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Expression and Thermostability study of Triose Phosphate Isomerase (TIM) from *Pseudomonas* π9, a Psychrophilic Bacterium

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ABSTRACT: Psychrophiles are organisms that grow rapidly below 20ºC. In order to overcome the inherent challenges in cold environment, evolution of cold-active enzymes with high catalytic efficiency at low temperature has been one of the psychrophiles adaptive strategies. In this study, triose phosphate isomerase (TIM) of *Pseudomonas* sp. π9, which was isolated from sea ice of Antarctic at Casey station, was expressed in *Escherichia coli* BL21 (DE3) host under IPTG induction and purified to homogeneity for subsequent biochemical characterization. The optimum temperature for the activity of this enzyme was found to be in the range of 35 to 40ºC. The thermostability study showed that this protein remained stable after 2 hr incubation at 40ºC and was gradually inactivated at 50ºC. The possessing of higher values of aliphatic index and grand average of hydropathy by *Pseudomonas* sp. π9 TIM as compared to other psychropilic TIM proteins could explain for its higher thermostability feature. Comparative protein sequence analysis performed on TIM sequences from psychrophilic, mesophilic, thermophilic and hyperthermophilic bacteria revealed the preference of tiny, small, aliphatic and non-polar amino acids in psychrophilic and mesophilic TIM as compared to thermophilic and hyperthermophilic TIM. In conclusion, our study showed that *Pseudomonas* sp. π9 TIM exhibited mesophilic properties instead of psychrophilic features. Nevertheless, the deeper understanding of strategies evolved by TIM enzymes that adapted to varied environments would provide contributive information for further studies on these valuable cold-adapted enzymes.

Keywords: Psychrophilic bacterium, cold-tolerant enzyme, triose phosphate isomerase

Introduction

Psychrophiles, a subset of extremophiles, are organisms that grow rapidly at about 15ºC or lower, having a maximum temperature for growth at about 20ºC, and a minimum temperature for growth at 0ºC or below (Morita, 1975). They are found at permanently cold terrestrial environment as well as at aquatic niche, snow, glacier, sea ice, and other cold ecosystems, which occupy more than three-quarters of the earth surface. Cold environments restrict growth of organisms. Hence, the ability of these organisms to survive and proliferate at low temperatures indicates a vast array of cold adaptations.

Low temperature slows down the rate of enzyme catalyzed biochemical reactions. To counteract the negative effect of cold on the activity of an enzyme, some enzymes of psychrophiles have evolved sufficient activities and efficiencies to support the growth of cold-tolerant organisms at low temperatures. These enzymes are known as psychrophilic enzymes or cold-active enzymes, which are enzymes that have high catalytic efficiency at low temperatures, and are inactivated at moderate temperature (D'Amico *et al.*, 2006; Gerday *et al.*, 2000).

In recent years, increased attention has been focused on psychrophilic enzymes. These enzymes are suggested to display high catalytic activities at low temperatures by having improved flexibility at active site and more rigidity at other protein regions that are not involved in the catalysis, as compared to their mesophilic counterparts. Psychrophilic enzymes offer considerable potential for fundamental research and biotechnological application. Their application in the detergent and food industries, and for the production of fine chemicals are significant (Gerday *et al.*, 2000). Industrial application of cold-active enzymes has greatly increased because of their active catalytic activity at extreme conditions. Besides that, they have energy saving advantage and consecutive economic benefits originate from their specific properties (Hoyoux *et al.*, 2004).

Triose phosphate isomerase (TIM) is a dimeric enzyme formed by two identical subunits each consisting of about 250 amino acid residues. It is a central enzyme in the glycolytic pathway. Beginning from glucose, glycolytic pathway is catalyzed by the sequential action of ten enzymes, in which, TIM enzyme catalyzes the interconversion of dihydroxyacetone

phosphate and D-glyceraldehyde-3-phosphate. It has been established that TIM is a very efficient catalyst as its reaction rates are diffusion-controlled. Neither cofactors nor metal ions are required in this reaction, and there is no evidence of allostery or cooperativity among the subunits (Alvarez *et al.*, 1998). Structurally TIMs do form a well characterized family. X-ray structures of TIM from several different sources have been solved (wild type or/and in complex with substrate analogues): chicken (Banner *et al.*, 1975), yeast (Lolis *et al.*, 1990), *Trypanosoma brucei* (Wierenga *et al.*, 1991), *Escherichia coli* (Noble *et al.*, 1993), human (Mande *et al.*, 1994), *Bacillus stearothermophilus* (Delboni *et al.*, 1995), and *Plasmodium falciparum* (Velanker *et al.*, 1997). In addition, 45 amino acid TIM sequences from a wide variety of organisms have also been determined and are available in the data bases (Alvarez *et al.*, 1998). Thus, TIM serves as a good candidate for the study on protein structure adaptation in order to carry out its function.

