinetic characteristics were determined. Even at temperatures as low as 0˚C, *S. psychrophila* LeuDH exhibits high specific activity and high k_{cat}/K_m value for leucine, indicating the enzyme has high catalytic efficiency under cold environmental conditions.

Main enzymological properties of *S. psychrophila* LeuDH are well similar to thermophilic *G. stearothermophilus* LeuDH and mesophilic *L. sphaericus* LeuDH, except that the *S. psychrophila* one has a lower optimum reaction temperature and less thermostability. The crystal structure of *S. psychrophila* LeuDH shows that substitutions of some amino acid residues may responsible to its low thermostability.

In general, the structures of some enzymes from psychrophiles are known to be clearly different from those of their mesophilic and thermophilic counterparts, reflecting adaptation to the cold, but *S. psychrophila* LeuDH has high sequence identity and structural similarity to its mesophilic counterpart. This kind of similarity can also be found in L-threonine dehydrogenase [88], aspartase [89], aldehyde dehydrogenase [90] from *Cytophaga* sp. strain KUC-1, and alcohol dehydrogenase from *Flavobacterium frigidimaris* KUC-1 [91].

The results obtained in this thesis showed that as the first LeuDH isolated from a psychrophilic bacterium, the *S. psychrophila* enzyme may be useful for practical

applications due to its catalytic efficiency even at low temperatures. In addition, the structural analysis of psychrophilic enzyme may afford us some information about relationships between the function and structure with respect to higher reactivity of psychrophilic enzymes at low temperatures, although much more detailed biochemical and genetic engineering analyses about the relationships are needed.

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