In this research, TIM enzyme of *Pseudomonas* sp. π9, an Antarctic psychrophile that was isolated from sea ice of Antarctic at Casey Station, was purified and characterized. The TIM gene from the psychrophilic bacterium had previously been isolated and cloned into a pET14b plasmid (See Too and Few, 2010). We aimed to find out whether this TIM enzyme possessed the characteristic of a psychrophilic protein by analyzing its biochemical characteristics as well as its thermostability in different temperatures. This study served to gather information on the enzyme produced by a psychrophilic bacterium in order to gain further insights of the adaptive strategies of cold-active enzymes. By having a deeper understanding of their evolved strategies in cold-adaptation, their application in industrial and biotechnological uses can be enhanced.

Materials and methods

Protein expression

E. coli BL21 (DE3) strain carrying recombinant plasmid was grown in 5 ml of LB broth containing 1% ampicillin and incubated on shaker at 200 rpm, 37ºC for overnight. One ml of the overnight culture was then inoculated into 100 ml LB broth containing 1% ampicillin and incubated at 37ºC. The culture was grown to OD_{600nm} 0.8-1.0. Then, 0.5 ml of the culture was taken out and spun down. Supernatant was discarded and the pellet was kept as the uninduced

sample for SDS-PAGE analysis later. Remaining culture was then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated on shaker at 200 rpm, room temperature for 7 hours. After 7 hours, 0.5 ml of the culture was taken out, spun down, and the pellet was kept as the induced sample for checking the protein induction by using SDS-PAGE analysis. Finally, cells were harvested from the remaining culture and the pellet was kept at -20ºC for the protein purification.

Cell lysis by sonication

Cell pellet was thawed on ice. It was then resuspended with 5 ml lysis buffer added with 3.9 µl β-mercaptoethanol. Then, the cells were lysed by sonicating at 20% amplitude for 6 to 8 cycles or until the suspension became clear, by using Branson Digital Sonifier (Models S-4500). Each sonicate cycle was set for 30 seconds with 0.5 seconds interval. After a clear lysate was obtained, it was spun down at 12 000 g, 4ºC for 20 minutes. Supernatant was pipetted out and kept at -20ºC for the protein purification step and an aliquot of 40 µl supernatant was kept for checking the solubility of protein by SDS-PAGE analysis. The pellet or cell debris was resuspended and 40 µl of aliquot were kept for SDS-PAGE analysis.

His-Tagged protein purification

The supernatant obtained in the sonication step was added with 200 µl Ni-NTA agarose resins were added. The mixture was mixed gently by rotating on a rotary shaker at 4ºC for 2 hours. After 2 hours, the mixture was spun down at 4000 rpm, 4ºC for 3 minutes. Supernatant was discarded. Ten ml of washing buffer were then added to the resin and the mixture was rotated on the rotary shaker for 30 minutes to wash away the non-specified proteins that bound to the resin. After 30 minutes, the mixture was spun down and the washing step was repeated for 3 to 4 times. After the washing step, the mixture was spun down and the supernatant was discarded. The resin was resuspended with 300 µl washing buffer and transferred into a 1.5 ml microcentrifuge tube. One µl of thrombin was added for His-tag cleavage and the mixture was mixed by placing on a rotary shaker at 4ºC for overnight. On the next day, the mixture was spun down and the supernatant containing the protein was separated out. The protein was aliquoted to 50 µl in each Eppendorf tube and kept at -80ºC. Purity of the purified protein was then checked with the SDS-PAGE analysis.

TIM Enzymatic assay (Modified from (Strauss and Gilbert, 1985)

Triose phosphate isomerase (TIM) activity was measured in the direction from glyceraldehyde-3-phosphate to dihydroxyacetone phosphate (DHAP) by coupling the product to a helper enzyme, α-glycerophosphate dehydrogenase, which catalyzed the conversion of DHAP into α-glycerophosphate with the oxidation of reduced nicotinamide adenine nucleotide (NADH). One ml assay mixture contained 100 mM Tris-HCl (pH 7.5), 0.2 mM NADH, 2 mM glyceraldehyde-3-phosphate, 1 U/ml α-glycerophosphate dehydrogenase and 1 µl purified TIM. Glyceraldehyde-3-phosphate was the last item to be added to initiate the reaction. The enzyme activity was measured by monitoring the decrease in absorbance of NADH at wavelength 340 nm.

Optimum temperature for TIM activity

The optimum temperature for the reaction catalyzed by TIM enzyme was determined by performing TIM enzymatic assay at various temperatures using Cary 100 Bio UV-Visible Spectrophotometer with a temperature controlled cell holder (Varian, USA), in which the temperature of the cell holder was controlled by Thermoscientific Cool Tech 320 refrigerated circulator. The activity of TIM enzyme was assayed at temperatures ranged from 20 to 45ºC. The decrease in absorbance of NADH at wavelength 340 nm per minute (ΔOD at 340 nm/min) was determined by Cary UV Win analysis software. Based on the readings, specific activity of TIM enzyme was calculated by applying (a) Beer-Lambert law, where Δc is the delta concentration of the substrate in solution per unit time that was expressed in µmol/min; ΔA is the delta absorbance of NADH at 340 nm per minute (Δ OD at 340 nm/min); ε is the extinction coefficient of NADH (6.22 M^{-1} cm^{-1}); and l is the pathlength of the cuvette (1cm), followed by formula (b) that divides Δc with the amount of TIM enzyme added into the assay mixture.

(a) Δc (µmol/min) $= \dfrac{\Delta A}{\varepsilon l}$

(b) Specific enzyme activity (µmol/min/µg) $= \dfrac{\Delta c}{\text{Amount of TIM enzyme (µg)}}$

Specific activity of TIM was defined as the conversion of the substrate catalyzed by 1 µg of TIM in 1 minute.

Thermostability study

Thermal stability of TIM enzyme was evaluated by determining the residual activity after incubation at 40, 50 and 60ºC for 30 and 120 minutes. Enzyme activity after the incubation was determined under the standard conditions as described before. The percentage remaining activity was as follows:

$$\text{Percentage Remaining activity} = \frac{\text{Specific enzyme activity after incubation}}{\text{Specific enzyme activity before incubation}} \times 100\%$$

Physico-chemical analysis of protein sequence

ProtParam tool available on ExPASy server (Gasteiger *et al.*, 2005) and Pepstats program of European Molecular Biology Open Software Suite (EMBOSS) (Rice *et al.*, 2000) were used to determine the molecular weight, amino acid composition, theoretical pI, aliphatic index and grand average of hydropathicity (GRAVY) of proteins.

Results

Expression and purification of TIM enzyme

Pseudomonas sp. π9 TIM enzyme was successfully expressed in *E. coli* BL21 (DE3) (**Figure 1**). Expected bands with molecular weight around 26 kDa were shown in lane 4, 5 and 6. It indicates the successful expression of TIM enzyme by *E. coli* under the induction of IPTG. Besides that, more evident bands were observed in lane 5 and 6, which shows higher yields of TIM enzyme were obtained after 5 and 7 hours of IPTG induction.

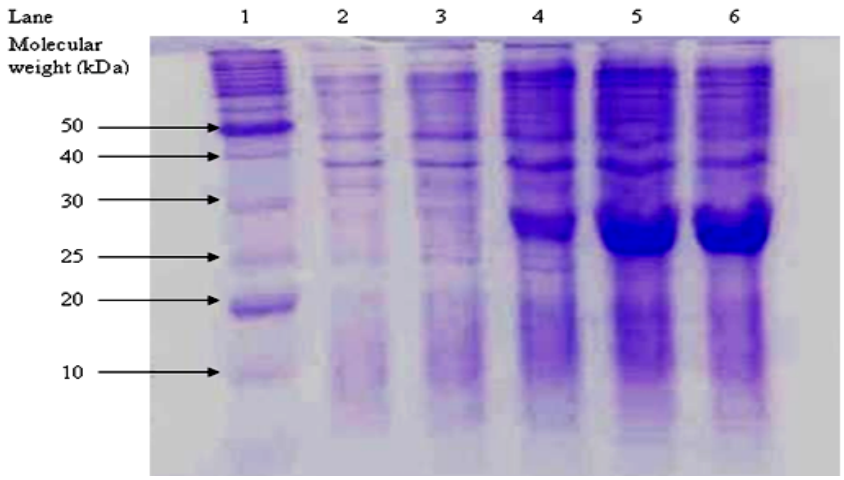

Figure 1: Expression of *Pseudomonas π9* TIM enzyme in *E. coli* host. SDS-PAGE (12.5%) gel was stained with Coomassie brilliant blue R-250. Expected bands with molecular weight around 26 kDa as observed in lane 4, 5 and 6 indicates the expression of TIM under the induction of IPTG. Lane 1: BenchMarkTM protein ladder; 2: Uninduced sample; 3: Induced sample after 1 hour induction; 4: Induced sample after 3 hours induction; 5: Induced sample after 5 hours induction; and 6: Induced sample after 7 hours induction.

TIM enzyme was successfully purified by using Ni-NTA agarose matrices. Purity of purified TIM enzyme was checked by SDS-PAGE analysis and the result was shown in **Figure 2**. A single band was obtained in lane 2. This indicates that the purified TIM was in a homogenous state.

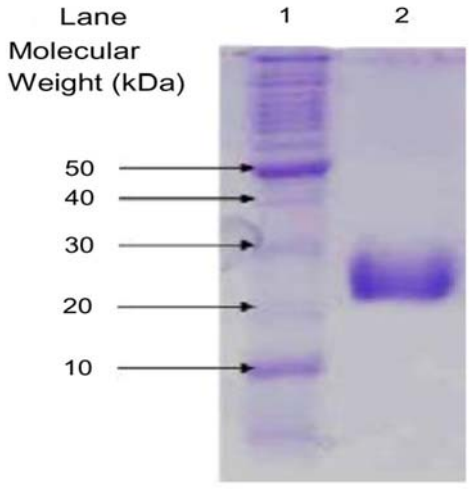

Figure 2: Purification of *Pseudomonas π9* TIM enzyme. SDS-PAGE (12.5%) gel was stained with Coomassie brilliant blue R-250. A single band with expected molecular size, 26kDa, was observed in lane 2. Lane 1: BenchMarkTM protein ladder; 2: Purified TIM.

Optimum temperature for TIM activity

Specific activity of TIM enzyme at temperatures ranged from 20 to 45ºC was determined. Enzymatic assay of TIM was performed as described in materials and methods (**Figure 3**). **Figure 3** show that the maximal activity of TIM enzyme occurred at temperature around 35 to 40ºC. Hence, the optimum temperature for TIM enzyme activity that was perceived from the result was between 35 to 40ºC.

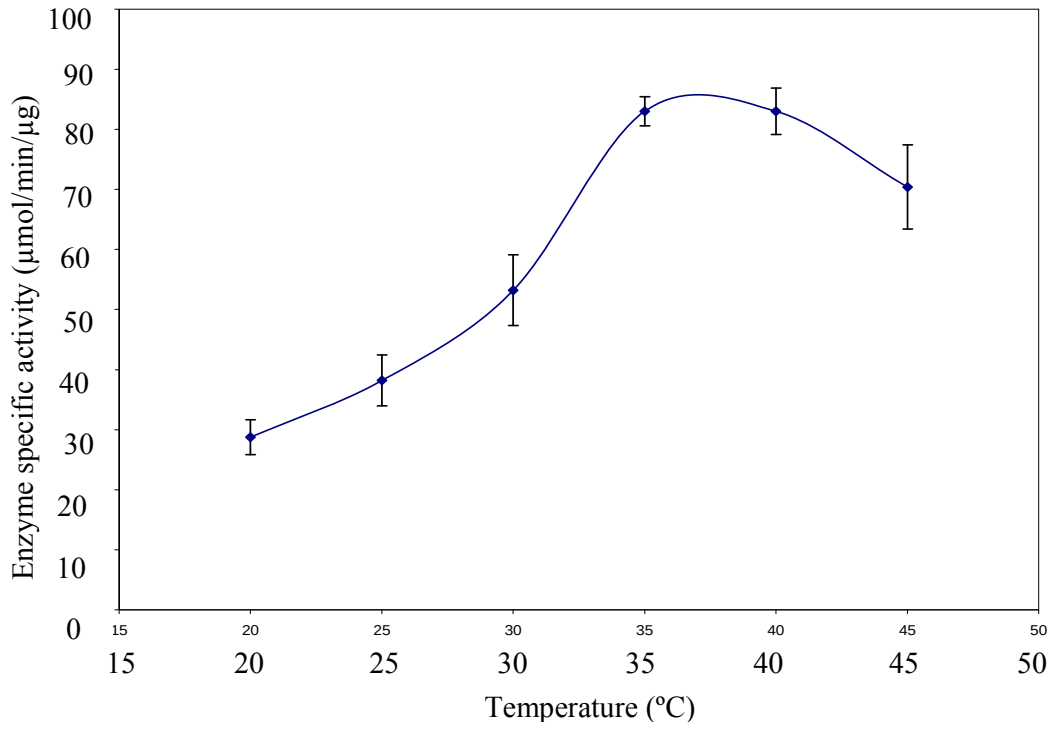

Figure 3: Optimum temperature profile of *Pseudomonas π9* TIM enzyme activity.

Thermostability study

The remaining activity of TIM enzyme after incubation at 40, 50 and 60ºC for certain time periods was determined. The residual activity of TIM enzyme was assayed and calculated as described in materials and methods (**Figure 4**). The residual activity of TIM enzyme after incubation at 40ºC for 120 minutes was slightly increased. While, a decrease in TIM enzyme activity was observed for the incubation at 50ºC and 60ºC. After incubation at 50ºC for 60

minutes, TIM showed a slight decrease in its activity. Incubation at 50ºC for another 60 minutes further decreased the enzyme activity to about 85%. Whereas, activity of TIM after incubated at 60ºC showed a greater decrease, in which only about 50% of the activity remained. This indicates TIM started to lose its stability at higher temperatures like 50 and 60ºC, while there was no detectable loss of activity at moderate temperature of 40ºC after 2 hours incubation.

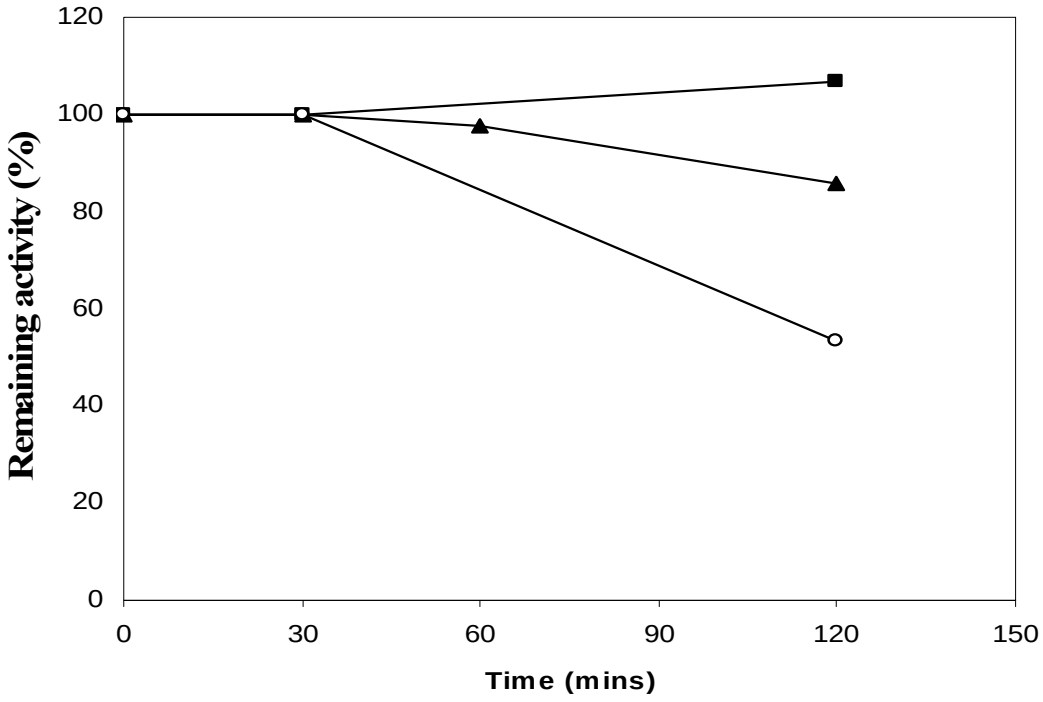

Figure 4: Thermostability study of *Pseudomonas π9* TIM enzyme. Remaining activity of TIM enzyme after incubation at 40, 50 and 60ºC over a time periods was plotted. Symbols: ■, incubation at 40ºC; ▲, incubation at 50ºC; and ○, incubation at 60ºC.

Physico-chemical analysis of protein sequences

TIM protein sequences from *Pseudomomas* sp. π9, *M. marina*, *E. coli*, *T. lettingae* and *T. maritima* were analyzed, in which their composition of amino acid (AA) and amino acid property groups were computed and shown in **Table 1** and **Table 2**. Comparison of the amino acid composition showed that some of the AA differed among the sequences, which include alanine (A), glutamic acid (E), glycine (G), isoleucine (I) and lysine (K). *Pseudomomas* sp.

π9, *M. marina* (psychrophile) and *E. coli* (mesophile) TIMs have higher frequency of Ala and lower frequencies of Ile and Lys as compared to *T. lettingae* (thermophile) and *T. maritime* (hyperthermophile) TIMs. For the comparison of amino acid property groups among the sequences, the result showed the frequency of tiny, small, aliphatic and non-polar AA was higher in TIM sequences of *Pseudomonas* sp. π9, *M. marina* and *E. coli* as compared to *T. lettingae* and *T. maritima*. Whereas, the composition of aromatic and polar AA found in *T. lettingae* and *T. maritima* TIM was higher as compared to other sequences.

Table 1: The composition of amino acids in protein sequences of *Pseudomonas π9, M. marina, E. coli, T. lettingae* and *T. maritima* TIMs analyzed by ExPASy ProtParam tool.

Amino acid	Composition (%)				
	Pseudomonas π9	***M. marina***	***E. coli***	***T. lettingae***	***T. maritima***
Ala (A)	13.9	18.8	17.6	5.8	5.9
Arg (R)	5.6	2.7	3.1	2.5	5.5
Asn (N)	4.0	4.7	3.5	5.0	2.4
Asp (D)	3.2	4.7	3.9	4.1	3.5
Cys (C)	2.0	0.8	1.2	2.1	1.2
Gln (Q)	5.2	3.1	4.3	5.8	3.1
Glu (E)	6.4	8.6	8.2	7.9	11.4
Gly (G)	12.7	9.0	8.6	8.7	9.4
His (H)	1.6	2.3	3.1	2.1	1.6
Ile (I)	6.0	6.6	7.5	10.8	9.0
Leu (L)	9.6	7.0	6.7	9.5	9.0
Lys (K)	2.4	5.1	6.3	7.1	7.5
Met (M)	2.4	2.3	2.7	2.9	2.0
Phe (F)	2.0	3.1	2.4	5.4	4.7
Pro (P)	3.2	3.1	2.7	3.7	2.7
Ser (S)	5.6	3.9	4.3	3.7	4.7
Thr (T)	3.2	4.3	3.5	2.9	3.9
Trp (W)	0.8	0.8	0.8	0.8	0.8
Tyr (Y)	1.2	1.6	2.0	2.5	2.4
Val (V)	9.2	7.4	7.5	6.6	9.4

Table 2: The composition of amino acid property groups in protein sequences of Pseudomonas *π9, M. marina, E. coli, T. lettingae* and *T. maritima* TIMs analyzed by EMBOSS pepstats.

Amino Acid Property Group	Composition (%)				
	Pseudomonas π9	***M. marina***	***E. coli***	***T. lettingae***	***T. maritima***
Tiny	37.5	36.7	35.3	23.2	25.1
Small	57.0	56.6	52.9	42.7	43.1
Aliphatic	38.65	39.8	39.2	32.8	27.5
Aromatic	5.6	7.8	8.2	10.8	9.4
Non-polar	62.95	60.5	59.6	58.9	56.5
Polar	37.1	39.5	40.4	41.1	43.5
Charged	19.1	23.4	24.7	23.7	29.4
Basic	9.6	10.2	12.5	11.6	14.5
Acidic	9.6	13.3	12.2	12.0	14.9

Discussion

Pseudomonas sp. π9 was an Antarctic psychrophilic bacterium isolated from sea ice at Casey station. In this study, *E. coli* host cell carrying plasmid (pET14b) with inserted gene of TIM from *Pseudomonas* sp. π9 was expressed to produce TIM enzyme. TIM enzyme was successfully purified to homogeneity. Due to the successful colonization of *Pseudomonas* sp. π9 at cold environment, enzyme produced by this psychrophile was expected to evolve and show cold-active properties, which have high catalytic efficiency at low temperature and low thermal stability. However, the result obtained in this study showed TIM enzyme of this bacterium does not possess the features of psychrophilic enzymes. There are possibilities that this organism evolved adaptations other than by having a psychrophilic enzyme to overcome the cold environment.

The optimum temperature for the activity of *Pseudomonas* sp. π9 TIM was observed in the range of 35 to 40ºC. Besides that, thermostability study showed it remained stable after 2 hours incubation at 40ºC and it gradually inactivated at a higher temperature, 50ºC. As compared to the identified psychrophilic TIM from *Moraxella sp.* that has been shown to has its maximal activity at 25ºC and half-life of 7 minutes at 45 ºC (Rentier-Delrue *et al.*, 1993), *Pseudomonas* sp. π9 TIM was found to be more active and stable at higher temperature.

Furthermore, *Pseudomonas* sp. π9 TIM also showed higher thermal stability as compared to psychrophilic TIM from *Vibrio marinus*, which has been characterized with a half-life of only 10 minutes at 25ºC (Alvarez *et al.*, 1998). Thus, *Pseudomonas* sp. π9 TIM was found to show the features of mesophilic enzymes instead of cold-active properties.

In other studies, some enzymes from psychrophiles have also been shown to display mesophilic features instead of cold-active properties. More strikingly, some enzymes have been characterized with thermophilic characteristics even although they were derived from psychrophilic organisms. Malate synthase isolated from a psychrophilic marine bacterium, *Colwellia maris*, has been reported to have a typical feature of mesophilic enzymes. The optimum temperature for its activity was 45ºC (Watanabe *et al.*, 2001). Whereas, in the study of aldehyde dehydrogenase isolated from a psychrophile, *Cytophaga sp.* KUC-1, the enzyme showed thermophilic properties, with its highest activity in the range of 55 to 60ºC and thermal stability at temperature above 40ºC (Yamanaka *et al.*, 2002). Hence, as has been presented by other studies, not all enzymes from psychrophiles were evolved to have their maximal activity coincide with the temperature optimum for the growth of organisms.

The adaptation of cold-active enzyme at low temperature requires a vast array of adjustments, where decrease in the stability of enzyme and enhanced conformational flexibility were known to have a close relationship with the cold-activity of psychrophilic enzyme (Zavodszky *et al.*, 1998). Several factors have been proposed contributing to the increased structural flexibility and the generally observed low stability of cold-adapted enzymes, which include a lower number of Pro and Arg residues; a higher number of Gly residue; less aromatic, hydrophobic and charge-mediated interactions; a larger accessible surface area; and a decreased number of hydrogen bonds (Violot *et al.*, 2005) .

The study by Metpally and Reddy (2009) showed that amino acids like Ala, Asp, Ser, Thr, and other amino acids with small/tiny and neutral property were favored in cold-active enzymes (Metpally and Reddy, 2009). Whereas, reduced number of Glu, Leu, charged, basic, aromatic and hydrophilic amino acids has been observed in cold-adapted enzymes.

In order to understand the adaptive strategies evolved in the *Pseudomonas* sp. π9 TIM and TIM family, TIM sequences from *Pseudomonas* sp π9, *M. marina*, *E. coli*, *T. lettingae* and *T.*

maritima, which representing psychrophilic to hyperthermophilic homolog, were analyzed in this study. The frequencies of amino acids occurrences and property groups in TIM sequences were computed. The result showed no obvious increase was observed in the frequencies of amino acids that have been suggested as preferable amino acids in cold-active enzymes, except an increase in Ala residue was observed in TIMs from psychrophiles (*Pseudomonas* sp. π9 and *M. marina*). However, *E. coli* TIM also showed a comparable high composition of Ala. As for the comparison of the frequencies of Glu, Leu, Arg and Pro occurrences, which have been suggested to be less abundant in cold-adapted enzymes, no difference was observed among the sequences.

By comparing the frequencies of amino acid property groups in TIM sequences, it showed that the composition of tiny, small and non-polar amino acids was higher in *Pseudomonas* sp. π9, and *M. marina* TIMs as compared to thermophilic and hyperthermophilic TIMs. Studies described tiny and small amino acids, for example glycine (Gly), were preferable in cold-active proteins because they provide greater rotational freedom and flexibility to the proteins, and capable of making cavities in the core parts of protein structures (Feller and Gerday, 2003, Panasik *et al.*, 2000). Whereas, the charged, polar and aromatic amino acids that contribute to weak interaction (ion pairs, aromatic interactions, hydrogen bonds and helix dipoles), which restricts and maintains the protein stability, were less abundant and not favored in cold-active enzymes (Metpally and Reddy, 2009). As has been shown by studies, reduced number of aromatic, polar, charged and basic groups of amino acids was observed in the TIM sequences of *Pseudomonas* sp. π9 and *M. marina*.

Apart from that, the main parameters resulting from biocomputation of the TIM amino acid sequences have also been compared. The result showed the aliphatic index for *Pseudomonas* sp. π9, *T. lettingae* and *T. maritima* TIM was higher as compared to *M. marina* and *E. coli* TIM. In fact, aliphatic index, which is defined as the relative volume of a protein occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine), has been regarded as a positive factor for the increase of thermostability of globular proteins (Ikai, 1980). The aliphatic amino acids have been widely accepted to contribute to the hydrophobic interaction for maintaining structural stability and rigidity in core region of the proteins. It has been shown to be significantly higher in thermophilic proteins (Pack and Yoo, 2004). Thus, this could

explain the finding that *Pseudomonas* sp. π9 TIM was showing a quite high thermal stability as compared to the other psychrophilic TIMs.

As for another calculated parameter, grand average of hydropathy (GRAVY), TIM sequence of *Pseudomonas* sp. π9 showed higher value (0.177) as compared to *M. marina* (0.058), *E.coli* (0.010), *T. lettingae* (0.053) and *T. maritima* (-0.038). Grand average of hydropathy was used to evaluate the hydrophilicity and hydrophobicity of each protein along its amino acid sequence. It is the average value of hydropathy index at each position in a sequence that indicates the hydrophobicity of protein. It has been shown that a higher average hydropathy was found in thermophilic proteins (Zhou *et al.*, 2008). Hydrophobicity interaction has been suggested as one of the factors contribute to the increase of thermal stability (Bae and Phillips Jr, 2004).

Table 3: Biocomputing parameters for *Pseudomonas* sp. *π9, M. marina, E. coli, T. lettingae* and *T. maritime TIMs* analyzed by ExPASy ProtParam tool.

Parameter	***Pseudomonas π9***	***M. marina***	***E. coli***	***T. lettingae***	***T. maritima***
No. of amino acids	251	256	255	241	255
Molecular weight	26.03	26.74	26.97	26.98	28.52
Theoretical pI	5.61	4.82	5.64	5.44	5.60
Aliphatic index	101.12	93.59	94.31	104.36	103.53
GRAVY	0.177	0.058	0.010	0.053	-0.038

Conclusion

In this study, TIM enzyme of *Pseudomonas* sp. π9 was successfully expressed in and purified from *E. coli* BL21 (DE 3) host cell under IPTG induction. Based on the results of thermostability study and the optimum temperature for enzyme activity, it was suggested *Pseudomonas* sp. π9 TIM exhibited mesophilic properties instead of psychrophilic features. Comparative protein sequence analysis performed on TIM sequences from psychrophilic, mesophilic, thermophilic and hyperthermophilic bacteria revealed the preference of tiny, small, aliphatic and non-polar amino acids in psychrophilic and mesophilic TIM as compared to thermophilic and hyperthermophilic TIM. Information of enzyme activities over a range of

temperatures in this study would be useful for future studies to reveal the cold-adaptation evolved in enzymes.

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